

## New molecular markers to genetically differentiate populations of *Didemnum vexillum* (Kott, 2002) - an invasive ascidian species

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### Abstract

Invasive ascidians can be a menace to both ecosystems and marine aquaculture in the coastal United States. One of the very recent introductions of an ascidian species in Puget Sound, a large inland waterway in the Northwest of the United States, appears to be the same species, *Didemnum vexillum* (Kott, 2002) that has caused significant economic and environmental harm in New England, California, British Columbia, northern Europe, and New Zealand. In light of such grave threats, identifying the vectors and sources of introduced organisms in order to prevent and possibly combat invasions is crucial. Distinguishing between primary introduction (source population from native range) and secondary introduction (source population from a previously colonized site) is fundamental to identifying pathways of introduction. We have developed three molecular markers that can be used to address these “source” questions as well as other basic questions of molecular evolution in *D. vexillum*. Two of these markers (Dnr1 and DL2.1A1) are polymorphic sequence regions which we found to each have four alleles. A third marker is a microsatellite locus, D6, which we observed has ten alleles. Including all samples characterized with these three markers, the proportion of heterozygotes for each marker was 0.682, 0.721, and 0.773 for Dnr1, DL2.1A1, and D6 respectively. Using these markers, we show significant genetic differentiation between population samples from New Hampshire and Puget Sound ( $F_{ST} = 0.226$ ,  $p < 0.05$ ). These markers will thus be helpful in genetically characterizing worldwide populations of *D. vexillum* and providing a means to reconstruct pathways of introduction by determining the genetic relationships of these populations. In addition, we report the discovery of multiple genotypes at microsatellite locus D6 found in DNA samples derived from larvae within single colonies of *D. vexillum*. This marker may prove useful in gaining insight into multiple paternity in *D. vexillum*, which is a topic rarely studied in ascidians.

**Key words:** aquatic invasive species, didemnid, *Didemnum* sp. A, invasion pathway, microsatellite, SNP, tunicates

### Introduction

Ascidians are sessile marine invertebrate filter feeders in the phylum Chordata (Swalla et al. 2000). There is an abundance of ascidians in harbors around the world and many of them are non-native (Lambert 2002). One of these non-native ascidians, a didemnid in the genus *Didemnum*, *Didemnum vexillum* (Kott, 2002), is of particular concern due to an alarming increase in range expansion and subsequent invasion of

marine communities that have so far included several in North America, Europe, and New Zealand (Bullard et al. 2007). The term “invasive” applies to *D. vexillum* because of its potential to do both economic and environmental harm. This harm has been demonstrated by an empirical study (a competition experiment showed *D. vexillum* was able to invade epifaunal communities in Connecticut in both shallow and deep water regardless of age of the community; Osman and Whitlatch 2007), present day

comparisons to historical records showing shifts in marine communities correlated with an increase in abundance of *D. vexillum* (e.g. Portsmouth Harbor, New Hampshire; Dijkstra et al. 2007; Georges Bank, offshore Maine; Valentine et al. 2007b), and reports that this species is fouling aquaculture facilities (e.g. in Puget Sound, WA; Bullard et al. 2007; and Japan, New Zealand, Ireland, and Canada; <http://woodshole.er.usgs.gov/project-pages/stellwagen/didemnum/>). The impact of this invasive species can be profound due to the organism's ability to rapidly overgrow and displace native sea grasses, sponges, hydrozoa, anemones, oysters, mussels, scallops, barnacles, bryozoa, and other species of ascidians (Raloff 2005; Bullard et al. 2007; Valentine et al. 2007b). This invasive didemnid continues to expand its range in the U.S. It was first reported on the U.S. East Coast in the early 1980's, and the U.S. West Coast in California in the 1990's, and it has now taken hold in the U.S. Northwest since its first report in Puget Sound, Washington in 2000 (Bullard et al. 2007; G. Lambert pers. comm.). The destructive potential of this species requires prompt countermeasures if ecological impacts are to be mitigated. However, state, tribal, and federal agencies may have difficulty addressing this issue effectively because basic information is still lacking regarding the taxonomic identity and number of species involved, origin of the invading populations, and vectors of transport.

Currently, the geographically separate introductions of this didemnid are thought to involve only one species due to the close morphological similarity of a large number of specimens that have been examined from around the world (Bullard et al. 2007; Lambert 2009). The characteristics of this newly arrived species in North America and Europe fit those in the description provided by Kott (2002) for a specimen from New Zealand, which she named *Didemnum vexillum* (Bullard et al. 2007). In addition, Stefaniak et al. (2009) have shown by sequencing mitochondrial COI and a nuclear gene *Tho2* that specimens from worldwide populations of this invasive didemnid share genetic conspecificity with *D. vexillum*. This evidence thus appears sufficient to refer to the invasive didemnid as *Didemnum vexillum*, which was previously referred to as *Didemnum* sp. A (Bullard et al. 2007; Lambert 2009).

*D. vexillum*, like all colonial ascidians, have lecithotrophic (non-feeding) larvae which are

typically short lived (didemnid larvae live just minutes, Svane and Young 1989; Hurlbut 1992) and thus are not likely to disperse long distances. The dispersal range of larvae of colonial ascidians has been estimated on the scale of several meters based on direct observation (most larvae settled within 2.5 m of parent colony for *Podoclavella moluccensis*; Davis and Butler 1989) and mark-recapture using natural tags (most larvae settle within a meter of parent colony for *Botryllus schlosseri*; Grosberg 1987). Upon settlement, *D. vexillum* larvae metamorphose and bud asexually to become a sessile colony composed of numerous zooids, which spread in a mat-like growth morphology that sometimes extends with rope-like lobes off overhanging substrate (Bullard et al. 2007). The colony of zooids sexually reproduces through release and capture of sperm and broods the resulting larvae within the colony (Bullard et al. 2007). Based on the typically low dispersal potential of ascidian larvae and sperm (Grosberg 1991), populations of *D. vexillum* would be expected to act as closed populations and thus would be genetically highly differentiated as has been found for other colonial ascidians (Grosberg 1991; Ayre et al. 1997). However, *D. vexillum* colonies are highly capable of dispersing asexually through fragmentation (Valentine et al. 2007a) and dispersal is also aided by human mediated transport [e.g. transport of oysters for aquaculture (Dijkstra et al. 2007) or hull-fouling (Coutts 2002; Coutts and Forrest 2007)] and habitat disturbance (such as scallop dredging; Bullard et al. 2007). Although the species' native range is unknown, it is hypothesized to have come from Japan (G. Lambert pers. comm.). It may have originally been transported to some areas via export of shellfish stocks in aquaculture, though at least in New Zealand it was most likely introduced via ship fouling (Bullard et al. 2007; Dijkstra et al. 2007). The high frequency of long distance movements among worldwide populations increases the challenge of resolving the details of the pathways of its introduction into new habitats.

Molecular genetic techniques using DNA markers are ideally suited to addressing important management issues related to invasive species such as clarifying taxonomic identity [e.g. as applied to saltcedar (Gaskin and Schaal 2003), ascidian (Turon et al. 2003), and fly (Yassin et al. 2008)] and pathways of introduction [e.g. as applied to feral pig (Hampton et

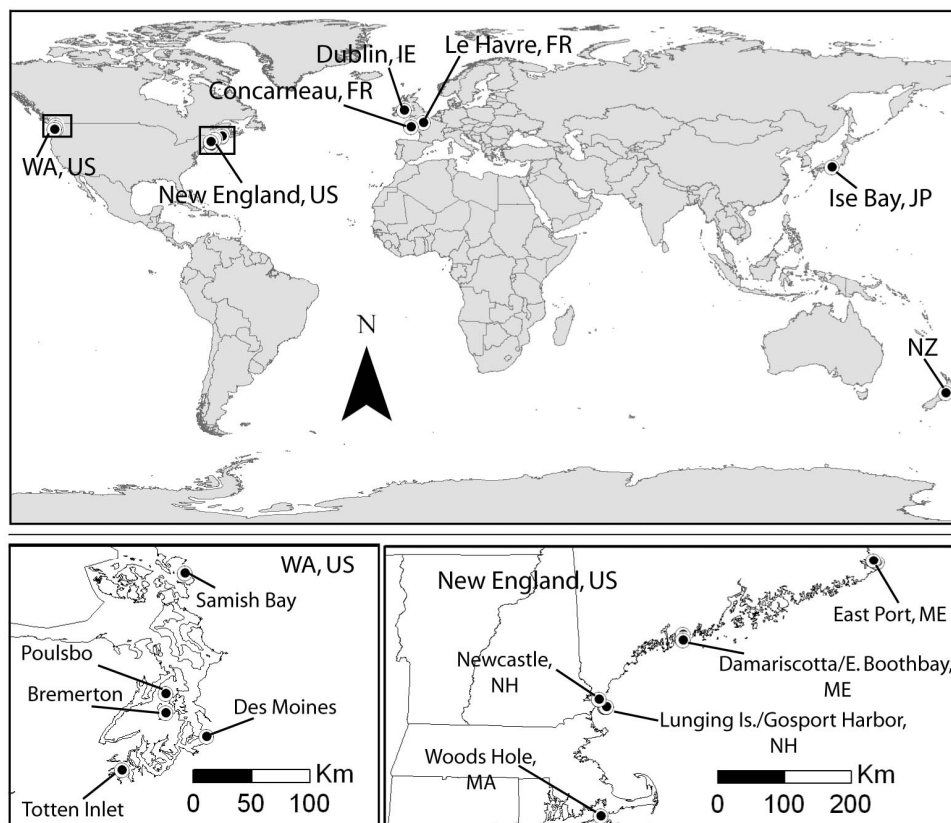
al. 2004), perennial weed (Gaskin et al. 2005), and ant (Corin et al. 2007)]. Newly developed genetic markers such as cytochrome oxidase subunit 1 and Tho2 genes for *D. vexillum* have been useful in demonstrating global specimens are likely all one species (Stefaniak et al. 2009). However, these markers may not have sufficient intraspecific variation to differentiate among populations. There are two main factors that complicate efforts to develop new genetic markers. First, there are a diverse array of organisms that associate with *D. vexillum* colonies (Carman 2007; Tait et al. 2007) and could be sources of DNA contamination. Second, there has been a minimal amount of sequence data published for close taxonomic relatives of *D. vexillum*. This last factor reduces the likelihood of success when attempting to use sequence from distant relatives (outside of the genus) to design primers that work in *D. vexillum*, and greatly increases the effort required to develop new markers.

In this study we have developed three new molecular markers to help resolve fundamental issues regarding the threat posed by *D. vexillum* on the west and east coasts of the U.S. and elsewhere in the world including the location of this species' native range, pathways of its spread, and vectors of introduction. We have taken an important first step toward addressing these issues in this study by demonstrating that these molecular markers have sufficient variation to detect population differences and describe methods to extract DNA while minimizing cross-species contamination.

### Material and Methods

#### *Biological Samples, DNA extraction, and Amplification*

Samples of *D. vexillum* were collected at twenty different locations around the world (Annex 1; Figure 1). These *D. vexillum* specimens were



**Figure 1.** The locations of our *D. vexillum* tissue samples. Ireland, France, New Zealand, and the United States are abbreviated with country codes, IE, FR, NZ, and US. U.S. sampling locations are enlarged at the bottom of the figure to show the Washington (WA) and New England populations where  $n > 5$  samples were collected. Within New England, we obtained samples from sites in the following three states: Massachusetts (MA), New Hampshire (NH), and two distantly separated sites in Maine (ME)

identified and distinguished from other didemnid species using morphological characters as detailed by Lambert (2009) and a subset of samples (those collected by G. and C. Lambert and all specimen collected outside the United States; Annex 1) have been further shown by Stefaniak et al. (2009) to have genetic conspecificity with known *D. vexillum* specimen based on mitochondrial COI and a nuclear gene *Tho2*. We obtained more extensive sample sets from three locations in the U.S.: Maine, New Hampshire, and Washington. In addition, we obtained samples from two distantly related species, *Styela clava* and *Ciona savignyi* collected in Puget Sound, WA; and two *Didemnum* congeners (*D. perlucidum* and *D. duplicatum*) to use as outgroups. For the entire *D. vexillum* samples used in our analyses, larvae and zooids were carefully dissected by hand from the colonies, put into 70% ethanol and stored at -20°C until DNA extraction. We used two approaches for isolating tissue for DNA extraction, which involved taking from a single colony either a pool of larvae or primarily zooid tissue. Each method has its advantages and disadvantages.

Extracting DNA from didemnids is complicated by the small size of their zooids (~0.5mm) and the presence of commensals living in the colonies (including foraminifera, nematodes, bacteria, etc). Isolating DNA from the non-feeding larvae, which are not yet associated with commensals, avoids potential contamination from other species. However, the size of the larvae necessitates pooling many individuals (a time-intensive process) to obtain DNA sufficient for analysis, and larvae in a colony may contain up to four different alleles per locus even from a single mating. This mixing of multiple larval genotypes complicates scoring the genotype of the parent colony and using these genotypes in subsequent population genetic analyses. On the other hand, obtaining DNA by isolating zooid tissue has the advantages of yielding a single *D. vexillum* genome and providing a source of DNA when larvae are not present. However, it is likely more prone to contamination by commensal DNA.

To obtain larval DNA from *D. vexillum*, we pooled about 100 larvae. To obtain zooid DNA we excised a nine mm<sup>2</sup> portion of tissue from a section of the colonial tunic (not the entire thickness of the tunic) incorporating approximately ten zooids. Genomic DNA from pools of larvae was extracted using the Sigma GenElute™

Mammalian Genomic DNA Miniprep Kit (Sigma, St. Louis, MO). Genomic DNA purification was completed as outlined in Swalla et al. (2000). Genomic DNA from zooids was extracted using QIAGEN tissue extraction kits and a QIAGEN BioRobot 8000 for fast sample throughput (QIAGEN, Inc., Valencia, CA).

#### *Construction of a genomic library enriched for microsatellites and screening*

Microsatellite enrichment was performed using a method based on Hamilton et al. (1999). We used a *D. vexillum* zooid extraction from a colony collected in Bremerton, Washington. The DNA was at approximately 50ng/μl concentration and digested with two different enzymes: PvuII and HincII. We used two different probes (GATA)<sub>4</sub> and (GATA)<sub>8</sub> for enriching the library for repeats. We used the TOPO TA Cloning kit for sequencing, version E (Invitrogen, Carlsbad, CA), and plated the clones out on agar plates selecting for ampicillin resistance. Clones were grown up in 1.3 ml of Luria-Bertani (LB) media with 50 μg/ml ampicillin in a shaker overnight at 37°C in plates provided by the QIAprep 96 Turbo Miniprep kit (QIAGEN, Inc., Valencia, CA). The minipreps were completed on a QIAGEN liquid handling BioRobot 8000. DNA yield was variable from 15ng/μl to 70ng/μl. Eight μl of this DNA was used in sequencing reactions to obtain 120-560ng total DNA. The miniprep template DNA was cycle sequenced using ABI Big Dye Terminator v.3 (Applied Biosystems, Foster City, CA). PCR cycle protocol was 96°C (30 sec.) followed by 30 cycles of [96°C (10 sec.), 50°C (15 sec.), and 60°C (4 min.)]. Sequence reactions were purified with Cleanseq (Agencourt Bioscience, Beverly, MA) and data was collected with a Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Forward and reverse sequences were aligned and edited using SEQUENCHER v4.6 for Mac. For all unique clone inserts that included a repeat with flanking DNA, we designed primers with the open source program PRIMER3 (available online at <http://primer3.sourceforge.net/>). These primers were tested by PCR amplification of a subset of individuals in 50 μl total reaction volumes containing 2.5 mM MgCl<sub>2</sub>, 1X Promega PCR buffer, 0.2 mM dNTPs, 0.5 μM of both primers, 0.625 U Promega Taq Polymerase, and approximately 20 ng genomic DNA template. PCR cycle

protocol was 94° C (2 min.), 35 cycles of [94° C (20 sec.), 54° C (20 sec.), and 72° C (2 min.)], and 72° C (10 min.). The PCR product was cleaned with a Millipore kit. The clean PCR template was then cycle sequenced as described above.

#### Microsatellite genotyping

Two microsatellite loci referred to as D6 and D17 (GenBank accession nos. **EU709739** and **EU709740** respectively) were genotyped on *Didemnum* samples using fluorescently labeled PCR forward primers (labeled PET and VIC respectively). The locus D6 is a tetra-repeat that yielded a [GTCC]<sub>11</sub>[GTCT]<sub>3</sub> repeat motif in the original clone based on genomic DNA from the Bremerton, WA specimen. The forward and reverse primer sequences are “D6F” 5'-TCGTGTATCGTATTTGCACCA-3' and “D6R” 5'-TAACCTGACGCAGACACCAA-3' respectively. The locus D17 yielded a [GACA]<sub>14</sub> repeat motif in the original clone and forward and reverse primer sequences are “D17F” 5'-CCCGCACTTAGAGCTGAACT-3' and “D17R” 5'-GTAAAGGAAACGGCGATCAA-3' respectively. PCR amplifications were carried out in 10 µl total reaction volumes containing 3.0 mM or 2.5 mM MgCl<sub>2</sub> (D6 locus at former concentration and D17 at the latter), 1X *GoTaq*<sup>®</sup> Flexi Buffer (without MgCl<sub>2</sub>) (Promega, Madison, WI), 0.2 mM dNTPs, 0.2 µM each of both forward and reverse primers, 0.25 U Promega *GoTaq*<sup>®</sup> Flexi DNA polymerase (Promega, Madison, WI), and approximately 5 ng genomic DNA template. The PCR cycle protocol was 94° C (2 min.), 35 cycles of [94° C (35 sec.), 55° C for D6 locus and 58° C for D17 locus (20 sec.), and 72° C (35 sec.)], and 72° C (5 min.). For D6 locus, an additional 55° C step for 45 min. was needed to adenylate the fragment for ease of analysis. The PCR reactions were diluted in water 2/160 and 1/160 for D6 and D17, respectively, before electrophoresis on the Prism 3100 Genetic Analyser using Genescan 500 LIZ as a genotyping basepair ladder standard (Applied Biosystems, Foster City, CA). Genotypic data were analyzed using GENE-SCAN and GENOTYPER version 3.7 software (Applied Biosystems, Foster City, CA). Data were organized in MICROSOFT EXCEL and formatted for various population genetic statistical programs using EXCEL MICROSATELLITE TOOLKIT version 3.1 (Park 2001).

#### Sequencing

We used two unidentified variable fragments DL2.1A1 and Dnr1 (GenBank accession nos. of the representative sequences are EU709735-38 and EU709733-34 for each locus respectively) for sequencing *Didemnum* samples. The locus DL2.1A1 was discovered as a PCR amplification artifact when using low stringency PCR conditions on *D. vexillum* DNA with a pair of published primers (locus DL2.1; Maclean et al. 2004) specific for *Diplosoma listerianum*, a member of the family Didemnidae. We directly sequenced the fragment that had been amplified from *D. vexillum* and then designed new primers that were specific to a 553 bp region within this fragment. Although we cannot reject the possibility that this fragment is not *D. vexillum* DNA, we found that it consistently amplified *D. vexillum* samples and failed to amplify a set of negative controls [including DNA from a nematode found in a *D. vexillum* specimen; DNA from distantly related ascidians, *Ciona savignyi* and *Styela clava*; and two *Didemnum* congeneric species (*D. perlucidum* and *D. duplicatum*)]. If the DNA sequence were from a commensal, a failure to amplify the DNA fragment would simply show that the same commensal is not shared among these other ascidian species and is not present in the nematode. On the other hand, if the sequence were truly *D. vexillum* DNA we would expect that out of all these species, the congeners would be most likely to amplify a DNA fragment because their priming sites may be the most similar to *D. vexillum*. The fact that we observed a failure of all other species to amplify is therefore still somewhat ambiguous. Our final test was to perform a BLAST search in Genbank, which in this case did not yield any significant similarities with other sequences.

The redesigned forward and reverse primer sequences are “DL2.1A1F” 5'-CATCGGGC-ATTGCGTAAGCTGATT-3' and “DL2.1A1R” 5'-ATCTGCAGCAGTTAGCCGTTGGAA-3' respectively. PCR amplification was carried out in 50 µl total reaction volumes containing 2.5 mM MgCl<sub>2</sub>, 1X Promega PCR buffer, 0.2 mM dNTPs, 0.5 µM of both primers, 0.625 U Promega Taq Polymerase, and approximately 20 ng genomic DNA template. PCR cycle protocol was 94° C (2:00), 35 cycles of [94° C (0:20), 54° C (0:20), and 72° C (2:00)], and 72° C (10:00). The PCR product was cleaned with a Millipore kit.

The clean template was cycle sequenced as described above.

The locus Dnr1 is a 678 bp fragment from a clone insert screened in our *D. vexillum* genomic library that is relatively long and lacks a conventional microsatellite repeat (See results for more details of the variation). Similar to the DL2.1A1 fragment, we can not reject the possibility that this fragment results from a commensal organism, rather than *D. vexillum*. However, the assay passed our tests mentioned above. In addition, a 102 bp region (220bp-321bp) within this sequence is 79% identical to a clone of *Botryllus schlosseri*, another colonial ascidian (Genbank accession no. AC139529.6). The forward and reverse primer sequences are “Dnr1F” 5’- CAAGCGCTCACAGTTTTTCAG -3’ and “Dnr1R” 5’- TTGGAAACGCAACAAAA-CAA -3’ respectively. This locus was PCR amplified and sequenced in the same way as locus DL2.1A1 above.

#### *Sequence Alignments and Population Genetic Analyses*

*D. vexillum* gene sequences were aligned using SEQUENCHER v4.6 for Mac. It was possible to deduce the gametic phase of the variable sites because variation was relatively limited and most of the haplotypes were found in homozygous form. Any heterozygous variants that could not be explained using existing combinations of known haplotypes were inferred by starting with the closest known haplotype match and reconstructing a new haplotype to explain the remaining variation.

The program FSTAT 2.9.3.2 (Goudet 2001) was used to calculate pairwise  $F_{ST}$  values among our population samples (Weir and Cockerham 1984), perform pairwise tests of population differentiation by randomizing multi-locus genotypes between the two samples, and calculate the proportion of heterozygotes. The p-values for the tests of differentiation are based on standard Bonferroni corrections at the 5% nominal level of significance. The sampling constraints in this study present multiple potential violations of assumptions related to estimating  $F_{ST}$ . Nevertheless, we use this commonly employed population genetic metric as a first approximation of genetic relationships in *D. vexillum*. The program GENETIX 4.03 (Belkhir et al. 1996-2006) was used to perform the principal component analyses on the allele frequency data, and values from the first two

principal component axes were used to graph the data in EXCEL.

#### **Results**

We sequenced two unidentified fragments DL2.1A1 and Dnr1 as well as genotyped D6 and D17 for all of the *D. vexillum* samples. The microsatellite locus D17 was not easy to score and appeared prone to null alleles or allele dropout. D17 allele sizes were rounded and reported in Table 1, but the data were not analyzed with the other loci. Each locus yielded a set of alleles that were shared among populations worldwide. Both loci Dnr1 and DL2.1A1 had four haplotypes, while the microsatellite locus D6 had ten or eleven. The possible eleventh allele is a 1 bp shifted allele (151 bp total size) that was present in one colony of Puget Sound, however we did not sequence to verify this variant. Most alleles were shared by at least two sites. However, allele 3 at locus DL2.1A1 was only found in a Puget Sound sample and allele 2 at locus Dnr1 was only found in the sample from Japan. The microsatellite locus D6 had three private alleles: allele 140 (only found in a Puget Sound sample), allele 148 (only found in a NH sample), and allele 151 (found as a homozygote in a Puget Sound sample). To be conservative, we chose to place the putative one-bp-shifted 151 allele into the 152 bp allele bin for the purposes of analyses. When all *D. vexillum* specimen from all sample locations are included, the proportion of heterozygotes for each marker was 0.682, 0.721, and 0.773 for Dnr1, DL2.1A1, and D6 respectively.

The variation found at the DL2.1A1 and Dnr1 loci was mainly in the form of single nucleotide polymorphisms (SNPs); eight and six SNPs were found for these two loci respectively (Table 2). However, in addition to SNPs, the variation at Dnr1 included two sites where insertion/deletions (in/dels) occurred. These in/dels involved a fourteen bp (GGATGTCCGCCTTT) and seven bp (GGTGGCA) length unit that were observed to be repeated twice in several haplotypes (Table 2). This first region was also observed to be deleted and replaced by (GGTGG) in haplotypes 2, 3, and 4. The second region was observed reduced to one unit in haplotype 4. These insertion/deletions greatly complicated scoring of the sequence data, which was obtained only through direct sequencing

from PCR. However, once the nature of this variation was understood it was possible to analyze. It may be useful to design new primers

for this locus that would amplify only these two sites for efficient scoring of these length variants.

**Table 1.** Genetic results of *D. vexillum* samples

Location	Tissue	Sample	Group n	D6Alleles	D6	Alleles by D17Alleles	Locus DL2.1A1	Dnr1
Le Havre, France	larvae	FR001	2	124, 124	3	113,113	1,4	1,3
Concarneau, France	larvae	FR002		144,152		113,178	4,4	1,3
Malahide Harbor, Dublin, Ireland	larvae	IE001	1	-	-	-	1,4	1,1
Ise Bay, Japan	larvae	JP001	1	136*,144,152,176*,180	5	113,113	1,4	1,2
Shakespeare Bay, Marlborough Sound, New Zealand	larvae	NZ001	1	128,136	2	113,113	2,2	1,3
Woods Hole, MA, USA	larvae	MA001	2	136*,144,160*	3	148,148	1,2	1,3
Maritime floats, MA, USA	larvae	MA002		136,144		143,148	1,4	1,3
Damariscotta estuary; ME, USA	zooids	ME001	10	144,152	3	135,148	1,2	1,3
Damariscotta estuary; ME, USA	zooids	ME002		144,152		135,148	1,2	1,3
Eastport, ME, USA	zooids	ME003		144,144		113,113	4,4	1,3
Eastport, ME, USA	zooids	ME004		144,144		113,113	4,4	1,3
Eastport, ME, USA	zooids	ME005		144,144		113,113	4,4	1,3
Eastport, ME, USA	zooids	ME006		144,152		113,113	1,4	3,4
Eastport, ME, USA	zooids	ME007		144,144		113,113	4,4	1,3
Half Moon Bay, Eastport, ME, USA	zooids	ME008		144,144		148,148	1,1	3,3
Half Moon Bay, Eastport, ME, USA	zooids	ME009		136,144		148,148	1,2	3,3
E. Boothbay, ME, USA	larvae	ME010		-		122,122	1,2	1,1
Newcastle, NH, USA	zooids	NH001	15	136,144	8	148,148	2,2	1,1
Newcastle, NH, USA	zooids	NH002		136,180		148,148	1,2	1,3
Lunging Island, NH, USA	zooids	NH003		136,136		148,148	1,4	3,3
Lunging Island, NH, USA	zooids	NH004		128,144		143,148	2,4	1,3
Lunging Island, NH, USA	zooids	NH005		144,144		143,148,156	1,1	3,3
Lunging Island, NH, USA	zooids	NH006		136,144		143,148	2,4	3,3
Lunging Island, NH, USA	zooids	NH007		144,148		148,148	2,2	3,3
Lunging Island, NH, USA	zooids	NH008		136,136		148,148	1,1	3,3
Lunging Island, NH, USA	zooids	NH009		136,144		148,148	1,2	1,3
Gosport Harbor, NH, USA	zooids	NH010		136,152		148,148	1,4	3,3
Gosport Harbor, NH, USA	zooids	NH011		136,144		148,148	1,2	1,1
Gosport Harbor, NH, USA	zooids	NH012		144,160		148,148	1,2	1,3
Gosport Harbor, NH, USA	zooids	NH013		136,152		148,148	1,4	3,3
Gosport Harbor, NH, USA	zooids	NH014		136,144		148,148	1,1	-
New Castle, NH, USA	larvae	NH015		136*,144*,176		148,148	1,4	1,3
Bremerton, WA, USA	zooids	WA001	6	152,152	6	178,178	4,4	1,4
DesMoines, WA, USA	zooids	WA002		140,152		178,178	1,2	1,1
Samish Bay, WA, USA	larvae	WA003		124,144*,176*		125,182,190	3,4	1,1
Samish Bay, WA, USA	zooids	WA004		152,152†		-	-	-
Poulsbo, WA, USA	zooids	WA005		136,152		178,178	1,2	1,4
Totten Inlet, WA, USA	zooids	WA006		124,144		166,166	4,4	1,1

Samples where >2 alleles were found in D6 genotypes have an \* marking the alleles with highest peak height (explained in results). DL2.1A1 and Dnr1 allele numbers refer to haplotypes in Table 2. The column labeled “D6” shows the total number of D6 microsatellite alleles observed for each population grouping.

† indicates the D6 alleles which may actually be a 1 bp shifted allele variant of size 151 (to be conservative we combined these alleles with the 152 allele bin).

**Table 2.** Gametic phase reconstructions and haplotypes of DL2.1A1 and Dnr1 sequence loci

DL2.1A1 sequence										
3	78	121	190	357	408	436	446	Haplotype	GameticPhase	Genbank No.
T	A	G	C	C	A	A	T	1	Homozygous	<u>EU709736</u>
T	A	G	C	C	G	A	T	2	Homozygous	<u>EU709737</u>
C	G	C	A	T	G	G	G	4	Homozygous	<u>EU709738</u>
C	G	C	A	T	G	G	T	3	Reconstruction	

Dnr1 sequence														
78	188-219		254	270	377-390		392	436	482	Haplotype	GameticPhase	Genbank No.		
C	(GGATGTCCGCCTT)		2	T	A	C	(GGTGGCA)	2	C	G	C	1	Homozygous	<u>EU709733</u>
C	(GGTGG)		1	A	T	(GGTGGCA)	2	T	G	T	3	Homozygous	<u>EU709734</u>	
C	(GGTGG)		1	A	C	(GGTGGCA)	2	C	G	C	2	Reconstruction		
G	(GGTGG)		1	G	C	(GGTGGCA)	1	T	A	T	4	Reconstruction		

The bolded numbers refer to the bp site or region of variation on the DNA fragment (numbering corresponds to alignment of the listed Genbank accession sequences). The haplotype number is given, along with information on whether this haplotype was found in homozygous form among our samples or if it had to be reconstructed by interpolating the gametic phase of observed variation.

There were allele frequency differences among populations as demonstrated by significant pairwise  $F_{ST}$  values (Table 3). Locus Dnr1 showed two significant pairwise  $F_{ST}$  values in comparisons of the Puget Sound sample to either New Hampshire or Maine samples ( $F_{ST}=0.305$  and  $0.434$  respectively). The other two loci did not have significant values, although for locus D6 the comparisons of Maine, New Hampshire, and Puget Sound were close to a 0.05 level of significance. When the data from all three loci were combined, the single comparison was highly significant, Puget Sound versus New Hampshire ( $F_{ST}=0.226$ ). This study is the first in our knowledge to demonstrate the ability to genetically differentiate *D. vexillum* populations, and we have done so despite small population samples and relatively few sites. It is likely that more of these population comparisons can be statistically differentiated when a greater number of samples are analyzed.

Another result worth noting comes from the microsatellite genotypes we obtained from thirty-six samples for locus D6. We observed greater than two allele peaks (the maximum number of alleles expected for a diploid individual) for four samples in which DNA was extracted from pooled larvae (three out of eight samples that had been dissected by Lambert and the single sample that had been dissected by Hess to obtain larvae). None of the other r genotypes from DNA extracted from zooid tissue

**Table 3.** Pairwise  $F_{ST}$  comparisons of the *D. vexillum* from U.S. populations. Each of three loci is shown analyzed separately and then in one combined analysis. Upper triangles are pairwise  $F_{ST}$  values and bottom triangles are p-values. Values outlined in grey are significant based on 28,000 permutations and indicative of adjusted nominal level 5%

D6			
	ME	NH	WA
ME	-	0.198	0.289
NH	0.006	-	0.175
WA	0.012	0.004	-

Dnr1			
	ME	NH	WA
ME	-	-0.018	<b>0.305</b>
NH	0.442	-	<b>0.434</b>
WA	<b>0.002</b>	<b>0.001</b>	-

D2.1A1			
	ME	NH	WA
ME	-	0.025	-0.073
NH	0.240	-	0.067
WA	0.550	0.105	-

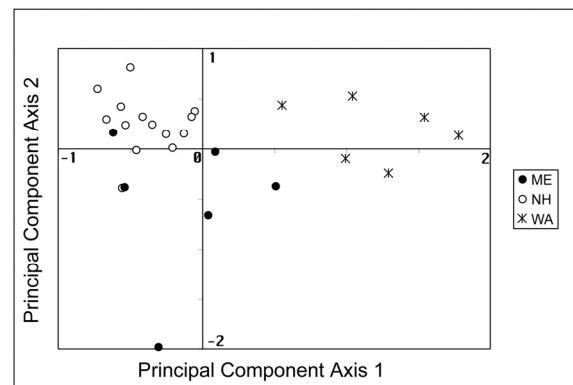
  

All3			
	ME	NH	WA
ME	-	0.080	0.182
NH	0.025	-	<b>0.226</b>
WA	0.007	<b>0.000</b>	-



( $n=28$ ) had greater than two allele peaks. This result implies that the larvae are the progeny of sexual reproduction by individuals with different genotypes. The four pooled larval samples were from Massachusetts, New Hampshire, Washington, U.S. and Ise Bay, Japan. Interestingly, the U.S. samples had no more than three alleles total, whereas the single sample from Japan had five alleles. These results support two main points: 1) the larvae are the progeny of sexual reproduction by individuals with different genotypes and 2) the zooids here are showing an absence of chimerism. The first point may be notable because it demonstrates that *D. vexillum* does outbreed at least at a high enough frequency we could detect it in this study with extremely limited sampling. It is important to resolve the degree to which the system of mating of *D. vexillum* is characterized by outbreeding or selfing (an extreme form of inbreeding which is available to some hermaphroditic ascidians). Selfing-avoidance mechanisms have been documented in ascidian species that act to promote outbreeding [e.g. *Diplosoma listerianum* (Bishop 1996), *Halocynthia roretzi* (Fuke and Numakunai 1999), and *Ciona intestinalis* (Harada et al. 2008)]. However, there may be advantages for selfing when organisms that have that capability are colonizing new habitats in which population density is often low. In fact, one non-indigenous solitary ascidian, *Corella eumyota*, appears to use this strategy in its non-native range (Dupont et al. 2007). Returning to the second point mentioned above, an absence of chimerism may be notable because this phenomenon in which zooids within the same colony have different genotypes as a result of fusion, has been found to be a common occurrence (up to 61% of colonies) in populations of another didemnid, *Diplosoma listerianum* (Sommerfeldt et al. 2003).

Supporting contingency tests and significance testing of  $F_{ST}$ , principal components analyses showed that the *D. vexillum* populations in the U.S. can be separated into groups according to geographic location (Figure 2). The first group includes samples from Puget Sound, Washington. The second group includes the two populations from the U.S. East Coast, Maine and New Hampshire. In order to perform this analysis, we had to make a decision on how to treat samples with more than two alleles as discussed previously. For these four multiple-



**Figure 2.** A principal component analysis of the combined genetic results for three DNA markers of *D. vexillum* samples

allele samples we chose the two highest peaks because we hypothesized these two peaks were most likely to originate from the maternal ascidian colony from which the DNA was extracted.

## Discussion

Our results suggest that these markers, specifically microsatellite locus D6 and nuclear sequence locus Dnr1, are variable enough to be useful to genetically differentiate *D. vexillum* populations and potentially characterize invasion pathways. According to our results, the East Coast sample collections from Maine and New Hampshire are very similar genetically. However, New Hampshire and possibly Maine (at least for locus Dnr1) can be described as significantly different from our West Coast Puget Sound collection. Possible reasons for observing significant genetic differentiation among these collections may include genetic drift, being founded by different source populations; or having had significantly different representation among founders from the same source population. With such low sample numbers, it is too early to conclude that other worldwide population samples are significantly differentiated from U.S. populations. The fact that many alleles were shared across samples from all over the world further supports the idea that *D. vexillum* is a single species (Bullard et al. 2007) and may have been somewhat homogenized by human mediated transport associated with activities such as oyster aquaculture (Dijkstra et al. 2007; Lambert 2009).

We suggest some interesting leads that could be followed by examination of more Japanese populations. First, the sample from Japan had the only private allele found at the Dnr1 locus. Second, the larval pool of DNA from the Japan sample harbored the highest apparent number of allele peaks of any sample. Because Japan is part of the putative native range of this species (G. Lambert, pers. comm.), future work should examine whether Japanese *D. vexillum* populations are, in general, more genetically diverse than introduced populations in Europe, North America, New Zealand, and elsewhere. Interestingly, Stefaniak et al. (2009) found that *D. vexillum* specimens from Japan had more differences in mitochondrial COI haplotypes than any other collections they sampled around the world. This finding is often the case for comparisons between native and introduced ranges of species in other systems (Neilson and Wilson 2005; Herborg et al. 2007). A population with high genetic diversity would be expected to have many alleles including rare alleles not found in other populations, which makes the single specimen from Japan interesting by meeting this expectation. However, clearly more *D. vexillum* colonies will need to be collected and analyzed from Japan in order to draw any firm conclusions.

The use of larval tissue rather than zooids will not be necessary for most population genetic studies once a set of markers are found that are confirmed to be specific to this organism. The option of using zooid tissue will greatly minimize the effort involved in extracting DNA from *D. vexillum*. However, an interesting range of questions regarding multiple paternity and gamete dispersal could be addressed if single larvae were genotyped (Johnson and Yund 2007). Genotyping single larvae is challenging due to the small quantities of DNA that can be isolated from them, but laboratory techniques are available to overcome this challenge (Sommerfeldt and Bishop 1999).

In summary, we have identified microsatellite and sequence markers that can genetically differentiate populations of *D. vexillum*. Our results support that this invasive didemnid is a single species with global distribution and shows some genetic differentiation between eastern and western U.S. populations. The next step that is required to determine crucial information regarding the location of this species' native range, pathways of its spread, and vectors of introduction will be to collect and analyze a

greater number of individual colonies of *D. vexillum* throughout its global distribution. Once these samples are genotyped and sequenced for these new molecular markers and any additional ones that are developed, we can begin to elucidate the phylogenetic relationships of these global populations and infer the chronological order of introductions.

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### Annex 1

Locations for samples of *D. vexillum*

Location	Samples	Coordinates		Sampling date	Collector
		Latitude	Longitude		
Le Havre, France	FR001	49.49170	0.09692	August 2005	G. and C. Lambert
Concarneau, France	FR002	47.86900	-3.91546	September 2005	G. and C. Lambert
Malahide Harbour, Dublin, Ireland	IE001	53.45462	-6.15412	October 2005	Dan Minchin
Ise Bay, Japan	JP001	34.55181	136.64520	July 2003	Teruaki Nishikawa
Shakespeare Bay, Marlborough Sound, New Zealand	NZ001	-41.27664	173.99880	January 2006	Ashley Coutts
Woods Hole, MA, USA	MA001	41.51700	-70.68300	August 2003	G. and C. Lambert
MA Maritime Academy floats, Buzzards Bay, MA, USA	MA002	41.74484	-70.61961	August 2003	G. and C. Lambert
Damariscotta estuary; ME, USA	ME001-002	43.93490	-69.58060	July 2006	Larry Harris
Eastport, ME, USA	ME003-007	44.90370	-66.98340	September 2006	Larry Harris
Half Moon Bay, Eastport, ME, USA	ME008-009	44.93430	-67.03550	September 2006	Larry Harris
E. Boothbay, ME, USA	ME010	43.86500	-69.58700	August 2003	S. Johnson
Newcastle, NH, USA	NH001-002	43.07190	-70.71180	October 2006	Larry Harris
Lunging Island, NH, USA	NH003-009	42.97750	-70.62690	February 2007	Larry Harris
Gosport Harbor, NH, USA	NH010-014	42.97880	-70.60930	February 2007	Larry Harris
Coast Guard pier, Newcastle, NH, USA	NH015	43.07190	-70.71180	August 2003	G. and C. Lambert
Port Washington Marina, Bremerton, WA, USA	WA001	47.57860	-122.64340	May 2006	G. and C. Lambert
DesMoines, WA, USA	WA002	47.39917	-122.32800	June 2005	Billie Swalla
Samish Bay, WA, USA	WA003-004	48.65125	-122.49355	October 2006	Jesse Schultz
Poulsbo, WA, USA	WA005	47.72450	-122.64010	May 2006	G. and C. Lambert
Totten Inlet, WA, USA	WA006	47.13967	-122.97917	October 2006	Jesse Schultz