Genetic variability of *Botryllus schlosseri* invasions to the east and west coasts of the USA

Douglas S. Stoner[1,2], Rachel Ben-Shlomo[3,4], Baruch Rinkevich[3,*], Irving L. Weissman[1,5]

[1]Hopkins Marine Station, Stanford University, Oceanview Boulevard, Pacific Grove, California 93950, USA

[2]Department of Biological Sciences, Program in Marine Sciences and Baruch Institute, University of South Carolina, Columbia, South Carolina 29208, USA

[3]Minerva Center for Marine Invertebrate Immunology and Developmental Biology, National Institute of Oceanography, Tel Shikmona, PO Box 8030, Haifa 31080, Israel

[4]Department of Biology, University of Haifa-Oranim, Tivon 36006, Israel

[5]Department of Pathology, Stanford University Medical Center, Stanford, California 94305, USA

ABSTRACT: Biological invasions are an important threat to the health and composition of coastal marine communities. One of the most important challenges for conservation biologists and ecologists is to develop methods for historically reconstructing the invasion process in order to better understand the tempo and mode of the invasion as well as to evaluate the biological consequences. Genetic markers provide 1 approach for documenting the temporal and spatial dynamics of recent invasions through characterization of the genetic structure of introduced populations. Here, we used microsatellites to determine the geographic origins of populations of the colonial ascidian *Botryllus schlosseri* from the east and the west coast of the USA. Specifically, we tested the hypotheses that the source of California populations was the east coast of North America. Eight populations were sampled from the east coast and California. This hypothesis was not substantiated. Rather, results suggest that either European or Asian populations are the most likely source for the introduction(s) of *B. schlosseri* to California.

KEY WORDS: Ascidians · Biological invasions · Heterozygote deficiency · Microsatellite · Population genetics

INTRODUCTION

Biological invasions are having a detrimental effect on native coastal marine communities (Carlton & Geller 1993, Ruckelshaus & Hays 1998, Cohen & Carlton 1998). Invasions typically occur either through dumping of ballast water or introduction of exotic species for aquaculture, although they may also be the result of intentional release or the importation of live marine organisms for consumption (Carlton 1996). Documented effects range from near extinction to complete modification of community structure (Cohen et al. 1995, Grosholz & Ruiz 1995), demanding a need to improve our understanding of the invasion process. Unfortunately, in most cases, there is no historical record of the nature of the introductions. Genetic markers provide an indirect approach for addressing the timing and mechanisms of invasion. By characterizing the genetic structure of introduced populations, one can draw a conclusion regarding potential sources of invaders, the number of individuals included in the initial population, and rates of growth in the early stages of invaders (Goff et al. 1992, Duda 1994, Geller et al. 1994, 1997).

A successful invader of marine communities is the colonial ascidian *Botryllus schlosseri* (Pallas 1766), a 'cosmopolitan' species found on all continents except

*Corresponding author. Email: buki@ocean.org.il

Antarctica (Van Name 1945, Kott 1985). *B. schlosseri* is believed to be a Mediterranean sea origin species (Berrill 1950). Many European populations grow in 'natural environments' such as rock outcrops in the low intertidal or subtidal to depths of 200 m (Berrill 1950, Millar 1969, B. Rinkevich unpubl. data). Elsewhere, the species is largely confined to ecologically impacted areas.

According to Van Name (1945), *Botryllus schlosseri* was probably introduced to the east coast of North America prior to the 1830s, presumably through European shipping traffic. On the California coast, *B. schlosseri* was first observed in San Francisco Bay in the mid-1940s (Cohen & Carlton 1995) and since then has become established in the west coast from San Diego to Alaska (Lambert 2001). The geographic origins of these introduced California populations is uncertain. Lambert & Lambert (1998) have suggested the introduction of this species into California waters by oyster mariculture, as it appeared at oyster farms. A likely candidate for the source area is the east coast of North America. Populations on the east coast were established at least 120 yr before the west coast, and during those years, there was considerable vessel traffic between the coasts and extensive movements of commercial oysters from the Atlantic to the Pacific coast (especially between 1870 and 1930; Cohen & Carlton 1995). The California *B. schlosseri* is morphologically similar to the Woods Hole, MA, *B. schlosseri* and inter-coast breeding protocols revealed fertile F_1 progenies (Boyd et al. 1990). However, allorecognition assays revealed no fusion between colonies growing in Woods Hole and Monterey as opposed to high levels of fusions of colonies growing in Monterey challenged with Japanese or Israeli Mediterranean coast colonies (Boyd et al. 1990, Rinkevich et al. 1992).

Identifying the source of a biological invasion requires constructing a phylogenetic tree that reliably depicts the genetic history of a species leading up to the introduction. A wide variety of genetic markers has been used to construct such phylogenetic trees. But increasingly, biologists have been turning to microsatellites to retrieve accurate phylogenies as (1) the genetic basis of their evolution is apparent (single- or multiple-step changes in repeat number); (2) they are selectively neutral; and (3) they have high mutation rates, are abundant within the genome and generally exhibit considerable intra-population variability (Goldstein & Pollock 1997). For example, microsatellites were successfully used to examine the geographic origins of humans (Bowcock et al. 1994, Goldstein et al. 1995, Shriver et al. 1995, Takezaki & Nei 1996). These studies tested the 'Out of Africa' hypothesis and found significant support for the the-

sis that modern man first evolved in Africa and later migrated out of Africa into Europe and Asia. Other microsatellite-based studies have identified the nest location of mating queen ants (Chapusat et al. 1997), the source of migrating Atlantic salmon (Nielsen et al. 1997), the origins of a West European subspecies of honeybee (Franck et al. 1998), the dispersal range of African populations of mosquitoes (Lehmann et al. 1997) and the migratory patterns of polar and brown bears (Paetkau et al. 1995, 1998). Here, we use microsatellites to compare genetic variability of *Botryllus schlosseri* between the west and the east coasts of the USA. Specifically, we address the question of whether California populations derive from introductions from the east coast of North America or from other sources.

MATERIALS AND METHODS

Sampling. Three populations from the east coast of North America and 5 from California were sampled from 1994 to 1997 (Table 1). Twenty-four colonies (51 in Maine) per population were collected along a linear transect at 10 m intervals and subsequently either frozen in liquid nitrogen or fixed in 100% ethanol.

Analysis of microsatellite loci. DNA extractions and PCR amplification were as described in Stoner et al. (1997). Genotypes were characterized at microsatellite loci BS811 (Pancer et al. 1994), and PB29, PB41 and PB49 (Stoner et al. 1997). Exact tests for deviation from Hardy-Weinberg expectations at each locus and for genotypic linkage disequilibria among polymorphic loci were computed within each population, and fixation index (Fst) estimates for population pairs were computed using GENEPOP 3.3 (Raymond & Rousset 1995).

Table 1. *Botryllus schlosseri.* Geographic locations, sampling sites and sampling dates of the 8 populations studied

Geographic location	Sampling site	Sampling date
California		
Bodega Bay	Snug Harbor	Apr 97
Santa Cruz	Santa Cruz City Boat Harbor	Oct 95
Moss Landing	Yacht Club Harbor	Apr 96
Santa Barbara	Santa Barbara City Boat Harbor	Aug 94
Los Angeles	Port of Los Angeles	Sep 97
East coast of North America		
Walpole, Maine	Damariscotta River	Aug 94
Woods Hole, Massachusetts	Eel Pond	Jun 95
Groton, Connecticut	Avery Point	May 97

RESULTS

We first tested the variability of the 4 microsatellite alleles in the sample populations. With 1 exception, all 4 loci were polymorphic within all 8 populations. Across individuals, we detected 5 alleles at locus PB29, 5 alleles at locus PB41, 11 alleles at locus PB49 and 36 alleles at locus BS811 (Table 2). The allele number varied from 17 to 31 per population and mean number of alleles per locus within populations varied from 4.25 to 7.75 (Table 2). There were no significant differences in allele numbers (A) between populations of the east and the west coasts. Observed heterozygosity (H_o) averaged across loci varied among populations from 0.21 to 0.51. In general, H_o were significantly higher in the west coast (0.49 ± 0.02) than in the east (0.32 ± 0.10). Highly significant deviation from Hardy-Weinberg expectation was found, where observed H_o was lower than expected in every locus for each population. Two populations possessed private alleles in the locus BS811: Maine (n = 6, east coast), Bodega Bay (n = 2, west coast). Two other west coast populations (Moss Landing, Los Angeles) represented a single, private allele, each, in the locus PB49. Many alleles were not shared among adjacent populations surveyed along the east and west coasts of North America. In some cases, unshared alleles occurred in high frequencies.

The inbreeding coefficient (Fis) was higher in populations of the east coast (ranged 0.3670 to 0.4445) relative to populations of the west coast (ranged 0.1881 to 0.3335; Table 3). Estimations of fixation index (Fst) of population pairs calculated for all loci summarized in Table 4. The lowest Fst (range between 0.0184 to 0.1390) appeared between paired populations of the west coast, while higher Fst (0.1860 to 0.1934) was evident between pairs of populations of the east coast. The highest Fst represent pairs of populations of both coasts (0.1319 to 0.4340).

Simple observations of allele frequencies within populations (Table 2) reinforce the conclusions generated by the Fst analysis (Table 4) that certain allelic combinations define 2 broad geographical groups (Table 2): east coast (alleles 174 and 177 of locus PB29, allele 193 of locus PB41 and allele 226 of locus PB49) and west coast (allele 168 of locus PB29, alleles 198 and 210 of locus PB41, and alleles 250 and 262 of locus PB49).

DISCUSSION

Founder(s) of USA populations

The analyses of the sampled populations of *Botryllus schlosseri* suggest that the California populations are probably derived from a single source, although we cannot rule out any region as a source. This conclusion is supported by an analysis of frequency distributions (Table 2). A similar genetic profile was also found in an additional Californian population sampled from the Monterey marina (Stoner et al. 1997). The almost complete absence of alleles diagnostic of the east coast populations within California populations suggests, however, that if there have been invasions from the east coast, they have had a minimal influence on the population genetic structure of California populations. The above conclusion is also supported by previous studies of genetic diversity based on cut colony assays which found 0 allorecognition fusions (i.e. the result of shared feasibility alleles on the Fu/HC locus) between colonies growing in Woods Hole, MA, and Monterey, CA (Boyd et al. 1990, Rinkevich et al. 1992). On the other hand, it is still possible that the east coast is the source population for California, but from as yet unidentified sites. Moreover, there could have been multiple invasions, given that different populations have private alleles.

The analyses clearly show that the east coast and California populations are genetically distinct (Tables 2, 3 & 4). One possible explanation for the large genetic distance between east coast and California populations is that the east coast and west coast populations represent different species. However, such a scenario seems unlikely given that Woods Hole *Botryllus schlosseri* have been successfully bred with Monterey *B. schlosseri* through to the F_2 generation (Boyd et al. 1990). A more reasonable explanation for the large genetic difference between coasts, therefore, may be that the populations on the 2 coasts were founded by 2 genetically disparate populations of the same species. If this latter explanation is true, then the question arises of where the genetically disparate source areas for these populations are.

Geography and records of ship traffic would suggest that the most probable source areas for the east coast populations are Western Europe and the Mediterranean. Both areas are geographically proximate to the east coast, and ship traffic between these areas and the east coast has been very heavy for more than 500 yr. This suggestion is also supported by the large number of successful introductions from these regions that have been recorded for other taxa, including solitary ascidians (Carlton 1993), molluscs (Carlton 1992), crustaceans (Cohen et al. 1995, Geller et al. 1997) and bryozoans (Berman et al. 1992).

The suggestion of European origin is supported by the fact that alleles equivalent in size to all alleles found in this study have been recorded in surveys of populations from the Mediterranean and western Europe (Rinkevich et al. 2001, B. Rinkevich unpubl. data). These surveys also suggest an optional origin of

Table 2. *Botryllus schlosseri.* Allele frequency, observed (H_o) and expected heterozygosity (H_e) and number of different alleles in populations from the west and east coasts of USA. (A = alleles per locus at specific populations)

Locus	Allele	Bodega Bay	Santa Cruz	Moss Landing	Santa Barbara	Maine	Woods Hole	Connecticut
PB29	167	0.167	0.125	0.313	0.208	0.078	0.042	0.125
	168	0.188	0.292	0.292	0.229	–	–	–
	171	0.625	0.583	0.375	0.563	0.206	0.271	0.271
	174	–	–	–	–	0.020	–	0.083
	177	0.021	–	0.021	–	0.696	0.688	0.521
H_o		0.333	0.500	0.625	0.583	0.333	0.250	0.292
H_e		0.558	0.571	0.691	0.600	0.471	0.462	0.646
A		4	3	4	3	4	3	4
PB41	193	–	–	–	–	1.000	0.188	0.354
	195	0.458	0.417	0.333	0.125	–	0.625	0.417
	198	0.396	0.208	0.250	0.375	–	–	–
	202	0.083	0.250	0.375	0.125	–	0.188	0.229
	210	0.063	0.125	0.042	0.375	–	–	–
H_o		0.500	0.417	0.458	0.542	–	0.500	0.333
H_e		0.636	0.720	0.699	0.702	–	0.551	0.662
A		4	4	4	4	1	3	3
PB49	226	–	–	–	–	0.324	0.250	0.083
	234	–	0.104	–	–	0.637	0.313	–
	236	0.333	0.146	0.042	0.021	–	0.063	–
	238	0.063	0.042	0.083	–	0.039	0.042	0.708
	240	0.208	–	0.021	–	–	–	0.021
	246	–	–	0.042	–	–	–	–
	250	0.063	0.083	0.146	0.271	–	–	–
	254	–	–	0.021	0.042	–	–	–
	256	0.208	0.396	0.542	0.583	–	0.333	0.188
	260	–	–	–	–	–	–	–
	262	0.104	0.229	0.104	0.083	–	–	–
H_o		0.667	0.458	0.417	0.458	0.255	0.500	0.417
H_e		0.800	0.766	0.677	0.590	0.493	0.738	0.465
A		7	6	8	5	3	5	4
BS811	238	0.063	0.125	0.125	0.208	0.069	–	0.146
	242	–	–	–	0.042	–	–	–
	244	–	–	–	–	0.020	–	–
	250	–	–	–	–	–	0.042	–
	254	–	–	–	0.042	0.049	–	–
	256	0.229	0.354	0.146	–	0.049	–	0.042
	258	–	–	–	0.521	0.029	0.042	–
	260	0.250	0.271	0.021	–	0.020	–	0.167
	262	–	0.021	0.021	–	0.127	–	–
	264	0.021	0.042	0.083	0.188	0.020	0.083	–
	266	0.021	0.042	0.042	–	–	0.021	–
	268	–	0.042	0.188	–	0.039	–	–
	270	0.042	–	–	–	0.078	–	–
	272	–	–	–	–	0.020	0.042	–
	274	–	–	–	–	0.069	–	–
	276	–	–	–	–	–	0.229	–
	278	0.063	0.042	0.021	–	–	–	–
	280	0.063	–	0.083	–	0.069	0.021	–
	282	–	–	–	–	–	0.021	0.021
	292	–	–	0.042	–	–	–	0.083
	296	0.042	–	–	–	–	–	–
	298	–	–	–	–	–	0.167	0.021
	300	–	–	0.063	–	–	0.021	–
	302	–	0.021	–	–	–	–	0.042
	304	0.104	0.042	0.104	–	0.029	–	–
	306	–	–	0.021	–	0.029	–	–
	308	–	–	–	–	0.020	0.167	–
	310	–	–	–	–	0.020	–	–
	312	–	–	–	–	–	0.021	0.292

Table 2 (continued)

Locus	Allele	Bodega Bay	Santa Cruz	Moss Landing	Santa Barbara	Maine	Woods Hole	Connecticut
	322	–	–	–	–	0.010	–	0.083
	326	–	–	–	–	0.108	0.042	–
	332	0.083	–	0.042	–	0.098	0.083	0.104
	340	–	–	–	–	0.010	–	–
	344	–	–	–	–	0.010	–	–
	346	–	–	–	–	0.010	–	–
	352	0.021	–	–	–	–	–	–
H_o		0.458	0.500	0.542	0.292	0.255	0.375	0.333
H_e		0.869	0.793	0.911	0.660	0.940	0.887	0.855
A		12	10	14	5	23	13	10
Summary								
H_o		0.49	0.47	0.51	0.47	0.21	0.41	0.34
H_e		0.72	0.71	0.74	0.64	0.48	0.66	0.66
Total A		27	23	30	17	31	24	21
Mean A		6.75	5.75	7.5	4.25	7.75	6	5.25

the west coast population from the same source, hence by different founders. As *Botryllus schlosseri* is believed to be a Mediterranean Sea origin species (Berrill 1950), it is not clear whether the introduction to the USA was directly from Europe, or through other introduced populations. Direct introduction from the Mediterranean to the east coast is the most straightforward explanation. However, concerning the west coast, as there are no direct seawater passages from the North Atlantic Ocean, their introduction can also be from other sources that originally settled by Mediterranean Sea origin individuals, such as populations in Asia. Candidate populations for such an introduction can be from Japan, Korea, Hong Kong or India.

There are records of *Botryllus schlosseri* in Japan prior to their proposed introduction(s) to California. *B. schlosseri* was first observed in Sagami Bay in 1929 (Tokioka 1953) and could have been introduced from Europe even much earlier, given that ships have been traveling between Europe and Asia since the 16th century. It could have been introduced to California from Japan either as a result of oyster imports or in the ballast of ships. *B. schlosseri* often grows on floating blades of *Zostera*, *Codium* and fucoids (*Fucus* and *Ascophyllum*) that could be jettisoned by boats along with their ballast (Cohen & Carlton 1995). Yet another possibility is that the California coast was colonized by introductions from both the east coast and Asia.

Support for an introduction from Asia comes from the fact that high levels of fusion between colonies growing in Monterey, CA, and Japan have been documented (Rinkevich et al. 1992). The suggestion that

Table 3. *Botryllus schlosseri.* Inbreeding coefficient (Fis) averaged for each population

Population	Sample size	Fis
Bodega Bay	24	0.3179
Santa Cruz	24	0.3335
Moss Landing	24	0.3116
Santa Barbara	24	0.2628
Los Angeles	24	0.1881
Maine	51	0.3776
Woods Hole	24	0.3670
Connecticut	24	0.4445

Table 4. *Botryllus schlosseri.* Fixation index (Fst) for population pairs estimated for all loci

Population	Bodega Bay	Santa Cruz	Moss Landing	Santa Barbara	Los Angeles	Maine	Woods Hole
Santa Cruz	0.0184						
Moss Landing	0.0658	0.0270					
Santa Barbara	0.1390	0.1130	0.0917				
Los Angeles	0.0957	0.0827	0.0642	0.0771			
Maine	0.3880	0.3831	0.3807	0.4257	0.4340		
Woods Hole	0.1860	0.1745	0.1658	0.2575	0.2292	0.2426	
Connecticut	0.1934	0.1904	0.1751	0.2748	0.2386	0.2847	0.1319

Japan might be the source area for the California populations mirrors the conclusions of other authors who have studied biological invasions of the California coast. Lambert & Lambert (1998) consider Japan as the likely source for several recent invasions of the California coast by non-native ascidians, and Cohen & Carlton (1995) implicate Japan as the source for the introduction of at least 22 invertebrate species to San Francisco Bay.

Heterozygote deficiency

Heterozygote deficiency was recorded in every locus for each one of the 8 populations tested (Table 2). Hardy-Weinberg exact tests for all loci, and all populations revealed a highly significant heterozygote deficiency. Such deviations from expected levels reflect a genuine biological phenomenon characteristic of *Botryllus schlosseri*. A similar outcome of heterozygote deficiency was found in another California population of Monterey (Stoner et al. 1997) as well as in the Mediterranean populations of Israel (Paz 1999), the Adriatic Sea, in Croatia, (Rinkevich et al. 2001) and in the introduced populations of New Zealand (Ben-Shlomo et al. 2001). Inbreeding or non-random mating as well as substructured populations in which gene flow is somewhat restricted are probable explanations of the deficiency (Chakraborty & Jin 1992).

Although *Botryllus schlosseri* colonies are hermaphrodites, sperm release from gravid colonies does not start until about 1 to 2 d after ovulation, effectively preventing self-fertilization (Yund et al. 1997). Furthermore, laboratory experiments in which inbreeding was enforced yielded a sharp decrease in vitality within a few generations (B. Rinkevich unpubl. data). Nonetheless, an aggregated settlement of sibling *B. schlosseri* planktonic larvae has been shown by Grosberg & Quinn (1986). Such aggregations may form small-scale subpopulations, a situation that promotes mating between sibling colonies (Sabbadin 1978, Grosberg & Quinn 1986, Rinkevich et al. 1998).

The fixation indices (Fis and Fst) support both inbreeding and subpopulation structure (Tables 3 & 4). Fis was generally higher in the east coast population, suggesting a higher level of inbreeding within each population. High levels of inbreeding characterize well-established populations of *Botryllus schlosseri* (R. Ben-Shlomo & B. Rinkevich), and was found to be the situation in the Mediterranean and Western Europe populations, while newly established remote populations exhibit lower levels of inbreeding. Relatively low levels of inbreeding, though, still show highly significant heterozygote deficiency, as was found in New Zealand populations (Ben-Shlomo et al. 2001).

Fst measures the degree of genetic differentiation of subpopulations. Theory and empirical evidence predict that for sets of populations not yet at equilibrium, Fst values should be consistently low over all distances (Hutchinson & Templeton 1999, Turgeon & Bernatchez 2001). Moreover, if drift becomes predominant, Fst should be highly variable. Indeed, lower and variable Fst estimates (0.0184 to 0.1390) were found among pairs of the west coast populations (Table 4), suggesting common ancestor and relatively few generations since introduction. Higher levels of differentiation (0.1860 to 0.1934) were found among the east coast populations, thought to be established 120 yr (several 100s of generations) earlier.

This and other recent studies (Ben-Shlomo et al. 2001, Rinkevich et al. 2001) are part of the first globally oriented molecular approach attempting to determine where *Botryllus schlosseri* first originated and by what routes its populations subsequently dispersed throughout the world. It further supports the idea (Van Name 1945, Berrill 1950, Lambert & Lambert 1998, Lambert 2001) that *B. schlosseri*'s global distribution is the result of multiple invasions from presumably multiple genetically disparate source areas. To better localize source areas, future studies should undertake more extensive sampling of *B. schlosseri* populations throughout Europe, the Mediterranean, Asia and the South Pacific. Taking multiple samples from these areas will increase the reliability of estimates of genetic diversity within each area and will also help eliminate some regions as potential source areas. Future studies would also benefit from taking samples at more than 1 point in time to reduce variation introduced by genetic drift resulting from population crashes.

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