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# Determining the native region of the putatively invasive ascidian *Didemnum vexillum* Kott, 2002

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ABSTRACT

Over the past 40 years, an increasing number of previously unrecorded populations of a colonial ascidian, recently identified as *Didemnum vexillum*, have been documented in most temperate coastal regions of the world, impacting aquaculture operations, natural rocky habitats, cobble/gravel substrates, and eelgrass beds. The earliest sample thought to be *D. vexillum* was collected in Mutsu Bay, Japan in 1926, but was not identified to species at the time. Lack of a published description led to widespread mis-identification of this species. Because of incomplete historical records and the numerous mis-identifications of this species, the native range of *D. vexillum* has not been conclusively known. To determine which portion of the current known range of *D. vexillum* is within its native region, we obtained DNA sequences of two genes, cytochrome *c* oxidase subunit 1(*co1*; mitochondrial) and THO complex subunit (*tho2*; nuclear), from 365 and 75 samples of *D. vexillum* from around the world, respectively. Both population genetics (*co1* only) and phylogenetics (*co1* and *tho2*) were used to measure and compare the amount of genetic variation contained in each region where *D. vexillum* is currently found (Eastern North America, Japan, New Zealand, Northwestern Europe, and Western North America). We found that genetic diversity in Japan was greater than in any other region. Northwestern Europe, western North America, and New Zealand all showed evidence of having recently undergone a genetic bottleneck. The gene tree for *co1* in *D. vexillum* was divided into three clades: one which is found globally; the other two found only in Japan. Analysis of a partial genomic sequence of the nuclear gene *tho2* confirmed that the *co1* clades belong to the same species. In agreement with the sparse historical data, the molecular evidence suggests that Japan lies within the native range of *D. vexillum*.


## 1. Introduction

There are important economic and ecological implications to identifying the native ranges of non-native species. Knowing the native range of an invasive species or whether a species is invasive to a particular region can influence management decisions. Delays in efforts to eradicate an invasive species can be caused by improperly designating the species as native or cryptogenic, which, in turn, can result in the establishment of costly pests (e.g. Coutts and Forrest, 2007). Species are often erroneously defined as either native or invasive because the fauna of a region was never fully characterized or because the baseline biota was determined during or after historical invasions (Carlton, 1996; Kott, 2004).

Since the early 1970s, numerous populations of a previously unrecorded colonial ascidian of the genus *Didemnum* have been documented in temperate coastal regions of the world [i.e., both coasts of North America, northwestern Europe, the UK, Ireland, New Zealand, and Japan (Bullard et al., 2007; Coutts and Forrest, 2007; Gittenberger, 2007; Lambert, 2009; Minchin and Sides, 2006)]. Recent morphological (Lambert, 2009) and molecular (Stefaniak et al., 2009) studies of worldwide populations have shown that only one species is involved in all of these non-native populations and that the most appropriate name for the species is *Didemnum vexillum* Kott, 2002.

Though *D. vexillum* is a relatively recent invader compared to other invasive colonial tunicates (e.g. Gittenberger, 2007; Lambert, 2009), in regions where *D. vexillum* becomes established, it readily overgrows a variety of artificial substrates, including dock pilings,

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boat hulls, and aquaculture equipment (e.g. Minchin, 2007; Osman and Whitlatch, 2007). *Didemnum vexillum* is capable of heavily fouling aquaculture gear (e.g. Coutts and Forrest, 2007), forcing oyster growers in some areas in the U.S. to switch from net- to bottom-culture (L. Harris, pers. comm.). This species rapidly colonizes natural surfaces as well, in both shallow (e.g. Gittenberger, 2007; Osman and Whitlatch, 2007; Valentine et al., 2007a) and deep water (e.g. Mercer et al., 2009; Valentine et al., 2007b) coastal sites.

While the native range of *D. vexillum* is not conclusively known, preliminary evidence suggests that Japan is the likeliest portion of the known range to be the origin of the species (Hess et al., 2009; Lambert, 2009; Stefaniak et al., 2009). The earliest known sample believed to be *D. vexillum* was collected from Mutsu Bay, Japan in 1926, 67 years before the next confirmed sample was collected from the Damariscotta River Estuary, ME, USA (Lambert, 2009). Unfortunately, the 1926 sample has degraded, precluding definitive identification (Lambert, 2009). However, in preliminary sampling, Stefaniak et al. (2009), Hess et al. (2009), and Smith et al. (2012) measured the greatest genetic diversity in mitochondrial DNA or microsatellites in samples from Japan compared to samples collected from other parts of the world. Therefore, we hypothesize that Japan lies within the native range of *D. vexillum*.

Given that *D. vexillum* is likely to have invaded its new locations relatively recently (<50 generations, Lambert, 2009), there has been a limited time for mutation, selection, and drift to obscure the molecular record. Molecular studies generally find that genetic diversity in invasive populations is lower than in native populations because relatively few individuals are transported from the native range during the invasion process, causing a genetic bottleneck known as the "founder effect" (e.g. Sakai et al., 2001; Wares et al., 2002). Therefore, invasive populations should have lower genetic diversity than their source populations (Dawson et al., 2005; Garnatje et al., 2002), particularly if the invasive population was the result of a single founder event (Roman, 2006). In this study, to determine the native range of *D. vexillum*, we used several population genetic tests to quantify the genetic diversity of *D. vexillum* populations throughout *D. vexillum*'s current known distribution.

## 2. Methods

### 2.1. Sample collection and DNA extraction

A total of 292 *Didemnum vexillum* colonies were collected from North America (east and west coasts), Japan, New Zealand, and Northwestern Europe (including Ireland and the United Kingdom) (see Appendix A for sampling details). Subtidal colonies were collected via SCUBA and snorkeling or from ropes hanging from both fixed and floating docks. Intertidal colonies were collected by wading at low tide. Colonies were preserved in 95% ethanol, thoraces were dissected from each sample prior to DNA extraction, and total DNA was extracted from the dissected thoraces following Stefaniak et al. (2009). Tissue vouchers for each sample were deposited with the Netherlands National Museum of Natural History: NCB Naturalis, Leiden, The Netherlands.

### 2.2. Amplification and Sequencing

PCR amplifications and sequencing were done according to Stefaniak et al. (2009). The mitochondrial gene, cytochrome *c* oxidase subunit 1 (*co1*), was amplified (Tun_for: 5′-TCGACTAATCATAAAGATATT AG-3′ and Tun_rev2: 5′-AACTTGTATTTAAATTACGATC-3′) and sequenced from 221 samples; the other 71 samples were previously sequenced by Stefaniak et al. (2009). A fragment of a nuclear-encoded putative RNA-processing gene, *tho2* (Jimeno et al., 2002), was amplified (DidTHO2F3: TGCCAAGTTCATCCACATTCTG and DidTHO2R3: TTGCTTTGCTGCTGCC-ATC) and sequenced from a subsample of 75 globally distributed colonies. In order to separate different *tho2* sequences amplified in the same sample, PCR products were cloned into a T-vector and analyzed by restriction digestion as in Stefaniak et al. (2009). Four to six plasmid inserts that were shown to have the predicted length were sequenced from each colony. Sequencing reactions were performed using BigDye terminator chemistry v3.1 and analyzed on an ABI 3730 automated sequencer (Applied Biosystems, Foster City, CA) at the DNA Analysis Facility on Science Hill at Yale University, New Haven, CT, USA. All of the sequences and the original chromatographs were manually checked for correct base calls. Unique *co1* haplotypes, *tho2* exon alleles, and full length *tho2* sequences were deposited in GenBank (*co1* accession numbers: JQ663508–JQ663517, *tho2* exon accession numbers: JQ653168–JQ653246, *tho2* full sequence accession numbers: JQ686393–JQ686617).

### 2.3. Phylogenetic analysis

In order to confirm morphological identification of samples, phylogenetic analyses were performed separately on the *co1* and *tho2* sequence datasets. For *co1*, 73 additional *co1* sequences from *D. vexillum* (Cohen et al., 2011; Smith et al., 2012, see Appendix A for details) as well as from two *Didemnum albidum* (EU419432; EU419456), one from *Didemnum* sp. B (EU419407; Stefaniak et al., 2009), and one from *Diplosoma spongiforme* (AY600972; Turon and López-Legentil, 2004) were obtained from GenBank. For *tho2*, exon sequences of one sample of *Didemnum* sp. B (EU419460) and one sample of *Didemnum psammathodes* (EU419461) were obtained from GenBank (Stefaniak et al., 2009). Sequences were aligned separately using CLUSTALX. Each alignment was manually checked. An unalignable poly-T region 26 bp in length in the first intron of the *tho2* alignment was removed. Sequences were then collapsed into haplotypes (*co1*)/alleles (*tho2*) using DNASP v5.1 (Librado and Rozas, 2009). When *D. vexillum tho2* sequences were aligned with sequences from other *Didemnum* species, only the exon sequences would align with *D. vexillum tho2* sequences; therefore, introns were removed and only exons used for the phylogenetic analysis. Of the 225 *tho2* exon sequences, 108 sequences of *D. vexillum* were previously published by Stefaniak et al. (2009). The *tho2* exons were further collapsed into alleles after removal of intron sequences. Using jMODELTEST (Guindon and Gascuel, 2003; Posada, 2008), the TIM + G (rate matrix = [0 1 2 2 3 0]; base frequencies = estimate) and TIMef (rate matrix = [0 1 2 2 3 0]; base frequencies = equal) nucleotide substitution models (Yang, 1994; Zharkikh, 1994) were chosen for the *co1* and *tho2* analyses, respectively. Maximum likelihood trees were generated using GARLI (Zwick, 2006) with 1000 pseudobootstrap replicates. *Diplosoma spongiforme* was used to root the *co1* tree, while *Didemnum* sp. B was used to root the *tho2* tree. The majority-rule consensus trees were generated separately using PAUP* (Swofford, 2003).

### 2.4. Population genetic analysis—co1

Using both *co1* sequences generated in this study and *co1* sequences obtained from GenBank ($n = 365$), samples were grouped into geographic regions (eastern North America (ENA), western North America (WNA), Japan, New Zealand (NZ), and northwestern Europe including Ireland and the UK (NW Europe); see Appendix A). Population diversity measures (number of haplotypes [total and private], relative frequency of haplotypes, and haplotype diversity) were calculated by region using DNASP v5.1 (Librado and Rozas, 2009). Differences in haplotype diversity among regions were tested with series of individual *t*-tests. To maintain overall experimental alpha at 0.05, the *p*-value cut off for significance was held at 0.005 for each *t*-test. Two indices, Tajima's D (Tajima, 1989) and Fay and Wu's H (Fay and Wu, 2000), were also calculated in DNASP v5.1 (Librado and Rozas, 2009) to test for the presence of genetic bottlenecks in each region. A haplotype network was calculated in

NETWORK v4.6.1.0 (fluxus-engineering.com; Bandelt et al., 1999; Forster et al., 2001). Because evenness of sample size among populations can affect the results of some analyses, all population genetic analyses, except the haplotype network, were run on both the entire data set and a randomized subset of sequences that held the number of samples in each region constant (each region $n = 46$).

## 3. Results

### 3.1. Co1 alignment and phylogenetic analyses

A 586-bp fragment of the mitochondrial gene *co1* was sequenced from 221 colonies of *D. vexillum*. Combined with the 71 *D. vexillum co1* sequences previously published by Stefaniak et al. (2009) and the 73 *D. vexillum co1* sequences obtained from GenBank, the total *co1* data set resulted in 23 unique haplotypes (Haplotypes 2, 3, 5, 6, 9–17 previously published by Smith et al., 2012) with 60 variable sites (10.2%). There was 92% bootstrap support for the clade including all *D. vexillum* haplotypes plus *Didemnum* sp. B (Fig. 1). The clade including only *D. vexillum* had 94% bootstrap support and was further subdivided into three subclades: Clade A (98% support) found globally, and Clades B and C (<50% support) found only in Japan. Clade C was found only at one site (Usujiri Fisheries Station) and is represented by only one haplotype.

### 3.2. Tho2—alignment and phylogenetic analyses

Once duplicate sequences were removed, one to six *tho2* alleles were isolated from each of the 75 samples of *D. vexillum* for a total

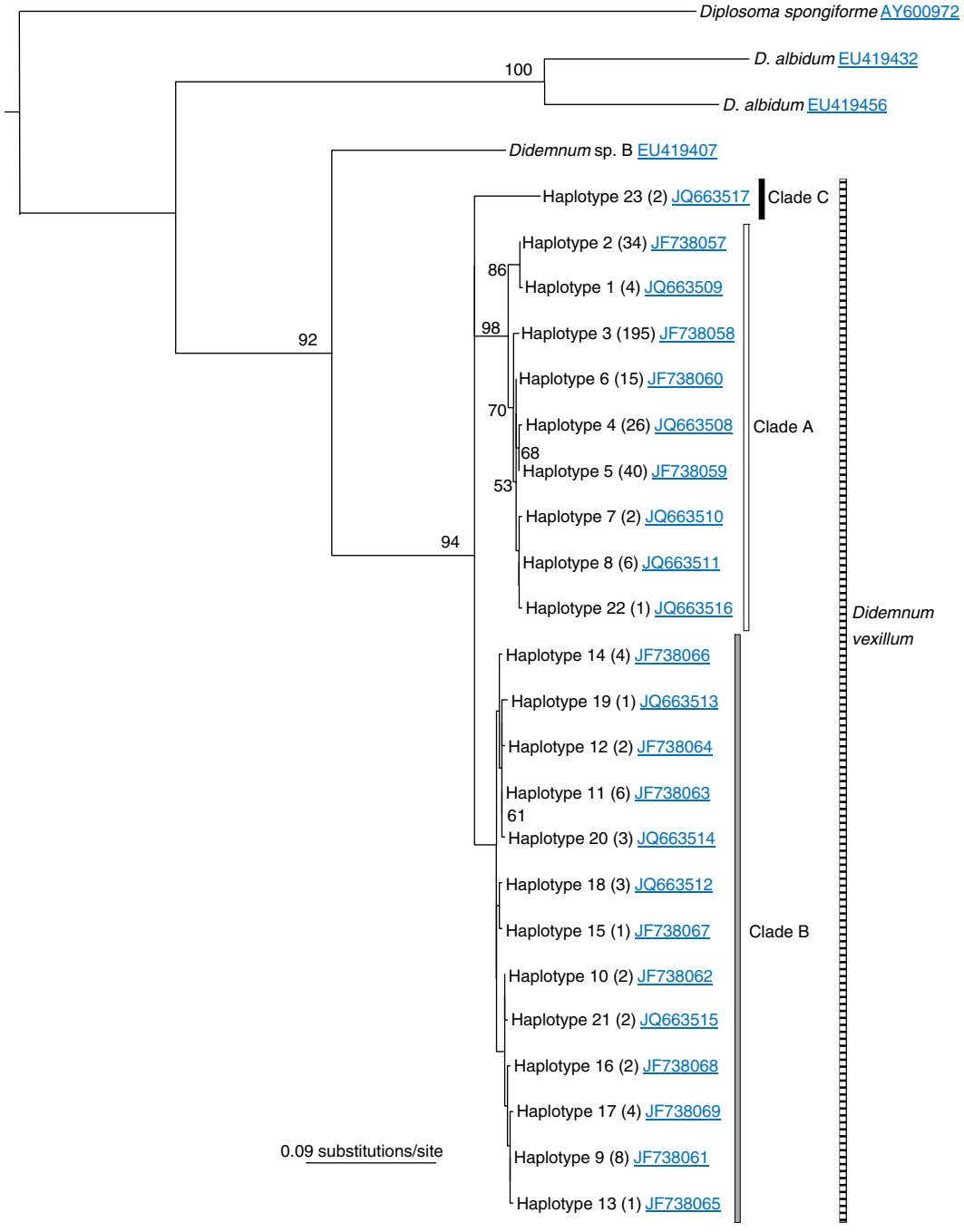


**Fig. 1.** Maximum likelihood tree of *Didemnum* spp. *co1* haplotypes (586 bp). One thousand bootstrap pseudoreplicates were run in GARLI using the TIM + G nucleotide substitution model. All branches with greater than 50% support are labeled. For *Didemnum vexillum* haplotypes, sequences are labeled as "Haplotype number (number of samples) accession number."

of 225 sequences. When aligned, the alleles could be categorized into six groups (Table 1, see Appendix B for alignment). Three groups (Tho2-1, Tho2-2, and Tho2-3, collectively Type 1) were primarily found in *D. vexillum* colonies with a Clade A *co1* haplotype, while the remaining three groups (Tho2-4, Tho2-5, and Tho2-6, collectively Type 2) were primarily found in *D. vexillum* colonies with a Clade B *co1* haplotype. Two colonies with the *co1* Clade B haplotype had *tho2* Tho2-1 alleles. Four colonies with the Clade A *co1* haplotype had at least one *tho2* Type 2 allele. Additionally, one colony from *co1* Clade A had one *tho2* allele that appeared to be the result of a crossover event between a *tho2* Type 1 and Type 2 allele (see Appendix B).

Once the introns were removed, the *D. vexillum tho2* alleles were collapsed into 79 unique alleles and aligned with sequences from *Didemnum* sp. B and *D. psammathodes* for phylogenetic analysis. Alleles can be divided into three categories: 63 alleles from samples that had *co1* haplotypes from clade A, 13 alleles from samples that had *co1* haplotypes from clade B, and three alleles from a mixture of samples that had *co1* haplotypes from clade A and B. All of the *D. vexillum* alleles were grouped into a single clade that had 57% bootstrap support. Within the *D. vexillum* clade, there was no obvious clustering of *tho2* alleles that are associated with *co1* haplotypes from clade A or from clade B, but most branches were not supported above the 50% bootstrap support threshold (Fig. 2).

### 3.3. Co1 population genetic analyses—complete dataset

A total of 23 unique *co1* haplotypes were isolated from the 365 *D. vexillum* colonies analyzed (Table 2). ENA, WNA, and NW Europe each had four haplotypes total. New Zealand had three haplotypes total. Japan had a total of 22 haplotypes, 17 of which were only found in Japan. While one of the 23 haplotypes was not found in Japan, only Japan had haplotypes found in no other region. A single haplotype (Haplotype 3) was most common in WNA, NW Europe, New Zealand, and ENA, accounting for 47.9% to 73.4% of the colonies in each region (Fig. 3). This haplotype was found in only 19.6% of the colonies collected in Japan. The 22 haplotypes found in Japan ranged from 1.1% to 19.6% of the colonies collected there (mean = 4.5%). Haplotype diversity was significantly greater in Japan ($0.925 \pm 0.013$ [Hd ± S.D.]; Fig. 4). NW Europe ($0.657 \pm 0.049$) and WNA ($0.680 \pm 0.044$) also had significantly greater haplotype diversity than ENA ($0.434 \pm 0.056$) and New Zealand ($0.404 \pm 0.048$; Fig. 4). While only NW Europe had a Tajima's *D* value significantly greater than zero, WNA and New Zealand also had Tajima's *D* greater than zero, and all three regions had Fay and Wu's *H* values of approximately zero (Table 2), suggesting that populations in all three regions had recently undergone a genetic bottleneck.

**Table 1**
*Tho2* allele type classification. Classification is based on unifying insertion/deletion mutations for each type. For complete details on other insertion/deletion and substitution mutations, see Appendix B.

| Overall type | Group | Classifying features | Range |
|---|---|---|---|
| Type 1 | Tho2-1 | No major insertions or deletions | Global |
| | Tho2-2 | Insertion: 51 bp at base 330 | ENA, Japan |
| | Tho2-3 | Deletion: 8 bp at base 662 | ENA, WNA |
| | | Insertion: 13 bp at base 775 | |
| | | Insertion: 7 bp at base 888 | |
| Type 2 | Tho2-4 | Deletion: 1 bp at base 135 | NW Europe, Japan, |
| | | Deletion: 5 bp at base 546 | New Zealand |
| | | Deletion: 30 bp at base 645 | |
| | Tho2-5 | Deletion: 1 bp at base 135 | Japan, New Zealand |
| | Tho2-6 | Deletion: 1 bp at base 135 | Japan |
| | | Deletion: 8 bp at base 662 | |
| | | Insertion: 13 bp at base 775 | |
| | | Insertion: 7 bp at base 888 | |

Like the *co1* maximum likelihood tree, the *co1* haplotype network divides into three groups corresponding to clades A (global), B (Japan only), and C (Japan only) (Fig. 5). Focusing on Clade A, it is readily apparent that most regions are missing a number of intermediate or linking haplotypes. Not including intermediate haplotypes which were not found in this study, NW Europe is missing one intermediate haplotype which is filled by haplotype 6 (found in Japan and WNA). ENA has two missing intermediate haplotypes filled by haplotypes 6 and 2 (found in Japan, WNA, and NW Europe). WNA and New Zealand are each missing one intermediate haplotype. In WNA, the missing haplotype is supplied by haplotype 5 (found in all other regions). For New Zealand, the missing intermediate haplotype is haplotype 6. Japan is the only region where all of the intermediate haplotypes were found. Additionally, the only haplotype not found in Japan is located terminally on the network and is only a single base pair different from a haplotype that is found in Japan.

### 3.4. Co1 population genetic analysis—standardized data subset

When the number of samples within each region was standardized, the general trends of the results were unchanged. The overall number of unique *co1* haplotypes was 16 isolated from 230 *D. vexillum* colonies (Table 2). The total number of haplotypes in ENA, WNA, New Zealand, and NW Europe remained the same, while the number of haplotypes found in Japan was reduced to 15, retaining 10 private haplotypes. Haplotype 3 remained the most frequent. Haplotype diversity continued to be significantly greater in Japan compared to all other regions (Fig. 4b). Results of Tajima's *D* and Fay and Wu's *H* continued to suggest that populations of *D. vexillum* in NW Europe, WNA, and New Zealand had recently undergone a bottleneck (Table 2).

## 4. Discussion

Japan was found to be the most genetically diverse region for *Didemnum vexillum*. Japan has the highest total number of haplotypes by more than a factor of five and has significantly greater haplotype diversity compared to all other regions. Japan is the only region with private haplotypes. NW Europe, WNA, and New Zealand show evidence of having undergone a recent genetic bottleneck, and haplotypes found in Japan provide missing links among haplotypes found in other regions on the *co1* haplotype network. While no one test can definitively determine the native range of a species, all of these data strongly suggest that Japan is located within the native range of *D. vexillum* and that *D. vexillum* should be considered non-native in ENA, WNA, New Zealand, and NW Europe. This finding agrees with the historical timeline; the first sample that is thought to be *D. vexillum* was collected in northern Japan in 1926, 67 years before the first confirmation of *D. vexillum* in other regions of the world (Lambert, 2009).

The phylogenetic analysis of *co1* and *tho2* was done to confirm the identification of *D. vexillum* samples because of the difficulty of identifying *Didemnum* species morphologically. While the *D. vexillum* clade on the *co1* gene tree was divided into three clades, the *tho2* tree supports that, at minimum, the *co1* clades A and B belong to the same species. Overall support on the *tho2* gene tree was low, likely due to the short length of the alignment (228 bp), but the *D. vexillum* clade is supported, and the *tho2* alleles associated with *co1* clade A or with *co1* clade B are not monophyletic.

However, the presence of greater than two alleles found in a single copy gene (unpublished preliminary data of the *D. vexilum* genome project) from a single colony suggests the *tho2* sequences isolated from any given sample may not have all come from a single genome. There are two possible explanations. First, colonies sampled may have been chimeras resulting from the fusion of two independent colonies.

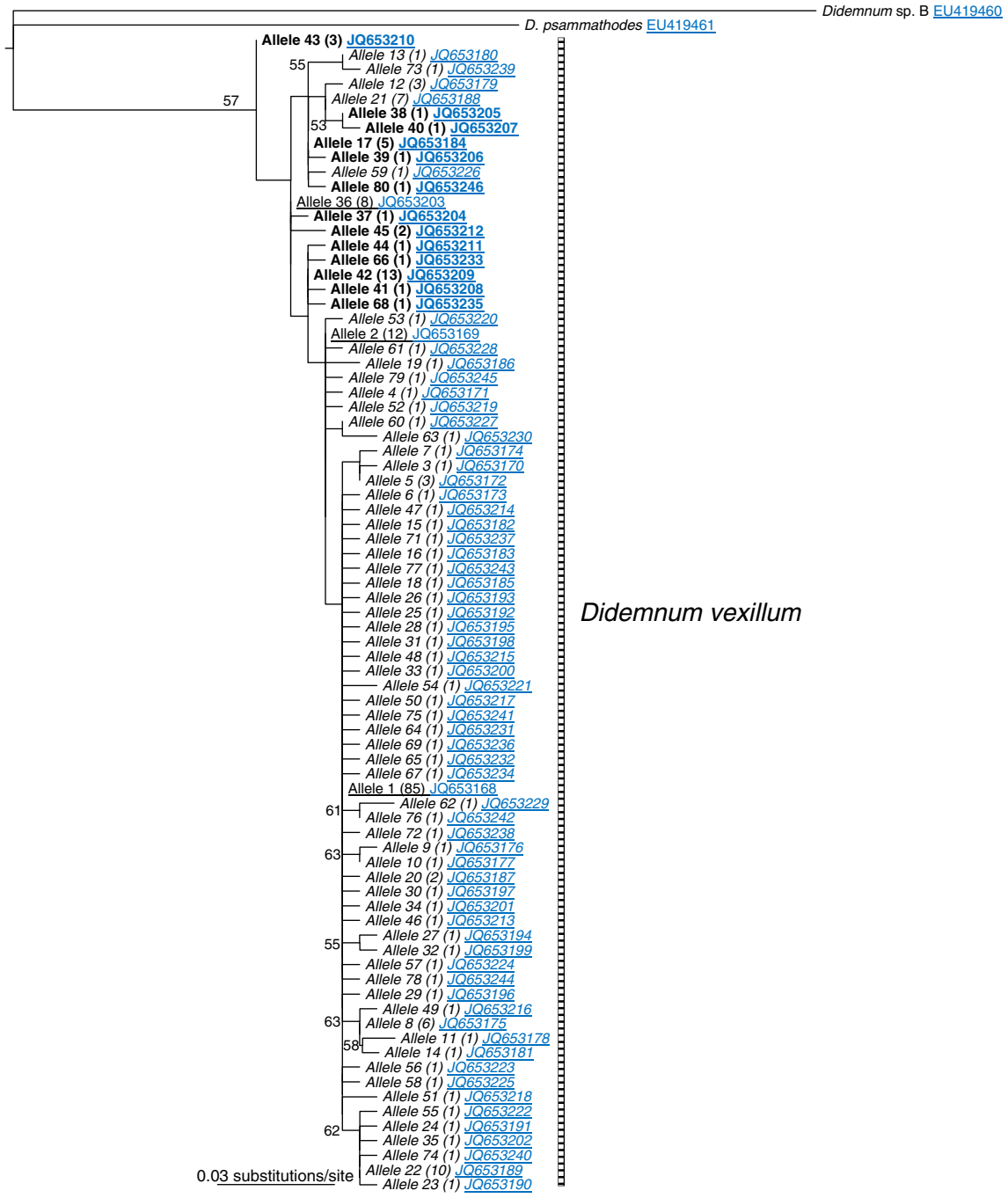


**Fig. 2.** Maximum likelihood tree of *Didemnum* spp. *tho2* exons (228 bp). One thousand bootstrap pseudoreplicates were run in GARLI using the TIMef nucleotide substitution model. All branches with greater than 50% support are labeled. For *Didemnum vexillum* alleles, sequences are labeled as "Allele number (number of samples) accession number." Alleles from samples with *co1* Clade A haplotypes are in italics, with Clade B haplotypes are in bold, and with a combination of Clade A and B haplotypes are underlined.

*Didemnum vexillum* colonies raised from separate larvae can fuse to form a single colony with indistinguishable borders (unpublished data). Zooids thoraces for DNA extraction were collected from a small area of each colony (~1 $cm^2$) to reduce the chance of sampling along a line of fusion. However, Sommerfeldt and Bishop (1999) found that zooids in the colonial tunicate *Diplosoma listerianum* can migrate through the tunic after fusion, interspersing themselves with the zooids from the other colony and thus increasing the chance of collecting zooids from different genetic origins. Another possibility is that the additional alleles were from stored sperm. Colonial ascidians have internal fertilization with sperm collected from the water column (Millar, 1971). Audioradiographs by Bishop and Sommerfeldt (1996) demonstrated that *Diplosoma listerianum* stores sperm in the lumen of the ovary. These sperm may remain viable for up to one month after storage (Bishop and Sommerfeldt, 1996). In our study, only thoraces were used for DNA extraction to avoid paternal DNA contamination from larvae, but this technique may not have avoided contamination from stored sperm cells. In either case, extra *tho2* alleles could be isolated from a single colony of *D. vexillum*.

Whatever the cause, if the Type 2/Japan *tho2* alleles are actually solely linked with the Clade B/Japan *co1* haplotypes and the Type 1/

**Table 2**
Population genetic analysis results by region. Standardized *N* data subset values in parentheses. Bold text indicates significant Tajima's *D* value.

| Region | *N* | Total haplotypes | Private haplotypes | Hd[a] | Tajima's *D* | Fay and Wu's *H* |
|---|---|---|---|---|---|---|
| ENA | 94 (46) | 4 (4) | 0 (0) | 0.657 ± 0.049 (0.677 ± 0.045) | −0.15836 (−0.67125) | −5.92084 (−6.27246) |
| WNA | 48 (46) | 4 (4) | 0 (0) | 0.680 ± 0.044 (0.680 ± 0.045) | 1.67551 (1.72699) | 0.10816 (0.2087) |
| New Zealand | 82 (46) | 3 (3) | 0 (0) | 0.434 ± 0.056 (0.428 ± 0.079) | 1.33754 (1.64983) | −0.84553 (−0.33623) |
| NW Europe | 49 (46) | 4 (4) | 0 (0) | 0.404 ± 0.048 (0.478 ± 0.052) | **2.07268** **(2.17542)** | 0.2602 (0.52174) |
| Japan | 92 (46) | 22 (15) | 17 (10) | 0.925 ± 0.013 (0.916 ± 0.021) | 0.54803 (1.79729) | −9.54181 (−5.58454) |
| Total | 365 (230) | 23 (16) | | | | |

[a] Haplotype diversity.

Global *tho2* alleles with the Clade A/Global *co1* haplotypes, direct sequencing of *co1* PCR products could have masked the presence of the alternate *co1* haplotype, while cloning of the *tho2* PCR product prior to sequencing would have allowed that signal to come through. In that case, the Type2/Japan *tho2* alleles found in samples with the Clade A/Global *co1* haplotype, and vice versa, may not be genetically linked to the sequenced *co1* haplotype, reducing the utility of the *tho2* gene tree to support the 2 *co1*clades as belonging to the same species. However, all *tho2* alleles from colonies morphologically identified as *D. vexillum* aligned well across both introns and exons. Comparisons to the solitary tunicate *Ciona intestinalis* and the sea urchin *Strongylocentrotus purpuratus* genomes (Dehal et al., 2002; Sea Urchin Genome Sequencing Corsortium, 2006), as well as to *tho2* sequences from *Didemnum* sp. B and *Didemnum psammathodes* generated in this study, indicate that while the locations of the introns in *tho2* are well conserved across deuterosomes, the intron sequence is not conserved, even among the three species of *Didemnum*. Taken along with the *tho2* allele that appears to be the result of crossing over between Type 1 and Type 2 *tho2* alleles, this supports the assertion that colonies having both Clade A and Clade B *co1* haplotypes belong to the same species.

It is interesting that while three distinct types of *co1* haplotypes are found in the native range of *D. vexillum*, only colonies with *co1* haplotype Clade A were apparently transported from Japan. One explanation is that Clade A haplotypes are associated with other characteristics that increase invasion success such as tolerance to extreme environmental conditions, a wider range of suitable environmental conditions, or increased predator deterrence. However, another possibility is that, by chance, only colonies with Clade A haplotypes were transported. From the samples analyzed in this study, Clade A and Clade B haplotypes were neither equally abundant (65% and 35% of total samples from Japan, respectively) nor equally distributed among sites. At four of the five sites sampled, Clade A was prevalent, accounting for 70.6% to 100% of colonies sampled. At the remaining site, Misaki Marine Biological Station (Sagami Bay), all colonies had Clade B *co1* haplotypes. Clade C was found only at the Usujiri Fisheries Station (Uchiura Bay). Since the probability of a haplotype being sampled during an invasion event is proportional to the frequency of that haplotype in the native population (Nei et al., 1975), if the invasion process originated at a site with a high proportion of colonies with the *co1* Clade A haplotypes, then it is more likely that colonies with Clade A haplotypes would be transported. This effect would be even more pronounced if the invasions to North America, New Zealand, and Europe were a series of secondary invasions rather than multiple primary invasions happening in parallel.

It should also be noted that while all current evidence indicates that Japan is within the native range of *D. vexillum*, it does not exclude the possibility that the native range of *D. vexillum* extends to continental Asia. This study was only able to sample *D. vexillum*

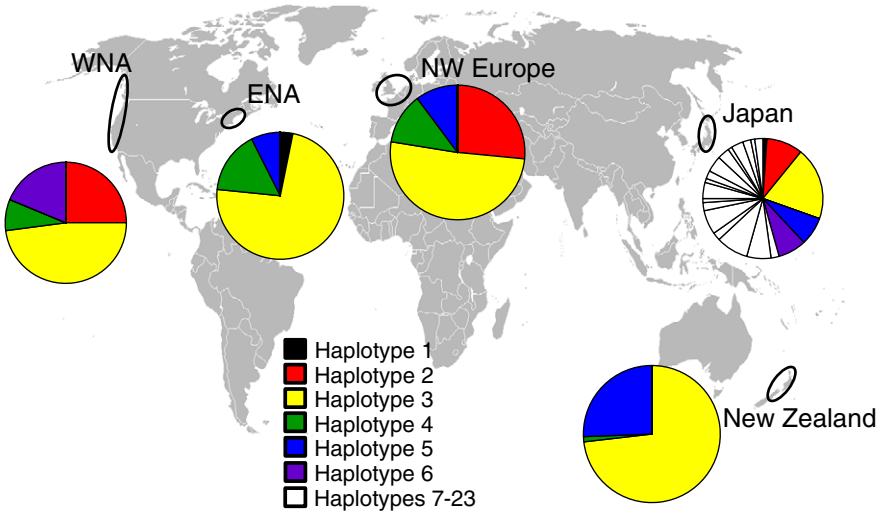


**Fig. 3.** Global distribution of *Didemnum vexillum* and haplotype frequency by region. The ovals in each region indicate the extent of the range of *D. vexillum* in each region. Each pie chart represents the total number of *co1* haplotypes found in each region with the slices of the pie showing the percentage of each haplotype present in the region. The legend at the bottom gives the pattern code for the haplotypes.

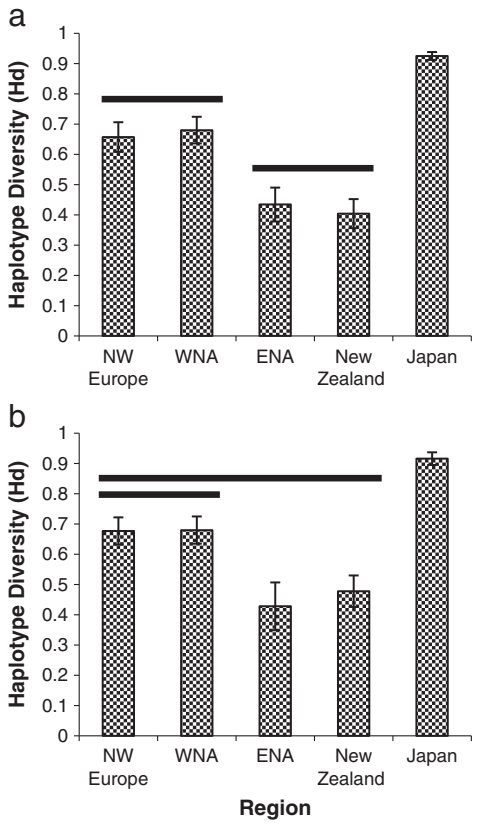


**Fig. 4.** a. Haplotype Diversity (Hd) among regions (total data set). 4b. Hd among regions (standardized subset, $n = 46$, each region). Horizontal black lines indicate Hd levels that are not significantly different. (Hd ± 1 S.D.).

colonies from the Pacific coast of Japan. One didemnid colony collected in South Korea was molecularly identified as *D. vexillum* (*co1* Haplotype 3, Clade A, unpublished data). Until such time as further sampling from the western coast of Japan as well as South Korea, China, and Russia is undertaken using more sensitive markers, the native region of *D. vexillum* should be described as the temperate Northwest Pacific Ocean.

Determining that the relatively recently discovered worldwide populations of *D. vexillum* belong to a single species opens opportunities for comparisons among populations and coordination of projects among ascidian researchers. Similarly, determining that Japan lies within the native range of *D. vexillum* will pave the way for comparative studies between populations of *D. vexillum* living in native areas with co-evolved interactions and populations in exotic environments exposed to a suite of novel interactions. Additionally, this study created a vouchered DNA bank of *D. vexillum* samples from many native populations, which will provide a baseline of native genetic diversity for use in population genetic studies investigating the spread of *D. vexillum* within invaded regions.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jembe.2012.04.012.

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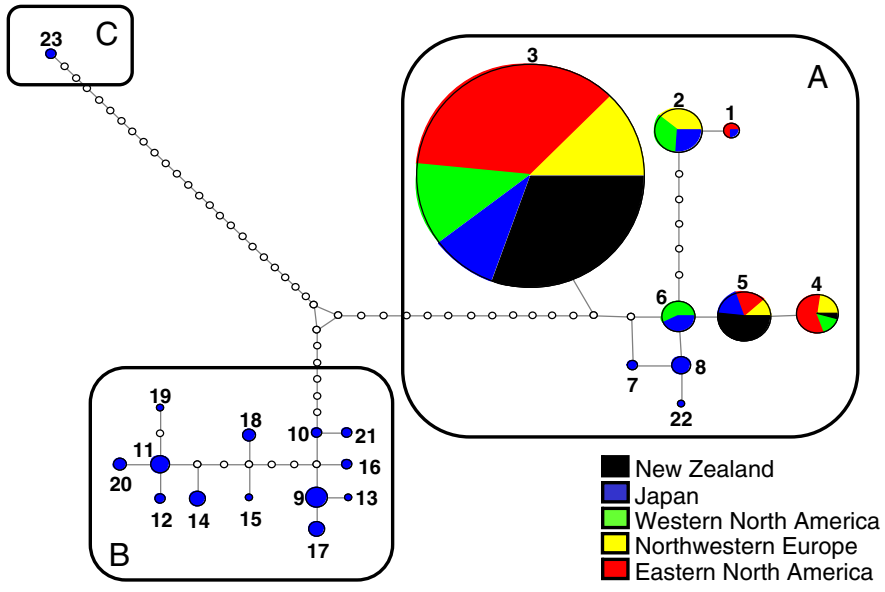


**Fig. 5.** *Co1* Haplotype network. Each circle represents one of the *co1* haplotypes found in *D. vexillum* and the relative size of each circle indicates the number of samples that had that haplotype. The different color slices indicate the percentage of that haplotype found in each region. White dots indicate missing haplotypes not found in our study.

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