Aquatic Invasions (2009) Volume 4, Issue 1: 29-44
DOI 10.3391/ai.2009.4.1.3

Special issue "Proceedings of the 2nd International Invasive Sea Squirt Conference"
(October 2-4, 2007, Prince Edward Island, Canada)
Andrea Locke and Mary Carman (Guest Editors)

Research article

Genetic conspecificity of the worldwide populations of *Didemnum vexillum* Kott, 2002

Lauren Stefaniak[1*], Gretchen Lambert[2], Adriaan Gittenberger[3,4], Huan Zhang[1], Senjie Lin[1] and Robert B. Whitlatch[1]
[1]*Department of Marine Sciences, 1080 Shennecossett Rd., University of Connecticut, Groton, CT 06340, USA*
[2]*University of Washington Friday Harbor Laboratories, Friday Harbor, WA 98250, USA*
[3]*National Museum of Natural History Naturalis, Leiden, The Netherlands*
[4]*Institute of Biology, University Leiden, Leiden, The Netherlands*
**Corresponding author*
E-mail: lauren.stefaniak@uconn.edu

Received 1 February 2008; accepted for special issue 19 April 2008; accepted in revised form 19 December 2008; published online 23 December 2008

Abstract

A colonial tunicate belonging to the genus *Didemnum* has recently been found in many temperate coastal regions throughout the world, as well as large areas of Georges Bank in the NW Atlantic. It continues to spread rapidly and compete aggressively with native, hard substrate species (e.g., mussels, barnacles, bryozoans, other ascidians). In addition, it can form dense mats on deep-water cobble-gravel substrates and influence the abundance and species composition of benthic epifauna and infauna. Thus, its ever-increasing presence is creating potentially severe detrimental economic and ecological impacts. This invasive species, referred to in recent publications as *Didemnum* sp. A, has been misidentified as five previously described species native to the regions where *Didemnum* sp. A has been discovered and has been described as two new species based solely on morphological characteristics. There are relatively few diagnostic characters and a great deal of variability in the relevant characters, making the task of identification very difficult. Adding to the confusion has been the widespread and apparently disjunct distribution of the species. Here, we present molecular data on both mitochondrial and nuclear genes from colonies sampled from Europe, east and west coasts of North America, Japan, and New Zealand. These data strongly indicate that *Didemnum* sp. A is a single species, possibly native to the northwestern Pacific Ocean, that has become established globally. Considering genetic and morphological evidence, the most appropriate name for this species is *Didemnum vexillum* Kott, 2002.

Key words: *co1*, *Didemnum*, invasive ascidians, taxonomy

Introduction

The identification and control of invasive species, both terrestrial and marine, are of increasing concern around the globe because of the economic and ecological toll exotic species can exact on their new habitats (e.g., Naeem et al. 1994; Pimentel et al. 2000). Invasive tunicates are often important spatial competitors and can alter community structure and local biodiversity (e.g., Stachowicz et al. 2002; Dijkstra et al. 2007; Osman and Whitlatch 2007). They can also cause economic damage by overgrowing aquaculture equipment (USGS 2008).

Over the last three decades, previously unrecorded populations of superficially similar colonial tunicates of the genus *Didemnum* have been discovered in many temperate coastal regions throughout the world (Minchin and Sides 2006; Bullard et al. 2007; Coutts and Forrest 2007; Gittenberger 2007; Lambert 2009, this issue). The invaders grow rapidly and are aggressive competitors with other hard substrate species both native and exotic (e.g., mussels, barnacles, bryozoans) and on both artificial and natural substrates (Figure 1a, Minchin 2007; Osman and Whitlatch 2007; USGS 2008). These tunicates are also of particular concern because of their unique ability to overgrow and cement

cobble/gravel substrates together (Figure 1b). Currently, over 230 km^2 of Georges Bank are covered with mats of *Didemnum* sp. A, causing concerns about potential impacts on food availability for economically important juvenile fishes (Valentine et al. 2007), and on other organisms residing in these habitats (Mercer et al. 2009).

Identification of this invader, or even the determination of how many species are involved, has been problematic. Various populations have been identified under as many as five different, previously described *Didemnum* species (*D. lahillei* Hartmeyer, 1909 – France; *D. helgolandicum* Michaelsen, 1921 – France and northern Europe; *D. lutarium* Van Name, 1910 – New England; *D. carnulentum* Ritter and Forsyth, 1917 – California and Washington; and *D. pardum* Tokioka, 1962 – Japan) as well as two new species described from New Zealand and New Hampshire (*Didemnum vexillum* Kott, 2002 and *Didemnum vestum* Kott, 2004, respectively) (Lambert 2009, this issue). However, morphological re-examination of material collected around the world and comparison with the type specimens of several of the above-mentioned species suggests that all of the recently discovered populations are in fact the same species (Lambert 2009, this issue), nominally designated *Didemnum* sp. A, and are not any of the previously described species suggested. Part of the confusion surrounding the identity of this species is due to the difficulty in identifying any species belonging to the genus *Didemnum*, since species of the genus show high morphological similarities. Many morphological characters are difficult to study due to the small size of zooids, larvae, and spicules. Poor preservation techniques, inadequate sampling, intra- and inter-colony variability, and even timing of collection during non-reproductive periods can obscure or eliminate many important characters, making identification difficult.

Molecular markers are therefore useful where there has been confusion using standard taxonomy because they are independent of the morphological characters in question (e.g. Tarjuelo et al. 2001; Nydam and Harrison 2007). Thus, samples that cannot be identified from morphological characters, such as lack of larvae or reproductive structures, can still be usable for molecular studies. Furthermore, molecular data from any one sample can be precisely recorded and stored for subsequent comparison with other samples at any other time. Finally, a great number of DNA sequences are publicly available (http://www.ncbi.nlm.nih.gov), allowing selection of markers with a level of interspecific variation that suit the required taxonomic resolution (e.g., rapidly evolving genes for intraspecific studies, slowly evolving genes for comparisons at the species-level or higher).

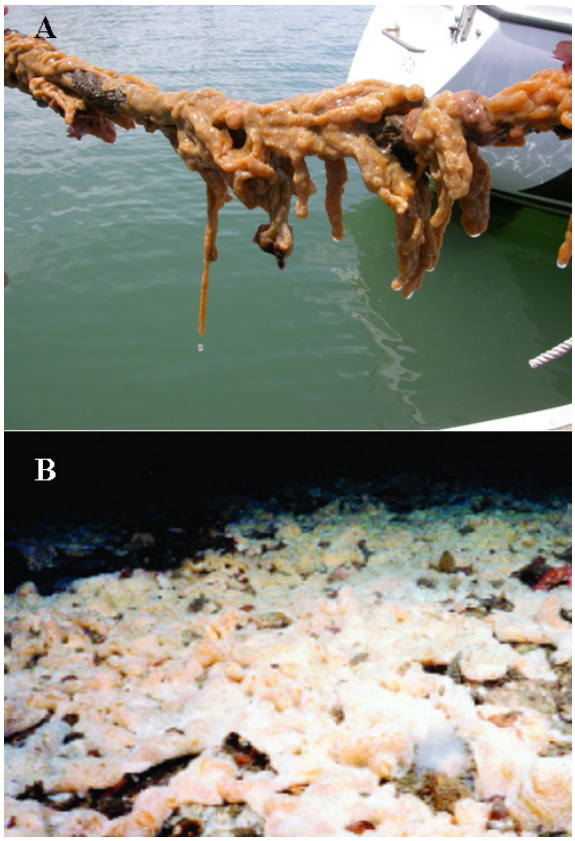

Figure 1. A- *Didemnum vexillum* overgrowing a rope in a marina at Sausalito, CA, USA. B - *Didemnum vexillum* overgrowing cobble substrate at Latimer Ledge, CT, USA

Due to the difficulty involved with identification of didemnids in general and the confusion surrounding the identity of *Didemnum* sp. A in particular, our objective was to develop and apply molecular markers for use as an additional character in order to identify the species *Didemnum* sp. A. In this study, we used a nuclear-encoded gene (*tho2*) and a mitochondrial-encoded gene (*coI*) as independent characters to test the morphologically-derived hypothesis of a single, broadly distributed species and determine what, if any, described species is genetically consistent with *Didemnum* sp. A.

Methods

Sample collection and DNA isolation

Colonies of *Didemnum* sp. A were sampled from around the world (Annex 1), including specimens of *D. vexillum* from New Zealand and two specimens from the type location of *D. vestum* (Kott 2002, 2004). Additionally, samples of three congeneric species were collected: *Didemnum albidum* Verrill, 1871 from the Gulf of Maine; *Didemnum psammathodes* Sluiter, 1895 from Panama; and a species designated *Didemnum* sp. B from New Zealand (see Annex 1). Colonies were collected subtidally from both natural and artificial substrates either via SCUBA or by pulling up ropes and other artificial structures hanging off of docks. Samples from Georges Bank were collected with dredges. Samples were preserved either directly in 95% ethanol or first in 70% isopropanol and then transferred to 95% ethanol. Fifty to one hundred thoraces were dissected from each colony and stored in 95% ethanol at -20°C until DNA isolation. Only the thoraces were used for three reasons: 1) to avoid contamination from including gut contents, 2) to remove most of the tunic which is primarily an acellular matrix of cellulose, and 3) to avoid larvae which could cause confusion with nuclear genes. Because *Didemnum* sp. A is capable of fusion (pers. obs.), thoraces were taken from a small area of each colony (<1 cm^2) to avoid sampling two genomes from a single colony. Forty-eight of the seventy-one invasive didemnid samples analyzed in this study were identified as *Didemnum* sp. A by G. Lambert before any molecular work was undertaken; the remainder of the samples were identified by L. Stefaniak. Voucher specimens for all samples sequenced have been deposited with the National Museum of Natural History Naturalis, Leiden, The Netherlands.

Ethanol supernatant was removed carefully from the tunicate thorax samples using a pipette, and the remaining ethanol was evaporated by heating at 55°C for 5-10 minutes. Thoraces were then resuspended in 200µl DNA extraction buffer (0.1 M EDTA, 1% sodium dodecyl sulfate, and 40 ng proteinase K [Invitrogen, Carlsbad, CA]) and incubated at 55°C for 72 hours. Total DNA was extracted using a CTAB method, following Zhang and Lin (2005), with slight modifications. DNA was isolated by adding 33 µl each of 5 M NaCl and 10% cetyltrimethylammonium bromide (CTAB, Sigma, St. Louis, MO) in 0.7 M NaCl and incubating at 55°C for 10 minutes, followed by one chloroform extraction. The DNA was purified from the aqueous layer using DNA Clean and Concentrator-25 columns (Zymo Research, Orange, CA), eluting twice each with 30 µl 10mM Tris, pH 8.0. DNA quality and quantity were measured using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

RNA isolation, cDNA library construction, and gene screening

Portions of live *Didemnum* sp. A colonies (<1 cm^2) were obtained from Avery Point, CT, USA and washed three times with 300 ml of 0.22-µm–filtered, autoclaved sea water and dried in a clean paper towel. The entire sample, zooids and tunic, was cut into small pieces and immersed into 1 ml of Trizol solution (Invitrogen, Carlsbad, CA), and total RNA was isolated according to Zhang et al. (2006). The cDNA library was constructed following the manufacturer's instructions of the SMART cDNA Library Construction Kit (Clontech, Mountain View, CA), with some modification. The Polymerase Chain Reaction (PCR)-amplified cDNA library was purified using a DNA Clean and Concentrator column (Zymo Research, Orange, CA), directly ligated into a T-vector, and transformed into bacterial competent cells as in Zhang and Lin (2002). Forty-eight of the resultant colonies were randomly picked up and their cDNA plasmids were isolated. Eight clones with insert DNA size of >500 bp were sequenced according to Zhang and Lin (2002). Sequences obtained were used to query GenBank databases, using BLAST to identify genes.

Primer design for coI *and* tho2

Previous attempts to amplify *coI* from *Didemnum* sp. A using the universal primers (Folmer et al. 1994) were unsuccessful (B. Swalla and J. Collie, pers. comm.). In this study, we designed a set of tunicate-specific primers. Tunicate sequences were obtained from GenBank and aligned using the ClustalW Multiple alignment function in BioEdit. Primer sites were selected by hand and tested for self-dimmers, pair-dimmers, and primer hairpins in the program PrimerSelect 4.03 (DNASTAR, Madison, WI). Specificity of the primers for tunicates was determined by BLAST analysis of

the primers against GenBank to make sure that they did not match the *coI* sequences of other taxa. One pair of primers was selected for further analysis (Table 1).

Table 1. Primer sequences for *coI* and *tho2* designed and used in this study

Gene	Primer Name	Primer Sequence (5' → 3')
coI	Tun_forward	TCGACTAATCATAAAGATATTA
	Tun_reverse2	AACTTGTATTTAAATTACGATC
tho2	DidTho2 F3	TGCCAAGTTCATCCACATTCTG
	DidTho2 R3	TTGCTTTGCTGCTGCCATC

A *tho2* gene homolog was obtained by cDNA library screening Several sets of primers were designed using Beacon Designer 3.0 (PREMIER Biosoft, Palo Alto, CA) (Table 1) and tested for specificity to *Didemnum* against genomic DNA from *Didemnum* sp. A and some phytoplankton. The primer set DidTho2 F3-R3 proved to amplify a 1-kb PCR fragment specifically yet efficiently from *Didemnum* genomic DNA and was selected for further analysis.

PCR and Sequencing

PCR amplifications were carried out in 25-µl reactions containing 0.2 mM each dNTP, 0.2 µM each primer, 1x buffer, 2.0 mM $MgCl_2$, 0.5 units of ExTaq polymerase (Takara Bio USA, Madison, WI), and 23-185 ng total DNA. Cycling conditions for *coI* consisted of an initial denaturing step of one minute at 94°C, followed by 60 cycles of 10-second denaturing at 94°C, 30-second annealing at 50°C, and 50-second elongation at 72°C, with a final elongation of 10 minutes at 72°C. To amplify *tho2*, the initial denaturing step was followed by 35 cycles of 20-second denaturing at 94°C, 30-second annealing at 56°C, and 1-minute elongation at 72°C. PCR products for *coI* were purified using DNA Clean and Concetrator-5 columns (Zymo Research, Orange, CA) with a single 8-µl elution by 10 mM Tris pH 8.0 and were then directly sequenced in both directions using the PCR primers with Big Dye v 3.1 Terminator chemistry and analyzed on an ABI 3130 automated sequencer (Applied Biosystems, Foster City, CA) at the University of Connecticut COMMA sequencing facility, Groton, CT. Because *tho2* is a nuclear gene with potentially multiple alleles in a single sample, PCR products for the *tho2* amplifications were cloned into a T-vector and transformed into competent cells -1 blue (Stratagene, La Jolla, CA). Eight of the resultant colonies were randomly selected and their plasmids were isolated (Zhang and Lin 2002). Insert length was analyzed by digesting plasmids with *Eco*RI and *Hind*III and running electrophoresis on a 1% agarose gel. Four plasmids from each PCR product were sequenced using the T3 universal primer with Big Dye v3.1 Terminator chemistry on an ABI 3730 automated sequencer at the DNA Analysis Facility on Science Hill at Yale University, New Haven, CT. Because *tho2* PCR amplifications for *D. psammathodes*, *D. albidum*, and *Didemnum* sp. B produced a number of different sized products, up to 24 clones were screened and sequenced for each species. Clones of *tho2* were identified via BLAST against GenBank. Base calls for all sequences were checked by hand and sequences deposited in GenBank (*coI* Accession numbers: EU419401-EU419459, EU742661-EU742677; *tho2* accession numbers: EU419460-EU419569). Single nucleotide polymorphisms found in only one of all *tho2* clones sequenced (>200 clones) were considered to be artifacts derived from PCR error.

Data analysis

Sequences for *coI* and *tho2* were aligned separately using ClustalX. Both analyses included sequences from *Didemnum* sp. A, *Didemnum* sp. B, and *D. psammathodes*. Sequences from *D. albidum* were also included in analyses of *coI*. For use as an out group in constructing phylogenetic trees, *coI* sequence for *Diplosoma spongiforme* was obtained from GenBank (Accession number AY600972). Both introns and exons of *tho2* aligned among *Didemnum* sp. A samples. However, while the locations of the introns in the amplified fragment of *tho2* were conserved among all *Didemnum* species in this study, the sequences and the lengths of the introns varied dramatically among species, making alignment difficult. Therefore, only the exon regions (227 bp) were used in this analysis. Maximum likelihood trees were generated with PhyML (Guindon and Gascuel 2003) using 1000 bootstrap replicates. Two nucleotide substitution models were used based on the results of modeltest (Posada and Crandall 1998): HKY85 (Hasegawa et al. 1985) and GTR (e.g. Lanave et al. 1984). For both models, the proportion of invariable sites was estimated, and for the HKY85 model, the transition:transversion ratio was also estimated.

Sequences of *coI* for *Didemnum* sp. A were collapsed in haplotypes using DNASP v4.0

(Rozas et al. 2003). Haplotypes from *Didemnum* sp. A were subsequently compared to *coI* sequences from *D. albidum, D. psammathodes*, and *Didemnum* sp. B to determine the percentage of variable sites in *co1* between *Didemnum* sp. A and other species in the genus.

Results

coI *sequences and phylogenetic trees*

Seventy-one *coI* sequences were generated from *Didemnum* sp. A colonies, one from *D. psammathodes*, and two each from *D. albidum* and *Didemnum* sp. B colonies. The 71 *Didemnum* sp. A samples analyzed in this study produced 18 unique *coI* haplotypes. Forty-six variable sites were found in the 586 bp *coI* fragment from the 18 *Didemnum* sp. A haplotypes (Figure 2). Haplotypes of *coI* from *Didemnum* sp. A averaged 28.7% variable sites in the 586 bp fragment compared to *D. albidum*, 14.7% variable sites compared to *D. psammathodes*, and 19.6% variable sites compared to *Didemnum* sp. B (Table 2).

Similar tree topologies resulted from the two substitution models. Branches with bootstrap support over 50% were the same between models, though the location of branches within a clade differed somewhat. Therefore, only the GTR model trees are presented here.

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                                  1111122222222223333334444444455555555555
Position                        1224782388913344666771668881233357900356778888
                               4028745702434584737814501135710131235866215014

Haplotype 1    (2)  ATAAATTCGGTCTTTGTTTCTTGTATGCTAGGGATTTTCTCAAATT
Haplotype 2    (4)  ........................................T....
Haplotype 3    (1)  ......A.....C...C........GAT.T..T........T....
Haplotype 4    (2)  ......A.....C...C..........T.T..T........T....
Haplotype 5    (4)  ...G..A.....C...C..........T.T..T......C.T....
Haplotype 6    (1)  ...G..A.....C...C..........T.T..T........T....
Haplotype 7    (4)  ......A.....C...C..........T.T..T......C.T....
Haplotype 8    (1)  C.G...A.....C...C..........T.T..TG.......T....
Haplotype 9    (1)  ......A.....C...C..........T.T..TG.......T....
Haplotype 10   (3)  .CG...A.....C...C..........T.T..A..............
Haplotype 11   (37) .CG...A.....C...C..........T.T..A........T....
Haplotype 12   (1)  .CG...A.....C...C......AT..T.T..A........T....
Haplotype 13   (1)  .CG..AA.....C...C..........T.T..A..A.....T.TA.
Haplotype 14   (1)  .CG...A.....C...C.C........T.T..A........T...A
Haplotype 15   (1)  .CG...A.....C...C...C......T.T..A........T....
Haplotype 16   (1)  .CG...A.....C...C.....A....TAT..A........TT...
Haplotype 17   (5)  ..G.G.ATAAAT.CCA.C.T.A.....T.TAAA.C.CCT.TT....
Haplotype 18   (1)  ..G...ATAAAT.CCA...T.A.....T.TAAA.C.CCT.TT....
```

Figure 2. Aligned variable sites of *Didemnum vexillum coI* haplotypes. Parentheses indicate the number of samples with each haplotype. Dots represent identity with first haplotype sequence

Table 2. Percentage of variable sites among *Didemnum* spp. for the 586 bp fragment of *coI* amplified in this study

	Didemnum sp. A	*Didemnum albidum* Georges Bank	*Didemnum albidum* Eastport, ME	*Didemnum psammathodes* Panama
Didemnum sp. A	---			
Didemnum albidum Georges Bank	29.4	---		
Didemnum albidum Eastport, ME	28.0	16.6	---	
Didemnum psammathodes Panama	14.7	26.6	25.4	---
Didemnum sp. B New Zealand	19.6	26.3	26.8	17.4

Similar tree topologies resulted from the two substitution models. Branches with bootstrap support over 50% were the same between models, though the location of branches within a clade differed somewhat. Therefore, only the GTR model trees are presented here.

All *Didemnum* spp. and *D. psammathodes* sequences formed a monophyletic clade to the exclusion of *D. albidum* sequences. The *D. albidum* clade branched off earlier than the *Didemnum* spp./*D. psammathodes* clade and had strong support (100%). The *Didemnum* sp. A clade grouped with *Didemnum* sp. B clade and the *D. psammathodes* sample with strong bootstrap support (97.4%); within the *Didemnum* sp. B cluster, the two samples were identical. Within the *Didemnum* sp. A clade, all 71 *Didemnum* sp. A sequences, including those identified as *D. vexillum*, grouped together in a single clade with 91.8% bootstrap support (Figure 3). The *Didemnum* sp. A clade was further subdivided into three subgroups, one with 93.6% support, containing sequences only from the Misaki Marine Laboratory in Japan; the second with 99.4% support, containing sequences from Sausalito, CA; Puget Sound, WA; Le Havre, France; and Asamushi, Japan; and the third with 62.7% bootstrap support, containing sequences from all sampling locations except the Misaki Marine Laboratory in Japan. The first subgroup containing samples from only a single sampling location is more divergent from the other *Didemnum* sp. A samples, however, the second subgroup does not reveal any exclusive geographic pattern at the present time, as samples from all four locations were also found in the third subgroup.

tho2 *sequences and phylogenetic trees*

Each colony of *Didemnum* sp. A produced one, two, or three alleles from the exons of *tho2*. Three alleles from a single colony are probably the result of accidentally including some abdomen tissue, fertilized eggs, or larvae in the DNA extraction. While there were four sequences from each sample, only one copy of each unique allele per sample was included in the analysis, resulting in 108 alleles of *tho2* from 57 colonies of *Didemnum* sp. A. Additionally, one *D. psammathodes* and one *Didemnum* sp. B allele were amplified and sequenced. No sequences of *tho2* were found from the total of 48 *D. albidum* clones screened.

All of the *tho2* sequences from samples identified as *Didemnum* sp. A grouped together in a single clade with 68% bootstrap support (Figure 4). Within the *Didemnum* sp. A clade, sequences from different geographic locations, including those identified as *D. vexillum*, formed several small (2 - 10 sequences) subgroups; however, these came with short branches and no bootstrap support, in general. In contrast, the clade was well-separated from *Didemnum* sp. B and *D. psammathodes*.

Discussion

The strong grouping of *Didemnum* sp. A and *D. vexillum* for both *coI* and *tho2* suggests that they are most likely the same species and that they are distinct from the other superficially similar and sympatric species included in the analysis. Two samples from the type locality of *D. vestum* (collected the same year as the *D. vestum* holotype), which were morphologically identified as conspecific to *Didemnum* sp. A, also fall into the same clade as *Didemnum* sp. A and *D. vexillum* for both genes. This suggests that all three species are, in fact, a single species, particularly given that the published descriptions of *D. vexillum* and *D. vestum* overlap to a large extent and that *D. vestum* was described from poorly preserved colonies that contained no larvae (Kott 2002, 2004). Morphological evidence based on examination of numerous samples including zooids and larvae also supports this grouping (Lambert 2009, this issue). Morphological and molecular data do not justify the specific separation between *Didemnum* sp. A and *D. vexillum* Kott 2002. Therefore, *Didemnum* sp. A, to date, must be designated as *D. vexillum* Kott 2002 (Lambert 2009, this issue).

Additionally, a comparison of 464 congeneric pairs of marine invertebrates produced an average interspecific divergence in *coI* of 15.9±0.2% (Nydam and Harrison 2007). This estimate may be low because of the inclusion of cnidarians, which generally have a lower interspecific divergence than other animals (Hebert et al. 2003); however, the amount of congeneric interspecific divergence between *D. vexillum* and the other species analyzed in this study (with the exception of *D. psammathodes*) is well above the average (Table 2) and *D. psammathodes* is highly distinct in both morphology and ecology to *D. vexillum* (Monniot 1983). Because of the minimal sampling in this study of non–*D. vexillum* species and *D. vexillum* from each of the study localities, the comparison of sequence variation within *D. vexillum* to that between *D. vexillum* and other *Didemnum* species alone may not be adequate to confirm that *D. vexillum* is

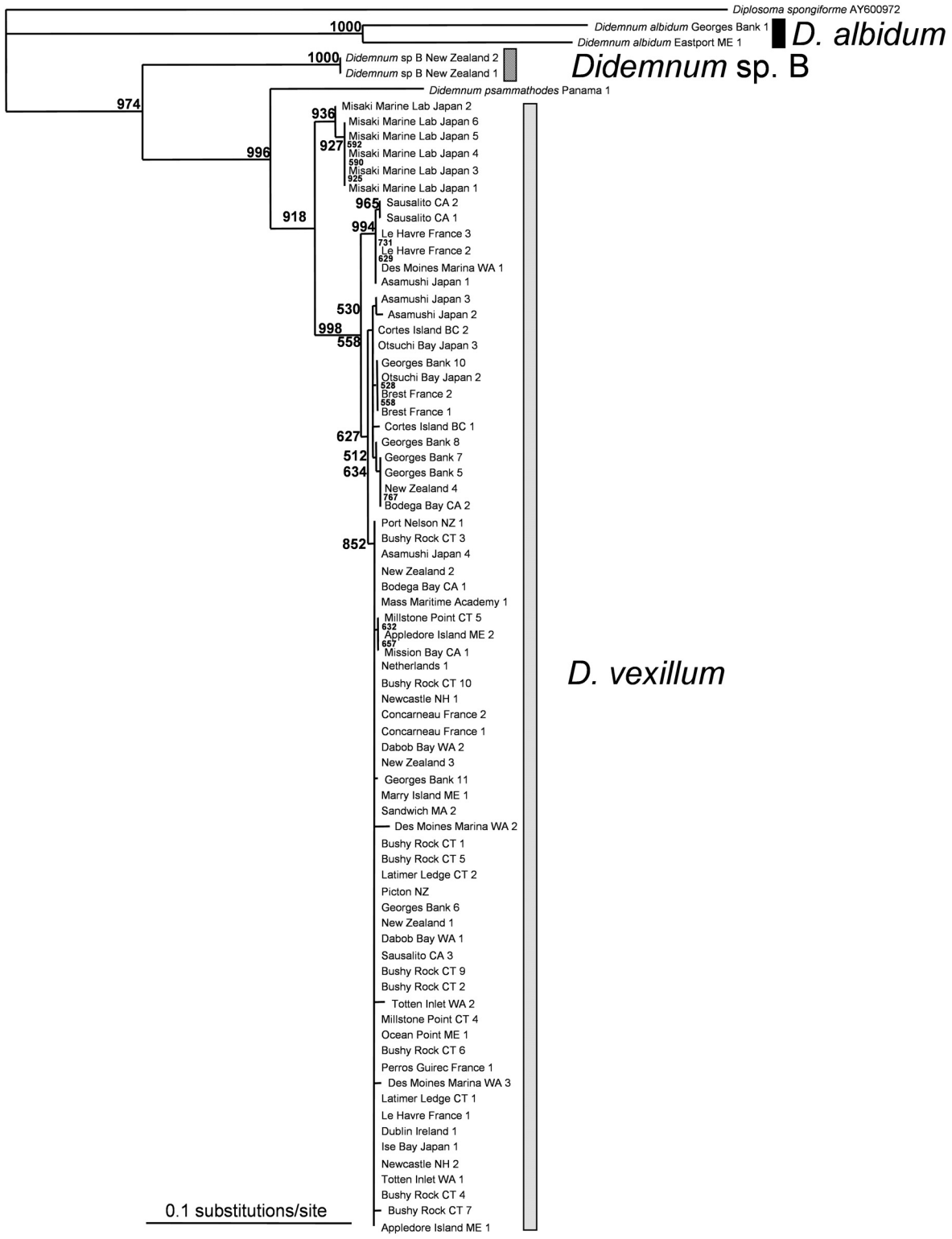

Figure 3. Maximum likelihood tree of *Didemnum* spp. *coI* (586bp). One thousand bootstrap replicates were run using the GTR nucleotide substitution model. All nodes with greater than 50 percent bootstrap support are labeled. For *Didemnum vexillum* samples, sequences are labeled as 'Sampling location sample number.' For all other species, species name is also included

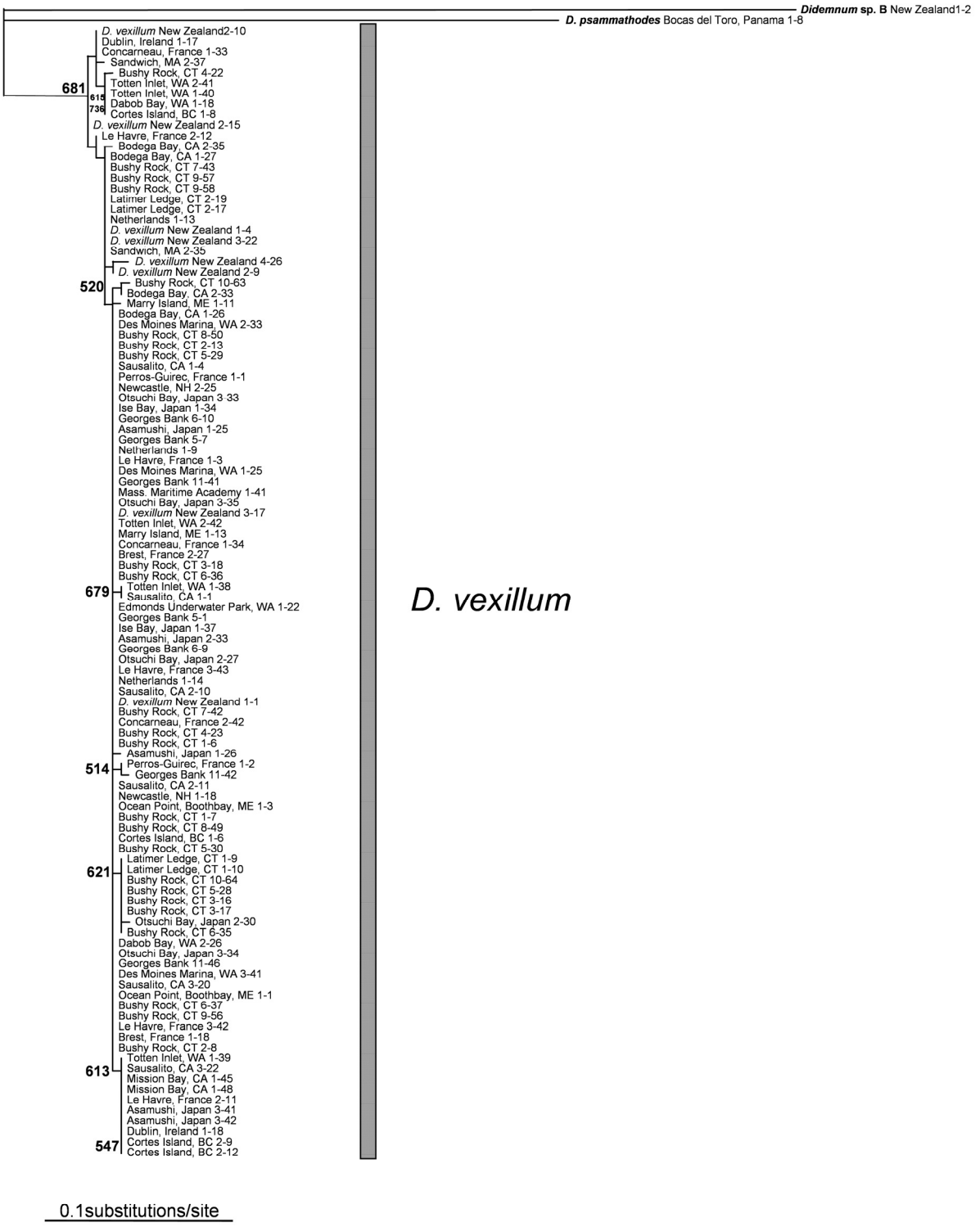

Figure 4. Maximum likelihood tree of *Didemnum* spp. *tho2* exons (227bp). One thousand bootstrap replicates were run using the GTR nucleotide substitution model. All nodes with greater than 50 percent bootstrap support are labeled. For *Didemnum vexillum* samples, sequences are labeled as 'Sampling location, sample number – clone number.' For all other species, species name is also included

a single and distinct species. However, the phylogenetic evidence here presented and morphological evidence (Lambert 2009, this issue) strongly supports *D. vexillum* as a single species. The recent discovery (<20 years ago) of *D. vexillum* in many parts of the world and the highly identical gene sequences suggests that this species has become globally distributed recently, probably as a result of a human-mediated invasion (Lambert 2009, this issue).

The initial populations of an invasion can undergo a phenomenon known as a founder effect: a genetic bottleneck caused by only a small number of individuals from the native range taking part in the invasion (Sakai et al. 2001; Wares et al. 2002; Lindholm et al. 2005). Thus, invasive populations should have relatively little genetic diversity compared to the source population (Garnatje et al. 2002, Dawson et al. 2005; but see Roman 2006 for a recent example of the effects of multiple invasions on signals of founder effects). Intraspecific variation in regions of *coI* congruent to the region amplified in *D. vexillum* in this study ranges from approximately 4-26% in other species of ascidians (Table 3). At 7.8% intraspecific variation, *D. vexillum* falls within the lower limit of this range. However, in the study that produced the lowest intraspecific variation (3.8%), samples of *Pseudodistoma crucigaster* were taken from a relatively small geographic distribution (<100 km on the Mediterranean coast of Spain and two offshore islands, Tarjuelo et al. 2004). In studies with larger range sampling, intraspecific variation is approximately 26% (Lopez-Legentil and Turon 2005; Lopez-Legentil et al 2006). A comparison of the intraspecific variation in *coI* of 45 species of marine invertebrates produced an average intraspecific variation of 10.9±1% (Nydam and Harrison 2007), which, again, is probably low because of the inclusion of cnidarians (Hebert et al. 2003). Finding low intraspecific variation in groups that tend to have a large amount of intra-specific variation could indicate that some of the populations are invasive. For example, Turon et al. (2003) found only 8.7% variation in *Clavelina lepadiformis* (Ascidiacea, Aplousobranchia) populations on both the Atlantic and Mediterranean coasts of Spain but determined that several harbor populations on the Mediterranean side were, in fact, introduced from the Atlantic. For a sessile organism such as *D. vexillum*, with short-lived, non-feeding larvae that could limit gene flow, to have a low genetic variation (based on *coI*) on a global scale, the most likely explanation is recent invasion in multiple regions, an explanation that is supported by the timeline of discovery of *D. vexillum* populations world wide (Lambert 2009, this issue).

Table 3. Intraspecific variation of *coI* in various colonial ascidian species

Species	Sampling Distribution	% variable sites	Source
Botryllus schlosseri	Mediterranean and Atlantic coasts of Spain and France	25.8	Lopez-Legentil et al. 2006
Clavelina lepadiformis	Mediterranean and Atlantic coasts of Spain	8.67	Turon et al. 2003
Cystodytes dellechaijei	Western Mediterranean	26	Lopez-legentil and Turon 2005
Didemnum vexillum	New Zealand, Japan, North America, and Northern Europe	7.8	This study
Pseudodistoma crucigaster	Meditteranean coast of Spain	3.8	Tarjuelo et al. 2004
Pycnoclavella communis	Meditteranean coast of Spain	16.8	Pérez-Portela and Turon 2008

It must be noted that the amount of interspecific variation between *D. vexillum* and *D. albidum* (Table 2) is only marginally greater than the intraspecific variation found in the colonial ascidian *Botyrllus schlosseri* (Lopez-Legentil et al. 2006) and the interspecific variation between *D. vexillum* and *Didemnum* sp. B and between *D. vexillum* and *D. psammathodes* are much less. This molecular evidence alone could suggest that all four *Didemnum* spp. are conspecific. However, morphological evidence shows them to be highly distinct. Additionally,

the amount of congeneric interspecific divergence can vary greatly within groups (Hebert et al. 2003). *Botryllus schlosseri* belongs to a different suborder than *Didemnum* and can be expected to have a somewhat different rate of mutation. Given that the interspecific variation in the genus *Didemnum* produced here is well above the average produced by Nydam and Harrison (2007), it is safe to conclude that the three superficially similar species are distinct; the interspecific variation between *D. vexillum* and *D. psammathodes* is less than the average produced by Nydam and Harrison (2007), however, the large morphological and ecological differences between these two species suggest that they are separate species.

Finally, the large (16.6%) intraspecific variation found in *D. albidum* compared to *Didemnum* sp. B and *D. vexillum* can be explained in two ways. The difference between *D. albidum* (16.6%) and *Didemnum* sp. B (0%) may be due to a sampling artifact. Both samples of *Didemnum* sp. B were collected at the same site, increasing the possibility that the two samples are closely related, while the two *D. albidum* samples were collected at sites over 100 kilometers apart. The distance between the *D. albidum* sites is relatively large compared to larval lifespans in colonial ascidians (e.g. Jackson 1986, Svane and Young 1989). As a result it is possible that considerable genetic differentiation has built up between these two populations of the native *D. albidum*. As a recently introduced species, *D. vexillum* is less likely to have accumulated as many mutations among populations. Also, while many colonies of *D. vexillum* were sampled globally, relatively few samples were collected within each region; it is possible that not all of the available genetic diversity in *D. vexillum* has been uncovered (e.g., Muirhead et al. 2008).

Identification of native or source ranges of species has important ecological implications. Understanding the ecology of an invasive species in its native range can help predict interactions with native biota and the environment in introduced ranges and determine effective modes of biological control (Wares et al. 2005). Information on the native range of an invasive species or even the definitive identification of a species as invasive can also influence management decisions. Incomplete characterization of the fauna of a region and/or records of baseline biotas before and during historical invasions can lead to species being erroneously defined as either native or invasive (Carlton 1996, Kott 2004). Improper designation of an invasive species as native or cryptogenic can delay or reduce nationally-coordinated efforts to eradicate or manage species that then become costly pests (e.g., *D. vexillum* in New Zealand, Coutts and Forrest 2007).

The globally low *coI* intraspecific variation found in the present study could suggest that the native range of *D. vexillum* has yet to be sampled. However, our sampling scheme was aimed at comprehensive global coverage with less than twenty samples per region. Except in areas that underwent a severe bottleneck event during the invasion process, it is unlikely that we have sampled the full range of genetic diversity in any given region. The earliest records of a didemnid tunicate exhibiting a combination of the lobe/tendril-forming growth form and other morphological characters peculiar to *D. vexillum* are from 1926 in Japan (Nishikawa 1990). That sample was unfortunately only identified to genus at the time and later identified as *Didemnum pardum* by Nishikawa (1990), but it is now considered to be conspecific to *D. vexillum* (Nishikawa, pers. comm., Lambert 2009, this issue). The greatest regional number of *coI* haplotypes was found in Japan (eight haplotypes from thirteen samples); however, many more samples will need to be analyzed from the entire current range of *D. vexillum* before the question of where is the native range of *D. vexillum* can be adequately addressed. Highly variable molecular markers such as microsatellites or introns may also be useful in that attempt.

Acknowledgements

The authors would like to acknowledge the invaluable assistance of the following people who collected the samples used in this study: G. Breton, J. Byrnes, M. Carman, C. Chiarizia, B. Coffey, A. Cohen, J. Collie, A. Coutts, M. Creuer, J. Culbertson, J. Dijkstra, K. Frick, B. Forrest, R. Gladych, J. Godfrey, L. Harris, S. Johnson, G. King, N. Lengyl, M. Lamare, M. Lilly, J. Mercer, G. Miller-Messner, D. Minchin, T. Nishikawa, T. Otake, J. Reinhardt, J. Rendall, K. Ritsu, J. Stachowicz, J. Swenarton, S. Takeda, and P. Valentine. We would also like to thank L. Miranda and Y. Hou for their help in the laboratory, B. Reijnen for his help designing the specific *coI* primers, and two anonymous

reviewers for their comments on an earlier draft of this paper. This project was funded by grants to L. Stefaniak from the University of Connecticut, Department of Marine Sciences and the Quebec Labrador Foundation/Sounds Conservancy and by development funds awarded to R. Whitlatch and L. Stefaniak from Connecticut Sea Grant. Additional funding was provided by the National Undersea Research Center.

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Annex 1

Summary of sampling locations, approximate geographic coordinates, sampling date, museum reference numbers, and sequence accession numbers. Haplotype number is also listed for *Didemnum* sp. A and *D. vexillum* samples.

(a) *Didemnum* sp. A

Sampling Location (geographic coordinates	Sampling Date	Museum number	*coI*	*tho2*	*co1* haplo-type
Floats, Le Havre, France 1 (49°29.3'N, 0°5.6'E)	26-Aug-05	RMNH.UROCH.689	EU419409	EU419528	11
Floats, Le Havre, France 2 (49°29.3'N, 0°5.6'E)	26-Aug-05	RMNH.UROCH.690	EU419426	EU419529, EU419530	2
Subtidal, Le Havre, France 3 (49°29.3'N, 0°5.6'E)	17-Dec-03	RMNH.UROCH.691	EU419427	EU419533, EU419534	2
Floats, Brest, France 1 (48°23.4'N, 4°25.5'W)	28-Aug-05	RMNH.UROCH.692	EU419423	EU419466	7
Floats, Brest, France 2 (48°23.4'N, 4°25.5'W)	28-Aug-05	RMNH.UROCH.693	EU419424	EU419467	7
Floats, Concarneau, France 1 (47°52.2'N, 3°54.9'W)	2-Sep-05	RMNH.UROCH.694	EU419403	EU419492, EU419493	11
Floats, Concarneau, France 2 (47°52.2'N, 3°54.9'W)	2-Sep-05	RMNH.UROCH.695	EU419425	EU419494	11
Floats, Perros-Guirec, France 1 (48°48.3'N, 3°26.5'W)	22-Jul-02	RMNH.UROCH.696	EU419430	EU419555, EU419556	11
The Netherlands 1 (51°44.4'N, 3°49.9'E)	Jul-06	RMNH.UROCH.697	EU419415	EU419542, EU419543, EU419544	11
Malahide Harbor floats, Dublin, Ireland 1 (53°26'N, 6°9'W)	12-Oct-05	RMNH.UROCH.698	EU419416	EU419499, EU419500	11
Georges Bank, USA 5 (41°55.4'N, 67°17.6'W)	29&30-Aug-05	RMNH.UROCH.699	EU419450	EU419507, EU419508	5
Georges Bank, USA 6 (41°59.3'N, 67°20.9'W)	29&30-Aug-05	RMNH.UROCH.700	EU419417	EU419509, EU419510	11
Georges Bank, USA 7 (41°57.7'N, 67°25.6'W)	30-Aug-05	RMNH.UROCH.752	EU742664		5
Georges Bank, USA 8 (41°57.2'N, 67°31.9'W)	24-Aug-05	RMNH.UROCH.753	EU742665		6
Georges Bank, USA 10 (41°55.5'N, 67°31.0'W)	10-Nov-04	RMNH.UROCH.755	EU742666		7
Georges Bank, Area 18 USA 11 (41°57.2'N, 67°30.9'W)	23-Aug-06	RMNH.UROCH.701	EU419433	EU419511, EU419512, EU419513	15
Bushy Rock, Groton, CT, USA 1 (41°18.9'N, 72°03.2'W)	2-Nov-06	RMNH.UROCH.702	EU419442	EU419468, EU419469	11
Bushy Rock, Groton, CT, USA 2 (41°18.9'N, 72°03.2'W)	2-Nov-06	RMNH.UROCH.703	EU419443	EU419470, EU419471	11
Bushy Rock, Groton, CT, USA 3 (41°18.9'N, 72°03.2'W)	2-Nov-06	RMNH.UROCH.704	EU419444	EU419472, EU419473, EU419474	11
Bushy Rock, Groton, CT, USA 4 (41°18.9'N, 72°03.2'W)	2-Nov-06	RMNH.UROCH.705	EU419445	EU419475, EU419476	11
Bushy Rock, Groton, CT, USA 5 (41°18.9'N, 72°03.2'W)	2-Nov-06	RMNH.UROCH.706	EU419446	EU419477, EU419478, EU419479	11
Bushy Rock, Groton, CT, USA 6 (41°18.9'N, 72°03.2'W)	2-Nov-06	RMNH.UROCH.707	EU419447	EU419480, EU419481, EU419482	11

Annex 1 (continued)

Sampling Location (geographic coordinates	Sampling Date	Museum number	*coI*	*tho2*	*co1* haplo-type
Bushy Rock, Groton, CT, USA 7 (41°18.9'N, 72°03.2'W)	2-Nov-06	RMNH.UROCH.708	EU419448	EU419483, EU419484	12
Bushy Rock, Groton, CT, USA 8 (41°18.9'N, 72°03.2'W)	2-Nov-06	RMNH.UROCH.709		EU419485, EU419486	
Bushy Rock, Groton, CT, USA 9 (41°18.9'N, 72°03.2'W)	2-Nov-06	RMNH.UROCH.710	EU419449	EU419487, EU419488, EU419489	11
Bushy Rock, Groton, CT, USA 10 (41°18.9'N, 72°03.2'W)	2-Nov-06	RMNH.UROCH.711	EU419453	EU419490, EU419491	11
Millstone Powerplant quarry, Niantic, CT, USA 4 (41°18.4'N, 72°09.9'W)	Mar-07	RMNH.UROCH.767	EU742674		11
Millstone Powerplant quarry, Niantic, CT, USA 5 (41°18.4'N, 72°09.9'W)	Mar-07	RMNH.UROCH.768	EU742675		10
Latimer Ledge, CT, USA 1 (41°18.2'N, 71°54.6'W)	6-Jul-07	RMNH.UROCH.712	EU419454	EU419526, EU419527	11
Latimer Ledge, CT, USA 2 (41°18.2'N, 71°54.6'W)	6-Jul-07	RMNH.UROCH.713	EU419455	EU419531, EU419532	11
Sandwich Tide Pool, Sandwich, MA, USA 2 (41°46.4'N, 70°29.4'W)	13-May-05	RMNH.UROCH.714	EU419410	EU419568, EU419569	11
Mass Maritime Academy floats, MA, USA 1 (41°44.9'N, 70°37.6'W)	5-Aug-03	RMNH.UROCH.717	EU419452	EU419539	11
Ocean Pt., Boothbay, ME, USA 1 (43°48.9'N, 69°35.8'W)	Aug-03	RMNH.UROCH.718	EU419418	EU419553, EU419554	11
Marry Island, Damariscotta River Estuary, ME, USA 1 (43°57.5'N, 69°34.6'W)	Aug-03	RMNH.UROCH.719	EU419451	EU419537, EU419538	11
Appledore Island, ME 1 (42°59.4'N, 70°37.1'W)	7-Sept-07	RMNH.UROCH.763	EU742662		11
Appledore Island, ME 2 (42°59.4'N, 70°37.1'W)	7-Sept-07	RMNH.UROCH.764	EU742663		10
Des Moines Marina, WA, USA 1 (47°23.8'N, 122°19.8'W)	20-Nov-04	RMNH.UROCH.720	EU419436	EU419504	2
Des Moines Marina floats, WA, USA 2 (47°23.8'N, 122°19.8'W)	9-May-06	RMNH.UROCH.721	EU419437	EU419503	13
Des Moines Marina floats, WA, USA 3 (47°23.8'N, 122°19.8'W)	9-May-06	RMNH.UROCH.722	EU419438	EU419505	14
Taylor Shellfish lines, Dabob Bay, WA, USA 1 (47°49.1'N, 122°49.5'W)	18-May-06	RMNH.UROCH.723	EU419419	EU419502	11
Taylor Shellfish lines, Dabob Bay, WA, USA 2 (47°49.1'N, 122°49.5'W)	18-May-06	RMNH.UROCH.724	EU419420	EU419501	11
Taylor Shellfish lines, Totten Inlet, WA, USA 1 (47°08.4'N, 122°58.8'W)	25-Oct-05	RMNH.UROCH.725	EU419439	EU419563, EU419564, EU419565	11
Taylor Shellfish lines, Totten Inlet, WA, USA 2 (47°08.4'N, 122°58.8'W)	30-Nov-04	RMNH.UROCH.726	EU419440	EU419566, EU419567	16
Edmonds Underwater Park, WA, USA 1 (47°48.9'N, 122°23.1'W)	26-Sep-04	RMNH.UROCH.727		EU419506	
Cass Marina floats, Sausalito, CA, USA 1 (37°51.8'N, 122°29.1'W)	28-May-03	RMNH.UROCH.728	EU419412	EU419557, EU419558	1

Annex 1 (continued)

Sampling Location (geographic coordinates	Sampling Date	Museum number	*coI*	*tho2*	*co1* haplo-type
Cass Marina floats, Sausalito, CA, USA 2 (37°51.8'N, 122°29.1'W)	28-May-03	RMNH.UROCH.729	EU419413	EU419559, EU419560	1
Cass Marina floats, Sausalito, CA, USA 3 (37°51.8'N, 122°29.1'W)	28-May-03	RMNH.UROCH.730	EU419431	EU419561, EU419562	11
Spuds Marina floats, Bodega Bay, CA, USA 1 (38°19.8'N, 123°03.5'W)	29-May-03	RMNH.UROCH.731	EU419414	EU419462, EU419463	11
Spuds Marina floats, Bodega Bay, CA, USA 2 (38°19.8'N, 123°03.5'W)	30-May-03	RMNH.UROCH.732	EU419422	EU419464, EU419465	5
Oyster farm, Cortes Island, BC, Canada 1 (49°12'N, 125°52'W)	2-Nov-05	RMNH.UROCH.733	EU419406	EU419495, EU419496	3
Oyster farm, Cortes Island, BC, Canada 2 (49°12'N, 125°52'W)	4-Nov-05	RMNH.UROCH.734	EU419441	EU419497, EU419498	4
Catamaran Dock, Mission Bay, CA, USA 1 (32°47.3'N, 117°15.7'W)	9-Feb-07	RMNH.UROCH.735	EU419404	EU419535, EU419536	10
Ise Bay, Japan 1 (34°54'N, 136°36'E)	31-Jul-03	RMNH.UROCH.736	EU419401	EU419519, EU419520	11
Otsuchi Bay, Marine Lab, Japan 2 (39°21.2'N, 141°55.1'W)	Jun-07	RMNH.UROCH.737	EU419434	EU419521, EU419522	7
Otsuchi Bay, Marine Lab, Japan 3 (39°21.2'N, 141°55.1'W)	Jun-07	RMNH.UROCH.738	EU419435	EU419523, EU419524, EU419525	4
Asamushi, Japan 1 (40°53.5'N, 140°51.6'E)	9-Jul-07	RMNH.UROCH.739	EU419457	EU419514, EU419515	2
Asamushi, Japan 2 (40°53.5'N, 140°51.6'E)	9-Jul-07	RMNH.UROCH.740	EU419458	EU419516	8
Asamushi, Japan 3 (40°53.5'N, 140°51.6'E)	9-Jul-07	RMNH.UROCH.741	EU419459	EU419517, EU419518	9
Asamushi, Japan 4 (40°53.5'N, 140°51.6'E)	13-Jul-07	RMNH.UROCH.756	EU742667		11
Misaki Marine Lab, Japan 1 (35°09.5'N, 139°36.7'W)	16-Aug-07	RMNH.UROCH.757	EU742668		17
Misaki Marine Lab, Japan 2 (35°09.5'N, 139°36.7'W)	16-Aug-07	RMNH.UROCH.758	EU742669		18
Misaki Marine Lab, Japan 3 (35°09.5'N, 139°36.7'W)	16-Aug-07	RMNH.UROCH.759	EU742670		17
Misaki Marine Lab, Japan 4 (35°09.5'N, 139°36.7'W)	16-Aug-07	RMNH.UROCH.760	EU742671		17
Misaki Marine Lab, Japan 5 (35°09.5'N, 139°36.7'W)	16-Aug-07	RMNH.UROCH.761	EU742672		17
Misaki Marine Lab, Japan 6 (35°09.5'N, 139°36.7'W)	16-Aug-07	RMNH.UROCH.762	EU742673		17

Annex 1 (continued)

(b) *Didemnum* sp. A from *D. vestum* type location

Sampling Location (geographic coordinates)	Sampling Date	Museum number	*coI*	*tho2*	*co1* haplo-type
Coast Guard floats, Newcastle, NH, USA 1 (43°04.3'N, 70°42.6'W)	14-Oct-02	RMNH.UROCH.715	EU419402	EU419540	11
Coast Guard floats, Newcastle, NH, USA 2 (43°04.3'N, 70°42.6'W)	14-Oct-02	RMNH.UROCH.716	EU419411	EU419541	11

(c) *D. vexillum*

Sampling Location (geographic coordinates)	Sampling Date	Museum number	*coI*	*tho2*	*co1* haplo-type
Mussel lines, South Island, New Zealand 1 (41°16.1'S, 174°0.2'E)	31-Jan-06	RMNH.UROCH.742	EU419405	EU419545, EU419546	11
Pilings, South Island, New Zealand 2 (41°16'S, 174°00'E)	Oct-04	RMNH.UROCH.743	EU419421	EU419547, EU419548, EU419549	11
Pilings, Port Shakespeare, New Zealand 3 (41°16'S, 174°00'E)	13-Aug-02	RMNH.UROCH.744	EU419428	EU419550, EU419551	11
Steel Mariner, Port Shakespeare, New Zealand 4 (41°16.4'S, 174°00.4'E)	18-Jan-02	RMNH.UROCH.745	EU419429	EU419552	5
Port Nelson Wharf Piles, New Zealand 1 (41°15.5'S, 173°16.6'E)	13-Jul-07	RMNH.UROCH.765	EU742676		11
Picton, New Zealand A (41°17.1'S, 174°0.5'E)	Jul-07	RMNH.UROCH.766	EU742677		11

(d) *D. psammathodes*

Sampling Location (geographic coordinates)	Sampling Date	Museum number	*coI*	*tho2*	*co1* haplotype
Bocas del Toro, Panama 1 (9°21'N, 82°12'W)	11-Aug-06	RMNH.UROCH.746	EU742661	EU419461	

(e) *Didemnum* sp. B

Sampling Location (geographic coordinates)	Sampling Date	Museum number	*coI*	*tho2*	*co1* haplo-type
Subtidal, Doubtful Sound, New Zealand 1 (45°16.8'S. 169°00'E)	26-Apr-04	RMNH.UROCH.747	EU419407	EU419460	
Subtidal, Doubtful Sound, New Zealand 2 (45°16.8'S. 169°00'E)	26-Apr-04	RMNH.UROCH.748	EU419408		

(f) *D. albidum*

Sampling Location (geographic coordinates)	Sampling Date	Museum number	*coI*	*tho2*	*co1* haplotype
Georges Bank, USA 1 (42°07.1'N, 66°44.8'W)	Jul-07	RMNH.UROCH.750	EU419432		
Eastport, ME, USA 1 (44°53.7'N, 67°0.1'W)	21-Jul-07	RMNH.UROCH.751	EU419456		