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# Discovery and significance of the colonial tunicate *Didemnum vexillum* in Alaska

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## Abstract

The colonial tunicate, *Didemnum vexillum* Kott, 2002, has a history of invading and overgrowing marine communities in temperate waters worldwide. The species can colonize and dominate remarkably large areas of benthic habitat, including coastal bays and outer coastal areas, causing concerns about potential long-term effects on community structure, critical habitats, and fisheries resources. We report here the confirmed occurrence of *D. vexillum* in Alaska, representing a dramatic 1000 km northward extension of this non-native species along the western coast of North America. The species was detected as part of a "bioblitz", engaging citizen scientists to survey local biota and detect non-native marine species incursions. Following detection, the identity of *D. vexillum* was confirmed with robust genetic methods, and morphological characters were also consistent with previous species descriptions. Although invasions have been relatively rare in Alaskan waters to date, it is now clear that *D.* vexillum is established in at least one site (Whiting Harbor) near Sitka, Alaska. Given the explosive growth and spread of this species in other global regions, and its potential for significant impacts across diverse habitats in Alaska, current efforts are underway to evaluate its distribution and options to eradicate or control the species.

**Key words:** *Didemnum vexillum*, ascidian, nonindigenous species, genetic species identification, citizen science, aquaculture, multilocus

## Introduction

The colonial tunicate *Didemnum vexillum* Kott, 2002 (Ascidiacea, Aplousobranchia) has attracted increasing attention as an invader in temperate marine communities around the globe, because it overgrows and dominates existing benthic communities, often over expansive areas (Coutts and Forest 2007; Lambert 2009). *Didemnum vexillum* occurs across a wide range of marine habitats, including exposed outer coasts and natural substrata at depths up to 81 m (Bullard et al. 2007), whereas most marine invasions appear restricted to bays and are associated with artificial or manmade structure (Wasson et al. 2005; Preisler et al. 2009; Ruiz et al. 2009). The capacity to colonize outer coasts is exemplified on Georges Bank off eastern North America, where the species is reported to occur over an area of 230 km$^2$ to depths of 60 m creating a continuous mat in some regions (Bullard et al. 2007; Valentine et al. 2007).

Concerns exist about potential local and regional impacts that may result from *D. vexillum*, due to its ability to spread over larger areas and dominate communities. The species has been damaging to shellfish aquaculture, where it can smother commercial stock in nets and lantern pens (Bullard et al. 2007; Carman et al. 2010; Valentine et al. 2007). It may also affect critical habitat and food resources for fisheries species (Lengyel et al. 2009; Mercer et al. 2009; Smith et al. 2010), although such direct and indirect effects are still poorly understood.

In western North America, *D. vexillum* was first documented in San Francisco Bay, California in 1993, and it has since appeared in additional locations in California, Oregon, Washington and British Columbia (Lambert 2009, Richard Emlet, personal communication, Sarah Cohen, unpublished data). As with many species, the actual date of invasion is uncertain, due to both a potential lag-time in detection as

well as the ability to confirm species-level identification. There are a number of morphologically similar species of didemnid tunicates, making taxonomic identification of existing specimens and links to historical records challenging.

In this paper, we report the first verified occurrence of *D. vexilllum* in Alaska. The species was detected in Whiting Harbor near Sitka, Alaska during a bioblitz, in which volunteers were organized to search intensively for particular non-native species (including *D. vexillum*) some of which had not yet been identified in Alaska but are present to the south (California to British Columbia). Here, we document this discovery of *D. vexillum* and the molecular genetic results to verify its identification, discussing briefly the current status of this species, past observations of a didemnid tunicate in the area, as well as the important role of volunteer citizen scientists in detecting invasions.

## Methods

On 12-13 June 2010, we conducted the Marine Invasive Species Bioblitz in Sitka, Alaska. One of our goals was to engage the public (i.e., volunteer citizen scientists) in searching for a suite of conspicuous non-native species that were previously unknown from Alaskan waters. The target species included the green crab *Carcinus maenas* (Linneaus, 1758) the Japanese kelp *Undaria pinnitida* ((Harvey) Suringar 1873, the bryozoan *Watersipora subtorquata* (d'Orbigny, 1842 ), and the tunicates *Ciona* spp, *Styela clava* (Herdman, 1881), and *Didemnum vexillum*; we also surveyed changes in the distribution of two recent invaders, *Botrylloides violaceus* (Oka, 1927) and *Botryllus schlosseri* (Pallas, 1766), which are established in Sitka (G. Ruiz et al. 2006; Wang 2011). The volunteers had a wide range of backgrounds, most with limited knowledge or experience involving invertebrate identification. Training was provided to participants through a slide presentation to assist with recognition of target organisms, a hands-on microscope examination of selected species, interactive discussion with experienced surveyors, and distribution of laminated field handouts with pictures and key characters of target species.

During the two days, ten sites were surveyed by two to ten volunteers per site (Figure 1). Each site was assigned a team leader, who coordinated a visual search of anthropogenic structures and natural substrata (Table 1). Up to four types of habitats were identified at each site and as many as possible were surveyed during a -0.6m low tide, including, a) shoreline between high and low tide line, b) docks (including structures hanging from docks such as lines, buoys, etc) c) boat hulls and d) aquaculture nets. The only aquaculture farm surveyed was in Whiting Harbor and was accessible only by boat. The other nine sites were reached by land, and six of them were harbors. Searches were conducted for one hour at Whiting Harbor and up to three hours at all other sites. Depths in the harbors ranged from 2 to 4 meters, while the depth at the Whiting Harbor survey site was approximately 13 meters.

Whenever one of the target non-native species was potentially found, representative samples were collected for further examination and preserved in 70-95% ethanol. Only one new putative target species, which was unknown to occur in Alaska, was detected during the bioblitz. It appeared to be *D. vexillum* based on gross morphology. Specimens of the organism were analyzed furter using molecular methods to confirm identification. Samples of *D. vexillum* for genetic comparison were also collected from floating structures in Sausalito, California in July 2010; from shell debris near seagrass in Tomales Bay, California in February 2009; and a near shore location in New Castle, New Hampshire in May 2002. Subsequently, the Alaskan Bioblitz samples were examined by microscope for diagnostic morphological characters.

For genetic analyses, genomic DNA extraction was carried out with Clontech columns, using three modified protocols that involved varying the method of tissue harvesting and initial processing. Tissue processing methods included a) soaking overnight in lysis buffer while shaking, b) drying out overnight at room temperature to evaporate the ethanol, or c) processing directly with 2-3 water rinses. Subsequently, the tissue was finely minced, without specifically isolating zooids from the tunic, prior to DNA extraction. All 3 methods of preparing sample tissue provided robust amounts of DNA and subsequent PCR products.

PCR methods and primers generally followed Stefaniak et al. (2009) and Hess et al. (2009) with slight modifications of PCR conditions (e.g., varying annealing temperatures to optimize reactions). Samples were prepared for


**Figure 1.** Map of Alaska showing inset of Sitka with the bioblitz survey sites: Ferry Terminal, Cove Marina, Eliason Harbor, Thomsen Harbor, ANB Marina, Sealing Cove, Crescent Harbor, Totem Flats, Sawmill Cove, Whiting Harbor Oyster Farm.

sequencing using a SAPexo reaction followed by cycle sequencing and analysis on an ABI 3130 Avant DNA Analyzer. Sequences were aligned in Sequencher 4.8 and basecalls were determined by eye with manual editing in comparison to reference sequences in Genbank.

*Didemnum vexillum* sequences from Whiting Harbor were compared to five San Francisco Bay samples, one New Hampshire sample, and to published references (Stefaniak et al. 2009; Hess et al. 2009). In total, comparisons were made among 77 apparent conspecific individuals and three congeneric species. The two anonymous nuclear loci were obtained with PCR primers that were designed by Hess et al. (2009) to selectively amplify *D. vexillum*, against a panel of two congeners (*D. perlucidum* and *D. duplicatum*) and some other tunicate and non-tunicate taxa. Reference samples of *D. vexillum* from Genbank are globally distributed, with a regional emphasis on current areas of invasive concern in Washington State, USA and the northwest Atlantic, USA (Hess et al. 2009; Stefaniak et al. 2009).

**Table 1.** Sitka survey sites and density data for *Didmenum vexillum* from the bioblitz in 2010: P= present. Habitat types: S=shoreline to low tide line, D=docks, B=boat hulls, C= aquaculture nets, NS=not surveyed, NA=not applicable as the habitat types in question were not present at that particular site.

| No. | Sites | Habitat | 2010 | Latitude | Longitude |
|---|---|---|---|---|---|
| 1 | Ferry Terminal | S | 0 | 57.12932 | -135.3798 |
| | | D | NS | | |
| | | B | NS | | |
| 2 | Cove Marina | S | 0 | 57.11723 | -135.3887 |
| | | D | 0 | | |
| | | B | 0 | | |
| 3 | Eliason Harbor | S | 0 | 57.05763 | -135.355 |
| | | D | 0 | | |
| | | B | 0 | | |
| 4 | Thomsen Harbor | S | NS | 57.05586 | -135.3512 |
| | | D | 0 | | |
| | | B | 0 | | |
| 5 | ANB Marina | S | NS | 57.04941 | -135.3418 |
| | | D | 0 | | |
| | | B | 0 | | |
| 6 | Sealing Cove | S | 0 | 57.0482 | -135.351 |
| | | D | 0 | | |
| | | B | 0 | | |
| 7 | Crescent Cove | S | NS | 57.05007 | -135.3282 |
| | | D | 0 | | |
| | | B | 0 | | |
| 8 | Totem Flats | S | 0 | 57.04521 | -135.3138 |
| | | D | NA | | |
| | | B | NA | | |
| 9 | Sawmill Cove | S | 0 | 57.04531 | -135.23 |
| | | D | 0 | | |
| | | B | NS | | |
| 10 | Whiting Oyster Farm | S | NS | 57.04554 | -135.3715 |
| | | D | P | | |
| | | B | NA | | |
| | | C | P | | |

## Results

### *Detection of Didemnum vexillum during bioblitz survey*

*Didemnum vexillum* was found at only one of the ten surveyed sites (Figure 1), the oyster farm in Whiting Harbor. All specimens were found on fouling lines or lantern nets hanging from the aquaculture docks within a few meters of the surface. The species formed extensive mats at three locations at the site, including two separate nets and one line, and occluded the mesh surface of the lantern nets (Figure 2). Colony growth and overall appearance resembled *D. vexillum as* described previously on the east and west coasts of North America (see Lambert 2009) and elsewhere. At the time of discovery in June, water temperature was 12$^{o}$C and salinity was 28 PSU in Whiting Harbor.

### *Confirmation of genetic identity for Didemnum vexillum*

The samples from Whiting Harbor were confirmed as *D. vexillum* based on comparison of DNA sequence variation at four loci, including the frequently used metazoan mitochondrial barcoding locus, cytochrome oxidase 1 (CO1) and three nuclear loci, including one coding locus, *tho,* and two anonymous loci, DL2.1A1 and Dnr1 (Table 2).

The sequence obtained from Alaskan samples for one of these loci, DL2.1A1 matches published *D. vexillum* haplotypes either directly or through visual phase inference from direct sequenced PCR products. Similarly, alleles obtained from Alaskan samples at the Dnr1 locus were found to match published SNP (single nucleotide polymorphism) haplotypes (i.e., genotypes based on patterns of variable nucleo-

**Figure 2.** *Didemnum vexillum* on oyster lantern net at Whiting Harbor Oyster Farm, Sitka Alaska, June 12, 2010, with close up. Photos by Linda Shaw NOAA.

**Table 2.** Genetic comparison of Alaskan allele identity to published global database alleles for one mitochondrial and three nuclear loci, with information from the global database on distribution records of the allelic matches.

| Type of locus | Locus name | # differing AK alleles (total # AK alleles sampled) | # global variants (total # global alleles sampled) | AK allele identity | global database locations matching AK samples | # global allele matches to AK alleles | Primer, database reference |
|---|---|---|---|---|---|---|---|
| Mito-chondrial | CO1 | 2 (2) | 18 (71) | 4 | Pacific only: Japan, BC oyster farm | rare (2/71) | Stefaniak et al. (2009) |
| | | | | 11 | global, both sides of Pacific and Atlantic | common (37/71) | |
| Nuclear, coding | tho2^ | 2 (4) | | | | | Stefaniak et al. (2009) |
| | exon | 2 or more (4) | 108 (114+) | NA | NA | NA | |
| | intron | 2 or more (4) | NA | NA | NA | NA | |
| Nuclear, anonymous | Dnr1 | 2-3 (4) | 4 (72) | 4 | NE Pacific and NW Atlantic: WA, ME | rare in Atlantic (1/58); Pacific (2/14) | Hess et al. (2009) |
| | | | | 1 or 2 | global (allele 1) or Japan only (allele 2) | AK allele 1: common (33/ 72); AK allele 2: rare, (1/72) | |
| Nuclear, anonymous | DL2.1 A1 | 2 (4) | 4 (74) | 2 | global, both sides of Pacific; west Atlantic | Common (19/74) | Hess et al. (2009) |
| | | | | 4 | both sides of Pacific and Atlantic | (26/74) | |

^ Indels complicate scoring in more detail without cloning.

tide sites) obtained through direct sequencing or phase inference (referred to as reconstructed genotypes in Hess et al. 2009, Table 2).

An additional robust confirmation of species identity with previously reported *D. vexillum* comes from a match of the *tho* intron obtained from the Alaskan and other new samples in this study. These samples show conserved flanking exon with the genbank *tho* exons and conserved flanking intron (445 nt intron with 7 SNP sites)

with the Alaskan sequence obtained in this study (S. Cohen, unpublished data).

*Geographic comparison of Alaskan haplotypes with global sampling*

Mitochondrial CO1 haplotypes found in Alaskan samples match 2 known haplotypes (4 and 11) in Genbank (Table 2). One is widely distributed, and the other is limited to occurrences at two known locations, including a single occurrence in North America at an aquaculture facility in British Columbia and a single occurrence in Japan (Stefaniak et al. 2009).

Nuclear loci provided PCR products and sequences that match existing *D. vexillum* sequences in Genbank and in newly genotyped samples (Table 2). However, rare allele matches were not found with these loci in samples available for comparison at this time.

*Morphological characteristics of Alaskan specimens*

Alaskan samples were examined morphologically by G. Lambert for diagnostic characters, including tunic, spicules, zooids, sperm duct, and larvae, all which matched published descriptions of this species (Kott 2002; Lambert 2009). Thus, morphological and genetic traits provide a consistent outcome in species identity.

## Discussion

*Identification of Didemnum vexillum*

We confirm the presence of *Didemnum vexillum* in Whiting Harbor near Sitka, Alaska. This is 1000 km north of the closest verified populations in British Columbia (Lambert 2009), extending the documented latitudinal range of this global invader to 57$^{o}$ latitude and the edge of the temperate zone. At this location, temperature ranged from 3.7$^{o}$C in February to a high of 15.1$^{o}$C in August from 1996-2011, over the 15 years data is available (NOAA NOS/CO-OPS ODIN 2011), and salinity is reported to range from 24 to 30 PSU in the Sitka area on an annual basis (Heather Meuret Woody, unpublished data).

The identification of *D. vexillum* in Alaska is robust for several important reasons. Firstly, using the traditional barcoding locus CO1, the 2 Alaskan samples genotyped match 2 known samples identically (haplotypes 11 and 4). This alone does not preclude identification error, since there are various known reasons for CO1 matches that do not reflect biological species; these include low rates of molecular divergence in some taxa (including *Didemnum*), incomplete lineage sorting, and hybridization (DeSalle 2006; Lou and Golding 2010; Ballard and Whitlock 2004). To avoid these errors we considered available molecular data at several loci to determine species identity. Secondly, primers used to amplify two of the loci (DL2 and Dnr1) were designed by Hess et al. (2009) to exclude two congeners, as well as more distant species, although there are currently few reference sequences for known congeners (Hess et al. 2009; Stefaniak et al. 2009). Finally, intron sequence from Alaskan samples at the *tho* locus matched intron sequence from non-Alaskan *D. vexillum*.

Combined use of these four diverse loci (CO1, DL2, Dnr1, and *tho*) in genetic analyses, *D. vexillum*, provides a robust answer to species identity. We show strong genetic similarities in both mitochondrial and nuclear genomes, including coding and noncoding sequence. Comparison of sequence data from both genomes additionally excludes interspecific hybridization that would be undetected with exclusive use of the mitochondrial CO1 barcoding locus. This information is ecologically relevant, because interspecific hybridization could occur between native and invasive species, and it has the potential to alter the population dynamics of an invading species.

Tunicates have high levels of genetic variation reportedly due to rapid molecular evolution (Tsagkogeorga et al. 2010), though this cannot be distinguished from morphological stasis and a long evolutionary history. Thus, the finding of shallow genetic divergence in *D. vexillum* CO1 and *tho* exon DNA sequence in global sampling appeared to highlight a genetic bottleneck due to founder effects during invasions (Stefaniak et al. 2009), and this limited variation appeared to be substantiated in several additional nuclear loci (Hess et al. 2009). Similarly, in this study, DL2 alleles found in Alaska (Hess haplotypes 2 and 4) are both previously reported in both Atlantic and Pacific Oceans at high frequencies (Stefaniak et al. 2009) and thus, they offer little discriminatory population information. Dnr1 revealed no new alleles, but some additional information related to geographic distribution is discussed below.

Surprisingly, however, a small number of new non-Alaskan samples revealed intriguing levels of variation at some of these same loci that may be useful for discovering invasion history and pathways. The *tho* locus intron alignment shows conspecificity, a lack of indels in a comparison of new samples in this study, and 7 SNPs were found in just 6 sequences. At the DL2 locus, additional samples from central California (n=6) and New Hampshire (n=1) revealed at least three additional DL2 alleles (3 in California and 2 in New Hampshire), suggesting that further use of this locus may provide additional source and vector information.

*Source*

The source of *D. vexillum* in Alaska is not immediately evident, due to limitations in the available data, including a) our small sample size to date from Alaska, b) the low level of variation that *D. vexillum* shows in global genetic variation at the CO1 locus, c) limited sampling in many regions including possible source areas such as Japan, and d) the global distribution of a common haplotype (11).

Despite these constraints, some important information is provided by an uncommon haplotype for CO1. The two CO1 haplotypes identified from Alaska include the relatively rare haplotype 4, matching just two samples (the marine lab at Otsuchi Bay, Japan and an oyster farm on Cortes Island, British Columbia) in 71 database samples (Stefaniak et al. 2009). Based on this limited sample size to date, Japan and British Columbia are at least two potential sources of introduction into Alaska.

In addition, there is a possibility of regional patterning suggested by the anonymous nuclear locus Dnr1. In the Alaskan samples, which are both heterozygotes, each contains an allele (Hess haplotype 4) that is only found in 3 other instances in the global database (72 individual samples, > 142 alleles sampled). All 3 cases are from the states of Maine and Washington, possibly suggesting there may be genotypes associated with northern latitudes, although more sampling is required. While the sample size in this report is extremely limited, the genotyping thus far of the Alaskan samples shows diversity at all four loci, both in haplotype variability and heterozygosity. The samples do not show genetic uniformity and are therefore not from a single clone. Thus, in addition to confirming the identity of *D. vexillum* in Alaska, the genetic variability reported here may also serve to increase the probability of high demographic performance (e.g., growth, reproduction, spread), since genetic diversity may confer advantages in this respect (Facon et al. 2006).

In sum, all loci indicate this newly detected population in Alaska is genetically diverse and that it contains genotypes that may show regional patterning. Although the geographic distribution of samples is still limited, it is possible that some genotypes may be well-suited to cold-water environments like Alaska. Increased sampling in Alaska and elsewhere, combined with experiments to examine performance, is needed to test for such a relationship. This approach could also provide critical insights into the potential source and transfer mechanism (vectors) for *D. vexillum* in Alaska.

*Current status, invasion history, and potential impacts in Alaska*

At the present time, the known distribution of *D. vexillum* in Alaska is restricted to Whiting Harbor near Sitka. It is now evident that the population is established here. Since the bioblitz, we have documented that *D. vexillum* covers relatively large areas of both natural and manmade substrate in Whiting Harbor, subtidally and intertidally to depths of 16 meters. We are now working with multiple agencies and organizations to determine the extent of the species' distribution by SCUBA, ROV and shoreline surveys, and to advance measures to eradicate or control the population as well as to prevent further spread of this species in Alaska; the latter includes strong public outreach and also exploring options to limit or minimize access to infested areas. Part of this plan includes additional sample collection to expand the number of alleles examined to get a clearer picture of how *D. vexillum* may have been transported to Alaska, and this in turn can shape our management strategies to prevent new incursions.

The timing of colonization of Whiting Harbor is uncertain. Following our recent discovery and confirmation of *D. vexillum*, we located anecdotal reports and even some photos of organisms from the same site that may be *D. vexillum* dating back to 2000 (T. Davis, unpublished data). However, to our knowledge, species level identifications are not available for any of these organisms, and we have not yet

been able to locate specimens that can provide taxonomic confirmation of earlier material.

It appears that *D. vexillum* is capable of extensive spread in Alaska's marine habitats, based on reports from eastern North America (Bullard et al. 2007; Valentine et al. 2007). Given its ability to overgrow resident biota, this species may pose a significant risk to Alaska's natural resources. Unlike other parts of North America, there are still relatively few non-native marine species at high latitudes, and Alaska has therefore experienced low impacts from such invasions to date (Ruiz and Hewitt 2009). Although several new invasions have occurred in Alaska in recent years (Ashton et al. 2008; Lambert et al. 2010), none of these are known to have severe impacts. In contrast, *Didemnum vexillum* has a history of explosive growth and spread. This species has the potential for severe local and regional impacts (given the potential areal extent) to benthic community structure, aquaculture, and fisheries resources.

One of the most important keys to management of invasive species is early detection as it can allow time for more management options, reduced costs and more successful outcomes (Anderson 2005). In terrestrial habitats, surveys can often be mounted with limited cost and logistical issues, and new species may be recognized relatively quickly. In aquatic habitats, it is sometimes costly and difficult to conduct extensive surveys. Aquatic invaders may often go undetected for years to decades (Grigorovich et al. 2003; Lambert 2009), particularly small invertebrates and species which have morphologically similar congeners throughout the world (Knowlton 1993; Geller et al. 2009).

In Alaska, the sheer size of the marine habitat relative to the small size of the scientific community and overall low population density there, further limits our ability to detect species invasions. We are now actively expanding a state-wide network for detection of non-native marine species, by engaging Alaskan citizens in the monitoring process. Our goal is to increase the participation of citizen scientists, from school children to adults, to address a significant gap that exists in our ability to track the arrival and spread of new invaders, such as *D. vexillum*. This approach increases opportunities for management response, through early detection, and also providing insights about where and how species are arriving to the state.

The lack of basic knowledge on colonial ascidians, in general, and of marine fauna in nearshore Alaska, highlights the issues with working in understudied regions and taxa. Lack of background information can severely hamper rapid response (Coutts and Forrest 2007), exacerbating invasion threats and complicating management issues. Baseline monitoring and surveys such as the bioblitz, are critical tools to be used to reduce the threat of invasive species. Surveying must be accompanied by significant attention to taxonomic and systematic data and comparison to global databases. This is particularly important for resolving issues of cryptic native versus invasive species, as well as in gaining information about potential population sources and vectors. The bioblitz and subsequent genetic analysis allowed us to detect an important invader that might otherwise have continued to go unnoticed for some time. Monitoring and bioblitz activities, involving communities and scientists working together, can provide a model for the early detection of conspicuous invasive species throughout the world.

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