

*Tunicate: Northward spread, diversity, source, and impact of non-native tunicates in Alaska:
Establishing a monitoring and education network*

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Final Report

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The opinions expressed in this PWSRCAC-commissioned report are not necessarily those of PWSRCAC.

PROJECT DESCRIPTION

Objectives

Our overall objective is to measure the source, species diversity, and impacts of nonindigenous tunicate species that are spreading northward along western North America. Our specific objectives are to:

1. Measure the arrival and spread of nonindigenous tunicate species in Alaska;
2. Identify the geographic source(s) and transfer mechanism(s) for tunicate species arriving to Alaskan waters;
3. Characterize the species diversity for botryllids, which may include cryptic (multiple) species that may be unrecognized at the present time;
4. Establish baseline data to measure changes in the number of invasions and their consequences at multiple sites in Alaska over time;
5. Use tunicates and fouling community monitoring as an education and outreach tool to engage local communities, including school and citizen groups.

Hypotheses

Using measures across a distributed network of sites, we wish to test the following hypotheses about non-indigenous tunicate species:

- Most newly established populations in Alaska are derived from multiple distant sources (California – Washington), instead of the nearest neighboring populations;
- Most newly established populations in Alaska occur in close proximity to oyster culture and/or derive from source bays with oyster culture;
- There are several cryptic species of botryllids along western North America that have not been recognized to date;
- The number of botryllid species decrease with increasing latitude;
- Once established, new populations undergo rapid increases in abundance (percent cover) and have significant effects on the species richness and abundance of native species.

Approach

Collections.

We will obtain collections of botryllids (*B. schlosseri* and *B. violaceus*) to test (using molecular analyses, below) for the presence of cryptic species, latitudinal gradients, and the source(s) of nonindigenous tunicates along western North America, with particular focus on Alaska. Specimens will derive from multiple sources. First, we will establish a network of citizen scientists in Alaska for detection and collection of botryllid tunicates. Second, we will also make extensive collections in San Francisco Bay, by visiting at least 10 different marinas/piers throughout the bay to make voucher collections. Morphological variation and some preliminary molecular data suggest that cryptic species may exist in this region (Cohen and Carlton 1995; S. Cohen, unpubl data). Moreover, the project will be based at SFSU, allowing easy access to this

bay. Third, we will utilize voucher material collected and preserved in our earlier surveys (Fig. 1; see Introduction). Fourth, we will augment these materials with collections as needed from bays in southern California, Oregon, and Washington.

Molecular sampling methodology.

Multiple individuals per geographic site will be characterized (genotyped). Sampling will be stratified so that sites will have ≥ 3 -5 individuals genotyped to provide species level identification. Some sites will have more intensive sampling to test for diversity within site (10-20 individuals per site). Sampling will also be stratified to accommodate any morphological anomalies. Locations with known introduction dates, due to previous SERC collections or other records, will additionally be emphasized. Levels of genetic variation (within species, and within and between population) will be used to infer possible patterns of invasion. For example, low genetic diversity may be interpreted as a single introduction or strong post-introduction selection. High genetic divergence between sites suggests multiple introductions. Further resolution of source and pattern will be achieved by comparison with samples from other locations including taxonomic vouchers.

Expected Outcome

This project advances several research and outreach goals. During the 1-year project, we will accomplish the following research goals:

- Test for the presence of cryptic species and a latitudinal gradient in botryllid species diversity along western North America, using molecular tools. The presence of a species complex, if confirmed, has significant implications for the distribution and impact of invading tunicates, since species may differ greatly in their environmental tolerance and interactions with resident communities.
- Characterize the source region(s) and possible vectors for nonindigenous species arriving to Alaska, using tunicates as a model system or indicator for a broader range of taxa.
- Establish a baseline of measures of native and non-native species diversity at several key sites in Alaska.
- Establish a citizen science network to understand invasion patterns and risks in Alaska. We expect to implement a pilot project that demonstrates the feasibility and opportunity to engage a distributed network of citizens to track invasion dynamics across Alaska and the western United States.

FINAL REPORT

Overview

We have achieved our project goals, both in establishing a network of sites (to provide baseline measures) and in using genetic tools to characterize botryllid tunicates invasive to Alaska in comparison to global population samples. These two core elements have laid the groundwork to effectively evaluate the status of introduced botryllids that are found, now and in the future, in Alaskan waters. Toward this end, we have completed extensive collections and genetic analyses of botryllids along western North America (and especially Alaska). Simultaneously, we have experienced a high level of interest, enthusiasm, and participation in the citizen science network, which shows great promise as a low-cost platform to advance diverse education and research goals. We are now working with our partners/collaborators to establish a sustained citizen network to track changes in the geographic distribution and abundance of these tunicates, and possibly other groups, with particular focus on northward spread of non-native species to Alaska.

Sampling Strategy & Citizen Network

Our project was focused on the US west coast and especially characterizing the northward expansion of botryllid tunicates into Alaska. As outlined in our original proposal, this requires the measurement of (a) the current and changing distribution of these organisms across large distances in Alaska and (b) comparisons of collections from multiple geographic regions using molecular techniques. Thus, a key component of our research was to establish a coherent sampling effort and standardized protocols, across a very broad geographic range.

To provide the necessary spatial and taxonomic coverage of samples for molecular analyses, we have developed multiple pathways to acquire specimens, including (a) a distributed network of sites in Alaska, (b) focused collections by our team from bays in California to Washington, (c) historical samples of non-native species collected throughout North America (from surveys funded by National Sea Grant, Regional Citizens' Advisory Council, and Department of Defense), and (d) selected collections by colleagues from other global regions. We discuss each of these in more detail below.

(a) Distributed Citizen Science Network

In June 2006, we initiated efforts to build a distributed network of sites in Alaska to measure presence and abundance of botryllid tunicates, and to provide specimens for molecular analyses (see next sections). This began with a 1-day, multi-agency planning meeting at Kachemak Bay, Alaska, to review and refine implementation. Included in this meeting were participants from Alaska Department of Fish & Game, Kachemak Bay National Estuarine Research Reserve, Prince William Regional Citizens' Advisory Council (RCAC), San Francisco State University, Smithsonian Environmental Research Center, and U.S. Fish & Wildlife Service. This meeting was designed to develop specific sites and details for a distributed network that involves citizen scientists, who can voluntarily implement standardized measures throughout Alaska. We initiated measures in June 2006 for several sites (Kachemak Bay, Port Valdez), as a model for broader expansion in 2007.

Based upon experience in 2006, we refined our protocols and have expanded the number of sites, In 2007, the sampling network included 8 participants in Alaska and 3 in California covering 15 locations. New participants have joined our effort, increasing the network to 11 participants in Alaska, 1 in British Columbia and 3 in California, covering 19 locations by September 2008 (Table 1).

Table 1. Geographic Distribution of Citizen Science Network Sites

Location	Date joined network
Dutch Harbor, AK	June 2007
Kodiak, AK	September 2007
Kachemak Bay (4 sites), AK	June 2006
Chenega Bay, AK	June 2008
Valdez, AK	June 2006
Cordova, AK	June 2007
Tatitlek, AK	June 2008
Yakutat, AK	December 2007
Glacier Bay, AK	June 2007
Juneau (2 sites), AK	June 2007
Petersburg, AK	June 2007
Sitka, AK	June 2008
Ketchikan (2 sites), AK	June 2007
Prince Rupert, B.C.	June 2008
Berkeley, CA	June 2007
Sausalito, CA	September 2007
Tijuana Slough National Estuarine Research Reserve, CA	June 2007

The citizen science network includes middle and high school students and their teachers, fisheries biologists, university professors and students, and state and federal agency biologists, many of whom had little to no background in marine biology. Organizations that have participated in the network to date include:

Alaska

- The Yakutat Salmon Board, Yakutat
- Yakutat High School, Yakutat
- Oceans Alaska Marine Science Center, Ketchikan
- Southern Southeast Regional Aquaculture Association, Ketchikan
- Alaska Sea Grant/University of Alaska, Ketchikan
- Alaska Sea Grant/University of Alaska, Fairbanks
- Kachemak Bay Research Reserve, Homer
- Prince William Sound Science Center, Cordova
- Glacier Bay National Park and Preserve, Gustavus
- Petersburg High School, Petersburg

Admiralty Island National Monument, Juneau
National Marine Fisheries Service, Habitat Conservation Division, Juneau
Prince William Sound Regional Citizens Advisory Council, Valdez
Kodiak High School, Kodiak
Chenega Bay EPA/Chenega IRA Council, Chenega Bay
Sitka Tribe of Alaska, Sitka
Tatitlek School, Tatitlek
Department of Transportation

British Columbia

Alaska Marine Highway, Prince Rupert

California

Pinole Valley High School, Pinole
Richardson Bay Audubon Center, Tiburon
Willow Creek Academy, Sausalito
Tijuana Slough National Estuary Research Reserve, Imperial Beach
California Polytechnical Institute, San Luis Obispo (anticipated 12/08)

Key to this expansion was (a) the involvement of the Alaska Department of Transportation Marine Highway, and (b) the enthusiasm and communication of network participants in Alaska. The DOT assisted with locating individuals in locations served by the ferry system, in transporting, free of charge, materials to these individuals, and in solving other logistical problems. A DOT employee in Prince Rupert (British Columbia) is also involved in deploying plates from the ferry dock there, providing coverage in an area that is likely to see increased shipping and invasive species over the coming years. The Alaska participants have established several modes to provide good communication and interaction, including regular phone conference calls that have been initiated and sustained by this group, taking significant ownership in advancing the project.

The protocol for 2007 called for each site to deploy 10 PVC settling plates in June 2007 and retrieve these in September 2007, sampling at least once per year. Nearly all participants have sampled quarterly, retrieving and redeploying plates in June, September, December, and March. Participants fill out data sheets for each plate, recording percent cover data and descriptions for all species or morphotypes present. In addition, each plate is photographed, and species of special interest are photographed in as much detail as possible. Participants sample Botryllid tunicates or suspected Botryllids and send these specimens to us. In addition to these biological measurements, participants deploy continuous water temperature loggers and take notes on nearby land and water use as well as any other relevant observations. Participants who have the equipment to do so also measure water column parameters such as salinity and turbidity.

Although the project has now ended, we are actively working to sustain and expand the citizen science network. Because of its distributed nature, both geographically and across many individuals, this provides a cost- and time-efficient approach to study the dynamics of invasions. Our next steps in advancing the citizen science network are to (a) develop a web-based portal for

participants to upload data and access a wide range of information resources on invasions, (b) create a forum for network participant discussion, (c) expand the number of sites (and participants), and (d) include a larger number of non-native species that are being examined through network activities.

(b) Collections in California - Washington

We implemented focused collections in bays from southern California to Washington. These collections are intended to provide high-quality photographic images for each vouchered (preserved) tunicate specimen, which is being sequenced, allowing us to link morphological characters to genetic data. In general, we sampled two different sites in each of the major bays in this region. At each site, we sought to obtain $n \geq 10$ colonies, selected to maximize differences in gross appearance and color. See attached protocol and Figure 1, below for further description.

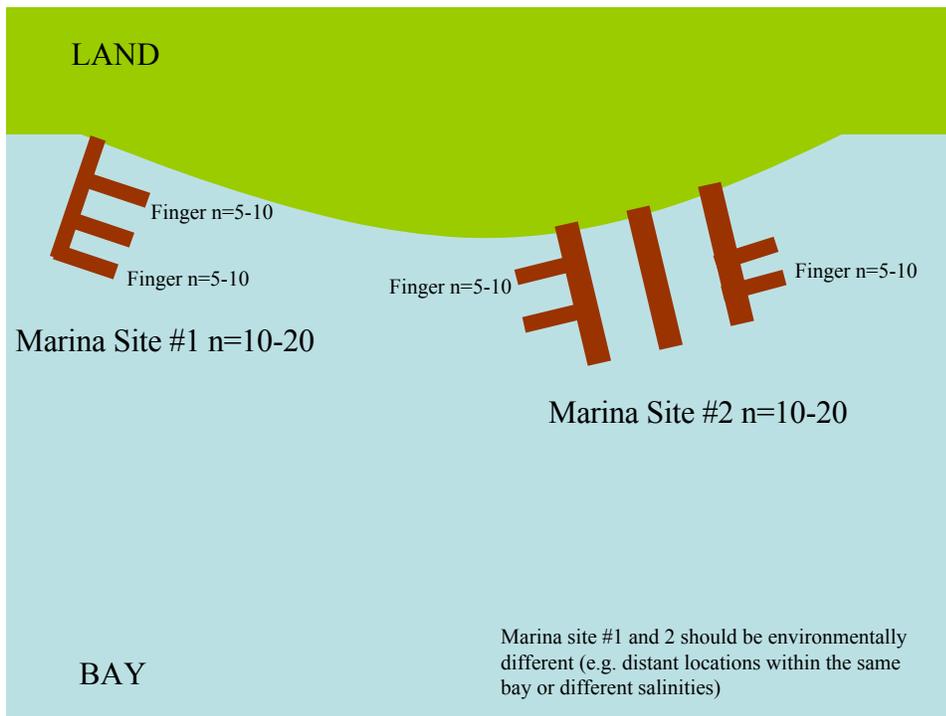


Figure 1. Collection strategy within bays to increase the diversity of ecophenotypes, genotypes, and potential species.

(c) Historical Samples from North America

As outlined in our proposal, we used voucher materials from previous surveys on North America (both coasts) for additional molecular analyses. While the coverage and amount of voucher material available here is great (see distribution of sampling sites in Figure 2, below), these specimens do not include photographs for linking morphological and molecular data. Instead, these previous collections are being used to increase sample size and geographic coverage in key areas.

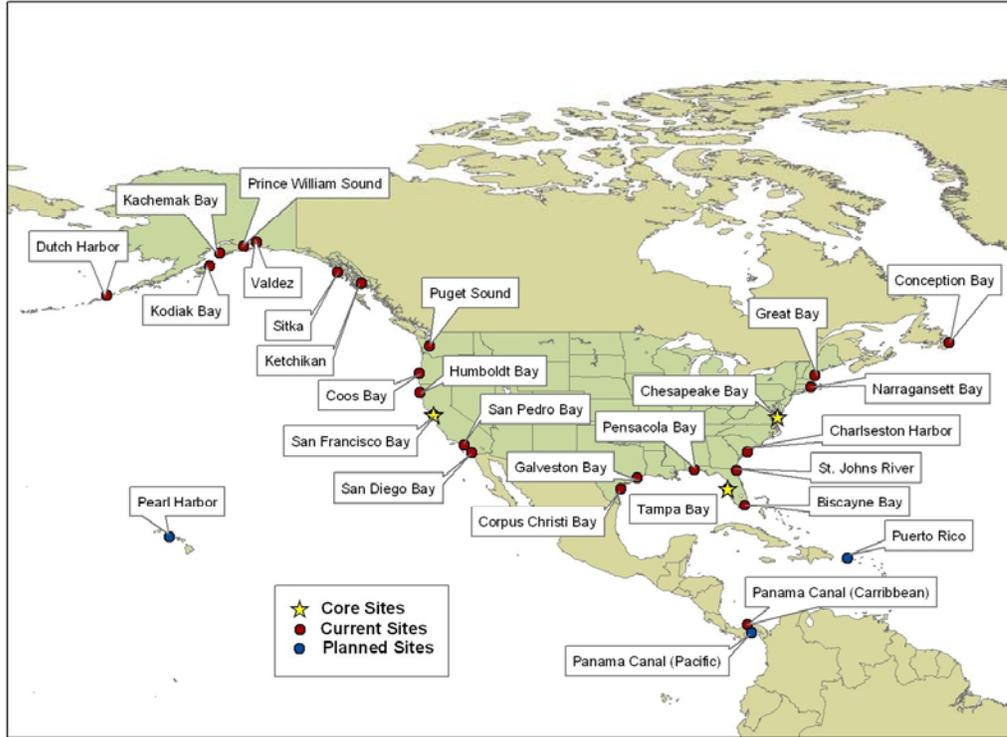


Figure 2. Location of Bays included in Nationwide Survey of Sessile Invertebrate Communities in North America. All sites were surveyed at least once, following identical protocols outlined in text. Core sites have been surveyed multiple times, across years and seasons.

(d) Selected Collections from Other Global Regions

Finally, and importantly, we have also obtained specimens from other global regions for our analysis. Without this material, our analysis of botryllid tunicates in western North America would be incomplete and leave key issues unresolved. Of particular importance are the specimens from Japan, where Dr. Yas Saito has provided detailed morphological, life history, and taxonomic analyses. We have used material from Japan to provide taxonomic identification, and as a basis of comparison, for specimens along the west coast of North America. At the present time, additional material has been provided for us from Europe, Africa, Australia, Panama, and South America.

Overview of genetics samples processed

As of September 2008, we have successfully analyzed > 300 specimens of botryllid tunicates, obtaining genetic sequence for CO1 (bar-coding locus). We are using these data in phylogenetic analyses along with additional data from Genbank, creating a total sample size of 350 botryllid sequences, and this number continues to expand weekly. Figure 3 shows the global distribution of samples that have been analyzed to date.

Non- US botryllids sequenced to date as vouchers

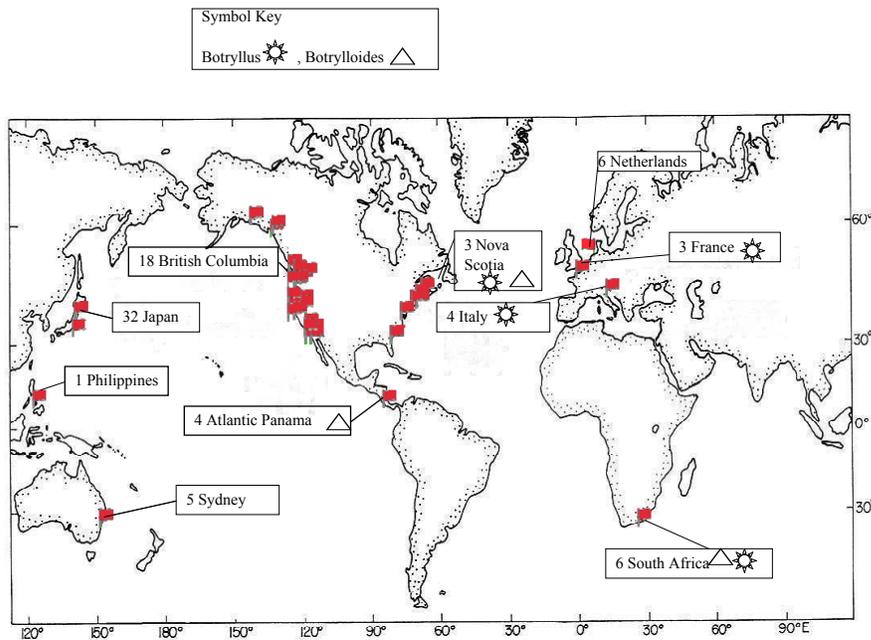


Figure 3. Geographic distribution of completed sequences. Note that only the samples from outside the United States. (Note that genus names are tentatively assigned by collectors providing samples and await further verification).

Genetics methods

Following protocols outlined in the proposal, genomic DNA is extracted from ethanol preserved specimens, derived as above. The preserved samples are soaked in water and buffer to remove alcohol before DNA extraction using a Nucleospin tissue kit with slight modifications. PCR is carried out using a customized alteration of the Folmer CO1 primers (Folmer et al. 1994) and other custom primers. PCR products are cleaned using a Shrimp Alkaline Phosphatase/Exonuclease I reaction followed by cycle sequencing using Big Dye Terminator v. 3.1 (ABI). Capillary sequencing is carried out on an ABI 3130 genetic analyzer at the Romberg Tiburon Center, SFSU. We re-extract, re-PCR, and re-sequence approximately 10% of samples as a control for contamination or human error.

PCRs are carried out with a variety of conditions depending on templates. We are using various PCR run conditions, buffers, taqs, and primers to obtain sequence from problematic samples. Most samples are straightforward to run, but where reruns are needed, the issues most likely relate to either template quality in a few cases, primer mismatches in more distant taxa, or possible competition with pseudogenes. We have not discovered any obvious pseudogenes in our analyses, i.e., sequences interrupted by stop codons or reading frame shifts. We have a few samples that show unexpected sequence divergence suggestive of pseudogenes.

For most samples we use single-pass automated sequencing using dye-labeled dNTPs run on an ABI 3130. In a certain number of cases, including some problematic cases, we do reverse sequencing on the same or a new PCR product to resolve ambiguities or confirm sequence reads.

Genetic Data Analysis Methods

Sequences were aligned and edited by eye using Sequencher 4.8 software (Gene Codes) and then exported to DnaSP 4.50.3 (Rozas et al. 2003), MEGA4 (Tamura et al. 2007), and Paup*4.0b10 (Swofford 2003) for divergence estimates and phylogenetic analyses using distance and character-based analyses in the neighbor-joining, parsimony, and maximum likelihood algorithms. Modeltest 3.7 (Posada and Crandall 1998) was used to find the best-fit model of nucleotide substitution for the full dataset, as well as various partitions. DNA divergence estimates were used to make preliminary species diagnoses, generally following bar-coding guidelines of at least 3 individuals with less than 2% sequence divergence amongst them to define a clade. Where possible, we compared these clades to known morphological vouchers so that taxonomic names could be established for our collections. Phylogenetic analysis is being used to examine historic relationships between individuals in different populations. This information allows us to infer likely sources and patterns of spread of novel species in Alaska and other locations.

Appropriateness of CO1 for botryllid invasion genetics

At the level of Styelidae, CO1 is predictably saturated at third base positions and this is likely causing some primer mismatches for more distant clades. This interpretation is inferred from issues with more basal botryllids (previously defined using both 18S and morphological analyses).

Resolution of genera

It is not clear whether CO1 analysis produces monophyly for each of the two genera, *Botryllus* and *Botrylloides*. Analyses continue to suggest a more complicated scheme (as also suggested based on non-genetic analyses by Saito et al. 2001).

Overview of Genetic Results to Date

Resolution of species and discovery of morphologically cryptic species

Using specimens from throughout the world, we have resolved 9-10 clades to date that appear to be distinct species. This relies on a criterion of having at least 3 individuals per clade as confirmation. If we include clades with less than 3 samples sequenced (including sequences from Genbank), our estimate of genetically distinct botryllid species from our sampling goes up to 14 highly divergent species level clades. Divergence levels of at least 9% are the rule between these clades with one exception of approximately 3% (where separate species status warrants further investigation).

Five clades match vouchers from Dr. Yas Saito (Japan) that include both morphological and most importantly life history characters based on rearing work. These five vouchered clades are: *schlosseri*, *primigenus*, *violaceous*, *fuscus*, and *simodensis*. Two uncertain clades cluster most closely with *simodensis* and include only warm water samples.

Another of the unknown clades (i.e., no accompanying described voucher species) was present in samples from southern California to Bodega Bay, the Netherlands, Sydney, and a sample from Japan that is currently being described by Saito's graduate student. This clade is the only one having samples described as having the "big zooid" morphology (probably not a size difference, but rather a color marking difference). However, not all samples in this clade show the "big zooid" pattern. A second unknown clade consists only of samples from Florida.

All samples presented by Saito as distinct species based on culturing studies have shown species status based on high divergence levels (minimum of 18%). Two clades were not distinguishable morphologically by Saito, but were found to be different species based on his rearing studies of life history characters and were subsequently confirmed by CO1 sequence divergence to be distinct species (*violaceus* and the unknown spp currently being described). Thus, morphological and life history characters are able to distinguish genetically differentiated species, though it may take a combination of detailed morphological and growth and reproductive studies for even a specialist to distinguish them at the current time.

Botryllid species in Alaska

To date, two species of botryllid tunicates have been confirmed in Alaskan waters, including *Botryllus schlosseri* and *Botrylloides violaceus*. No botryllid tunicates have been detected or collected north of Sitka in southeastern Alaska during this study. Given that the extent of sampling was relatively limited during the current project, lack of detection should not be taken as absence; a more detailed survey is needed to evaluate the northern range limits of these taxa, and we hope the citizen science network will develop further for this purpose.

(a) Summary of *Botryllus* findings. Sitka samples of *B. schlosseri* were limited in diversity in comparison to other west coast samples (Oregon and California). They occurred in fewer clades and showed less divergence between haplotypes, compared to samples from further south on the Pacific coast of the US. Of the two haplotypes currently found in Sitka, one (the less common one in Sitka) is also found in warmer waters (San Diego) while the other (more common haplotype in Sitka) has not been detected south of San Francisco. The two Alaska haplotypes together only show a match with a single east coast sample from Maine. Three additional clades found on the U.S. Atlantic coast were not found in Alaska haplotypes.

Our genetic data for *Botryllus* suggest:

- *Botryllus schlosseri* invasion into Alaska is derived from Pacific locations.
- The northward expansion of southern populations is the most plausible source, rather than the western Pacific, based on low genetic diversity and shared (common haplotypes).
- Genetic diversity in Alaska is limited compared to more southerly locations both in species numbers, clade diversity, and haplotype diversity. ***However, haplotype diversity has increased since the Smithsonian originally collected in 2001.***
- Compared to Alaska, haplotypic or nucleotide diversity is higher in all other sampled regions, including Oregon, California, the East coast, the Mediterranean, and Japan, despite lower sampling intensity in many of these regions.
- No other *Botryllus* species, other than *schlosseri*, have been found in Alaska to date.

- *Botryllus schlosseri* was not detected during our analysis of samples from Ketchikan, where another botryllid occurs (see below).
- Water temperature is unlikely to limit this species' distribution in southeastern Alaska, given its occurrence in Sitka; thus there is a high likelihood of spread in this region.

(b) Summary of *Botrylloides* findings. Twenty-eight specimens of *Botrylloides* from Alaska were successfully sequenced for our analysis. *Botrylloides* was found in Sitka and Ketchikan and 14 samples from each location were sequenced. All sequences fell within 2 clades that are presumed to represent *B. violaceous*. Nearly all (27/28) samples belong to the most abundant clade of botryllid in our analysis (147 individuals share this haplotype out of our total botryllid database of 351 sequences, approximately 40%). This clade matches the *violaceous* morphological voucher from Y. Saito. One Ketchikan sample falls in a separate clade from this main one, and shows less than 3% divergence indicating it also is *violaceous*.

The large clade that most Alaskan samples match is widely distributed throughout the Pacific US coast, from San Diego to Coos Bay, and then Alaska. It also occurs along the Atlantic US coast, from Connecticut to Nova Scotia (sampling beyond this range is limited and may under-represent the current distribution along this coast). The smaller clade with one Ketchikan representative is found globally in Holland, in the northwestern Atlantic (Rhode Island and north), and in the northeastern Pacific from San Francisco north. This pattern mirrors the 2 clade pattern seen in *B. schlosseri* haplotypes in Alaska: one clade shows broad distribution across temperature zones and the other shows a more northern distribution.

Our genetic data for *Botrylloides* also are consistent with an eastern Pacific (i.e., western US) source region. Only one species has been detected in Alaska to date, whereas additional species have invaded California. It appears the scope for spread is great for *B. violaceous*, both within southeastern Alaska and likely throughout south central Alaska.

(c) Conclusions on Alaskan botryllid invasions.

- At the present time, only two distinct botryllid species are known to occur in Alaskan waters.
- The pool of botryllid species and haplotypes along the western US, and worldwide, suggest diversity may increase through time.
- Non-native botryllid tunicates currently have a very restricted distribution in Alaska but should be expected to spread. There is a great deal of apparently suitable habitat in Alaska that is not yet colonized by botryllids. In addition, the opportunity for human-mediated transfers (especially due to hull-fouling on vessels and aquaculture) is increasing through time.

Appendix 1

Additional Project Information

Training

This project has provided training in genetics, collection and identification methods including field IDs for collecting and detailed morphological identification methods in the lab. A Master's thesis at SFSU will also result from this project and associated funding. Also, adjunct to the project and with support from NSF REU, UMB, and SFSU College of Science and Engineering funding, additional undergraduates (5 students including 2 under-represented minority students) and a local high school teacher and high school student have learned about field collection, field experimental design, morphological IDs, and genetic work. Material developed in this project is also used in Cohen's Marine Ecology courses on main campus and at RTC, including information about invasions, biology of the botryllids, fouling communities, and sampling with fouling panels, and use of molecular markers in invasion research to trace patterns of invasion and carry out taxonomic and systematic studies.

Presentations on aspects of this project have been made or are anticipated in the following venues (*Undergraduate NSF fellow, **Graduate Sea Grant trainee):

Rodelo*, A. and **S. Cohen**. Salinity tolerance in invasive ascidians. COSE, SFSU. 2007. 5th prize undergrad poster.

Rodelo, A* and **S. Cohen**. Estuarine Research Federation, November 2007, Providence, Rhode Island, "Salinity tolerance in invasive ascidians".

Donald, J* and **S. Cohen**. Growth rates of invasive colonial ascidians *Botryllus* and *Botrylloides* spp. in San Francisco Bay. COSE, SFSU, 2007.

RTC, SFSU 2 seminars by graduate student Verena Wang**

Donald, J* and S. Cohen, Western Society of Naturalists Meeting, Vancouver, BC, November, 2008.

Additional students (undergraduate and post-bac) working on topics related to the project (sources of support are indicated):

Amy Rodelo*, SFSU, NSF REU, UMB—Salinity tolerance of invasive botryllids; genetic variation in SF Bay area samples.

Jessica Donald*, SFSU, NSF UMB – Growth rates of invasive botryllids from estuarine and outer coast environments

Ritchelle Quiambao*, SFSU, NSF REU -- Preservation protocols for invasive botryllids; systematic characters

Patrick Lee*, SFSU – Preservation protocols for invasive botryllids; Systematic characters

Anton Horwath*, SFSU, NSF REU—Preservation protocols for invasive botryllids, systematic characters, characterization of behavioral variation in lab cultures.

Additional funding sought and obtained

We have applied to NOAA for additional funding to augment the scope of the project and provide additional student support and we are planning a major submission to NSF.

We have successfully obtained funding to study related questions of botryllid invasions in San Diego from California Sea Grant as a one year Rapid Response award to Cohen and Ruiz, with a Sea Grant graduate traineeship to Verena Wang (2008-2009). With this award, she is continuing to compare west coast botryllids using additional loci for higher resolution of spatial patterning from Alaska to San Diego.

Appendix 2

Tunicate Sampling Protocol *For special targeted collections of Botryllids*

Developed by Sarah Cohen, Karen Alroy & Greg Ruiz
(Version: July 2007)

An Introduction to Tunicates

Tunicates are marine invertebrate (without a backbone) animals that are commonly found attached to rocks in the coastal ocean. If you spend time around harbors and marinas you are likely to have seen them attached to docks and pier pilings. Tunicates have a swimming larval stage, but most species undergo metamorphosis to turn into adults that are permanently attached to substrate. Tunicates may be solitary (single animals, like humans) or colonial (living attached to one another).

Both solitary and colonial tunicates have soft, sac-like bodies and feed by filtering water. Water is drawn in through an incurrent siphon and expelled through an out current siphon. The siphons are visible on the larger solitary tunicates, which will sometimes expel water when touched, earning them the nickname “sea squirts.”

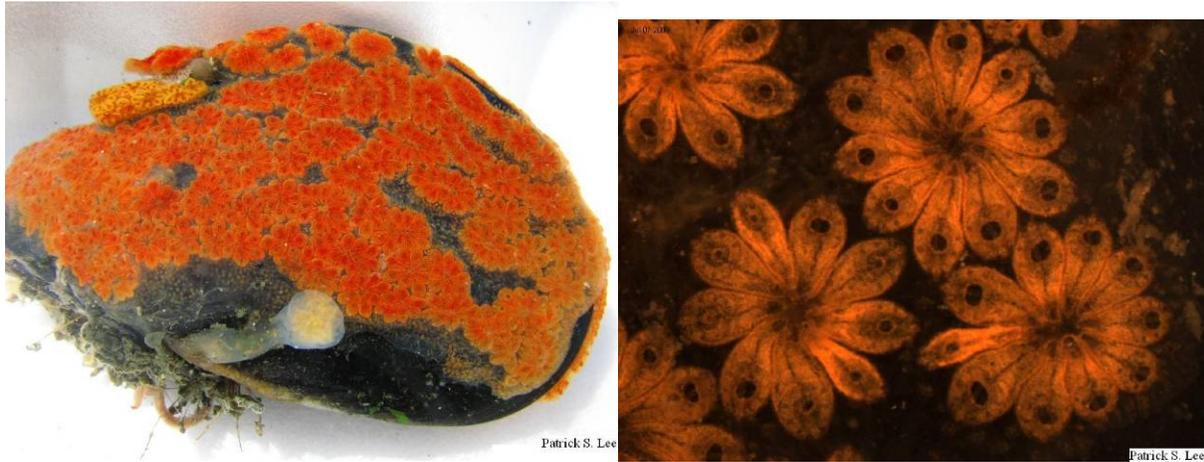
Colonial tunicates are much smaller and live embedded in a common “tunic”; their siphons are difficult to see without a microscope. Colonies start when a larva attaches to substrate and undergoes metamorphosis to become an adult tunicate, called a zooid. The first zooid then replicates itself asexually through a process called budding, creating additional zooids all connected via the living tissue of tunic, which may be translucent and gelatinous or thick and leathery. While the individual zooids are small, colonies can be quite large. In some tunicate species, zooids are arranged in patterns, such as clusters in the shape of flowers or stars.

In the field, tunicates can sometimes be difficult to distinguish from sponges. In general, sponges feel “spongy” – compressing and then springing back when touched, while tunicates tend to resist compression. The siphons on solitary tunicates will usually retract if the animal is touched; sponges may have openings that resemble siphons, but these are not usually paired, and they do not retract. Colonial tunicates generally are slick and shiny, while the surface of sponges is usually dull or porous.

Colonial tunicates are highly variable in color, size, shape, and attachment substrate. Botryllids, the type of colonial tunicate in which we are particularly interested, may be any color including orange, yellow, black, cream-colored, red, blue, and others. They may have striking patterns with multiple colors.

Our Focus is Botryllid Tunicates

Botryllid tunicates are commonly found on a variety of submerged and occasionally intertidal surfaces including docks, ropes, boats, mariculture pens, trays, and nets, pilings. They are also found on seagrass and rocky substrate in intertidal and subtidal habitats including boulders, walls, and even small cobble.



In left photo above, a mussel with two *Botryllus* colonies: a large orange sheet, and a small yellow/orange colony in upper left. Close-up photo of a *Botryllus* is shown in above right photo, showing the flower-shape arrangement of modules (zooids). Also present (bottom middle of upper left photo), small *Distaplia* colony (a different type of colonial tunicate that often forms stalked buttons, mushroom or club-shaped colonies, or large mounds). In contrast to *Distaplia*, botryllid tunicates are generally more sheet-like and thinner, though they may also grow in larger gelatinous blobs or even strings depending on substrate availability).

The photo below shows a piece of a *Botrylloides* colony. By comparison with *Botryllus*, note the meandering, less flower-shaped arrangement of modules (zooids).



We are interested in obtaining samples of both of these types, *Botryllus*, with its flower-shaped zooid arrangements, and *Botrylloides*, with its more meandering zooids.

It is important to note that both *Botryllus* and *Botrylloides* colonies occur in a wide diversity of colors and shapes (see figure below). Some of these differences are thought to reflect species-level distinctions. We are therefore seeking samples of different colors and shapes for each *Botrylloides* and *Botryllus*, as available at each collection location.



Collecting Protocol for Botryllids

Overall, we are interested in obtaining samples of any botryllid-like colonies for molecular and morphological identification, to examine geographic variation. We are obtaining specimens from localities around the globe for comparison.

A. Collecting Location.

Within a single location (e.g., an individual bay, estuary, or harbor), our collecting protocol is to select two different marinas or other hard substrate that are considerably distant from each other. Ideally, we wish to maximize possible environmental conditions (e.g., salinity, temperature, exposure, contaminant influences, shipping proximity, etc.) that may affect species and genotype composition. Since environmental conditions may differ with distance, and these tunicates have very limited larval dispersal, we are using distance in our sampling strategy.

At each marina, select two sampling sites (e.g., two docks in a marina) that are far apart. Collect 5-10 samples from each dock. In total we would like to collect 10-20 tunicate samples per marina. The individual specimens can be collected haphazardly from anywhere on the dock. One of our major goals is to gather specimens representing different morphologies, so please keep your eyes open for differences in color, zooid arrangements or shape and include these in your collection.

Colonies of each tunicate genus are usually visible just below the waterline at low tide or on floating docks. Floating docks or even lines/ropes in the water offer the easiest access, since colonies can be accessed at any tidal condition.

B. Collecting the Tunicate Colonies.

Tunicates can usually be removed easily from the hard substrate, using any tool such as a razor, knife, or tweezers. Slide the tool underneath a colony edge. Tweezers can also be used to detach the colony edge. After you have lifted a good portion of the colony edge, you will be able to pull most of the colony from the substrate for photographing and preservation.

Once removed, colonies should be photographed and placed into ethanol as quickly as possible. Animals that are not detached from the substrate, like colonies on mussel shells, settlement plates, or small cobbles can be kept safely in a tub or bucket of water for a few hours as long as they stay cool. As a general rule, it is best to take ethanol and vials with you into the field for rapid preservation following collection and photographing of live samples.

There should be no safety concerns with the collection of tunicates outside from the general issues that arise in any marina/harbor/bay environment. While you can touch the tunicates without worry, some people are sensitive to some types of sponges, which may co-occur with tunicates, and stinging hydroids may be present at some sites. In general, these are concerns for more tropical, rather than temperate waters. If you are concerned, wear rubber gloves or dive gloves while reaching under docks to retrieve organisms.

C. Photograph of Specimen.

Please take a close-up digital photograph of the tunicates BEFORE storing in ethanol. Ideally, we would like two different shots per colony: One that shows the entire colony and one that shows details of the zooid system for each specimen you collect (see first figure in this protocol for example of each). If you have to choose one, please take the close-up.

Number each specimen you collect, starting with 1 for the first one you collect, up to 40. If possible, it's best to place a label in the photograph, indicating the sample number, date, and location. Place the number that matches your specimen number in the closeup shot. The labels can be placed in the vial with the specimen after the photos are taken.



We have found that tunicates are best shot in shallow tubs of water, although very flat colonies can be photographed out of water. In both cases, the most difficult problem to overcome is glare, either on the surface of the colony (out of water) or on the surface of the water for specimens in water. We have had the best results shooting in bright light with an umbrella or other light shade over the area to be photographed. Sometimes simply positioning yourself to shade the tunicate will be sufficient. Placing the tunicates or tub on a dark background gives the best contrast for your shot. If you have this feature on your digital camera, play the shot back and zoom in to make sure that you have crisp details before moving onto the next photo.

D. Data Sheet.

Please fill out a data sheet, indicating where and when you collected and how many specimens you collected. Ideally, this would be a simple table that includes Per Specimen: sample number, location (name and lat/long, if available), photo number(s), date, water depth.

In addition to the data requested on the sheet, please make notes about whether the tunicates you collected are abundant at your location, hard to find, or extremely hard to find. If you know either how long they have been in the area (= first date recorded there), and at that specific location, that would be very helpful.

If you have equipment on hand, we are also interested in knowing the temperature and salinity of the water at the time you collected as well as the GPS coordinates for your collection sites. This is not required.

E. Preservation of Specimen.

After a photograph of each specimen is obtained, please place each one in a separate vial (5-20ml), filled with ethanol. Each vial should be labeled with the specimen number, ideally with an internal label in pencil. The specimen should be moved to ethanol as quickly as possible after collection, and should spend little time out of water.

Ideally, 90-95% ethanol is best. If this is not available, please try to get as high a concentration as possible. Also, please do NOT use denatured ethanol.

When available, we would like samples of at least 2 cm square per colony, but we can use much less for DNA (as little as a few zooids, in theory). The extra material will aid in morphological work and provide backup tissue. However, don't hesitate to collect small colonies, particularly if they are different-looking.

Most importantly, the specimen must not take up more than 1/3 of the volume of the collection tube – more than this and it is not likely to preserve well, making DNA extraction more difficult (see photographs and explanation below). Similarly, the number of specimens collected will depend upon your access to high grade ethanol and collection vials.

[Whenever possible, in the U.S., we will attempt to get ethanol and tubes to you in sufficient quantities. We are limited in the amount of ethanol we can mail (30 ml/package). If we send you ethanol through the mail, you will have fewer and smaller tubes, which means you will have to adjust your collection accordingly. We (probably Sarah) will work with you to guide your collection strategy, depending on what preservation resources you have.]

Tubes should not be overfilled because the material will not preserve well. When the sample is placed in the tube, it should be able to slosh up and down the tube if it is tilted back and forth. The ethanol should be able to flow easily around the sample. Samples may be broken in half within a tube to facilitate ethanol mixing around the sample, but please make a note if you do break a sample into pieces in the vial. If your samples are being returned to our lab by car, we may give you larger tubes to facilitate return of larger, unbroken colonies

Samples to be mailed/shipped back to us should be well wrapped in parafilm or duct tape and triple-bagged in plastic bags to catch any leakage. Available transport mechanisms may vary by location, especially for overseas collection, so we may need to discuss available options.

For genetics and collection questions please contact Sarah Cohen, Sarahcoh@sfsu.edu 415-338-3750 and give your name and contact information.

If you have any questions regarding shipping please contact Greg Ruiz, ruizg@si.edu or Chela Zabin, zabinc@si.edu 415-435-7128.