Long-term population genetic dynamics of the invasive ascidian Botryllus schlosseri, lately introduced to Puget Sound (Washington, USA) marinas

Jann Zwahlen[1], Eitan Reem[2], Jacob Douek[3], and Baruch Rinkevich[4]

[1]Universita del Salento
[2]Israel Oceanographic & Limnological Research
[3]Israel oceanographic and Limnological Research
[4]Israel Oceanogrphic and Limnological Research

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Abstract

Invasive species are of increasing concern to the local biodiversity and ecology as the magnitude of biological invasions is increasing globally. The genetic structures of newly established invasive populations may reveal insights towards the invasion processes, making population genetics an important tool for understanding current invasions. Here we study newly established populations (<10-20 years before first sampling) of the cosmopolitan alien ascidian Botryllus schlosseri in four Puget Sound (Washington, USA) marinas, using eight polymorphic microsatellites. Up to seven sampling sessions over a period of 19 years revealed populations with fluctuating allelic richness (AR=2.693-4.417) and gene diversity (He=0.362-0.589). The populations were well differentiated on spatial and temporal scales and subjected to moderate genetic drift (Fs'=0.027-0.071). The obtained significant heterozygote deficiencies, positive inbreeding coefficients (Fis) and population structure measures (Fst) revealed that no population was under the Hardy-Weinberg equilibrium. Comparing these parameters with those from two Californian sites (Moss Landing and Santa Cruz, 1200 km southerly; invaded by Botryllus during 1940's) revealed a connection between Moss Landing and Puget Sound, while Santa Cruz remained isolated. On the US west coast scale, this study revealed no major difference in invasive population dynamics between recently and decade long established populations, except for fewer alleles and lower He. When comparing ten worldwide sites, only few microsatellite loci displayed strong regional differences. With globally the lowest numbers of alleles and lowest genetic indices, the Puget Sound Botryllus populations exhibit genetic characteristics of recently established populations, further emerging as one of the youngest B. schlosseri populations, worldwide.

Introduction

Biological invasions have become more frequent driven by increasing globalisation, anthropogenic activities and climate change (Hulme, 2009; Van Kleunen et al., 2015), inflicting severe negative impacts on local biota, while imposing economic aftermaths (Gallardo, Clavero, Sánchez, & Vilà, 2016; Walsh, Carpenter, & Van Der Zanden, 2016). The footprints of biological invasions can be traced in the changes of species diversity, the dramatic alterations in communities, habitats, top-down and bottom-up control modifications, pandemic statuses, shifts in food chains, nutrient cycling, and in attenuations of ecosystem services (Anton et al., 2019; David et al., 2017; Simberloff & Rejmánek, 2011). Like in terrestrial ecosystems, biological invasions in the marine/oceanic realm are major drivers of ecological and evolutionary shifts, altering community structures while restructuring ecosystem functions with direct and indirect impacts on ecosystem services (Carlton & Geller, 1993; Darling et al., 2017; Katsanevakis et al., 2014). With the increasing impacts of biological

invasions in marine environments, more interest is being devoted for the inclusion of genetic, phylogenetic and evolutionary aspects in the research, with parameters that may improve the resolution and cost effectiveness of monitoring biological invasion (Darling et al., 2017; Rius, Turon, Bernardi, Volckaert, & Viard, 2015) and describe changes of population genetics and adaptation properties of important invasive species in better details (Barrett, 2015; Tepolt, 2015).

Botryllus schlosseri (Pallas, 1766) is a common Mediterranean Sea/European Atlantic colonial ascidian species, notoriously invasive to temperate zones worldwide (Reem and Rinkevich, 2014; Freeman, Frischeisen, & Blakeslee, 2016; Lin and Zhan, 2016; Lord, 2017), currently inhabiting all continents except Antarctica, including the coasts of Japan, New Zealand, India, South Africa, Chile, Argentina, USA, and Canada (Van Name, 1945; Rinkevich, Shapira, Weissman, & Saito, 1992; Ben-Shlomo et al., 2001, 2010). Populations of this species were assigned to five highly divergent clades on the Cytochrome Oxidase subunit I (COI) by Bock et al. (2012) with clade A being cosmopolitan, revealing significant differentiation between native and invasive populations (Lin & Zhan, 2016), while the other clades are restricted to European waters. Reem, Douek, Paz, Katzir and Rinkevich (2017) pointed, however, to the possibility of admixture within two Mediterranean populations between individuals from different clades, further revealing that the origin of *B. schlosseri* , particularly the cosmopolitan clade A, is still under debate. Early theories (Van Name, 1945) have suggested that the species originated in European waters, a proposal supported by Reem et al. (2017), while Yund, Collins and Johnson (2015) proposed that at least one haplotype in clade A is native to the northwest Atlantic. Carlton (2005) proposed, yet without supporting documentations, a possible Pacific origin.

In the United States, *B. schlosseri* was already present on the east coast in 1841 (Gould, 1841), while west coast documents (e.g., San Francisco Bay, Port Hueneme and San Diego, and Bremerton, WA) were anecdotally recorded around 1944 – 1947 (US Navy, 1951; taxonomic validation is yet to be performed). In San Francisco area it remained at low frequencies for at least a decade (Cohen & Carlton, 1995), while becoming common in San Diego region in the early 1960s (Lambert & Lambert, 1998). In the Washington area, *B. schlosseri* was not reported for the next 36 years, following the first report in 1951 (US Navy, 1951), until its first validated documentation (Lambert, Lambert & Kozloff, 1987). Lambert and Lambert (1998) reported an anecdotal earlier observation of *B. schlosseri* (late 1960s or early 1970s) in an oyster farm on San Juan Island, just north of the Puget Sound. However, subsequent publications did not mention *B. schlosseri*(Kozloff, 1973, 1974, 1983; Lambert, 1969) in the Washington area. This species was not recorded by James T. Carlton that surveyed the biofouling communities in Washington during 1976-1977, and the mentioning of *B. schlosseri* by Wonham and Carlton (2005) was a taxonomic error (J.T. Carlton, personal communication, August 2019). Furthermore, during 1987, one of the authors of this paper (B. Rinkevich; unpubl.) performed a survey of marinas along the US west coast and could not find *B. schlosseri* north of Coos Bay, Oregon (an area that was still under active invasion process, as *B. schlosseri* failed to establish yet in the upper Coos bay communities; Hewitt, 1993), nor in several marinas surveyed in the Puget Sound. In contrast, *B. schlosseri* was very common in some of the sampled marinas on his next survey conducted in 1999. The above suggests possible patchy distributions of early *B. schlosseri* introduced to the Seattle area. It is unknown yet whether the current widespread populations of *B. schlosseri* in this area (Cohen et al., 1998; Pleus, Leclair, & Schultz, 2008) originated from earlier invasions, reported during the 1940's and 1970's, or reflect later introductions to this area.

As an invasive species *B. schlosseri* negatively impacts aquaculture organisms (Arens, Christine Paetzold, Ramsay, & Davidson, 2011; Carver, Mallet, & Vercaemer, 2006). Having a short larval duration of no more than two hours (Grave & Woodbridge, 1924; Grosberg, 1987; Rinkevich & Weissman, 1987), that allows just a limited larval dispersal (Grosberg, 1987), *B. schlosseri* relies on floating objects for long-distance dispersal. It is commonly observed on ship hulls and aquaculture submersed infrastructure which move between marine sites (Berrill, 1950; Lambert & Lambert, 1998; Skerman, 1960) and also spreads to different marinas, probably by boating activity (Lacoursière-Roussel et al., 2012; López-Legentil, Legentil, Erwin, & Turon , 2015).

Long-term monitoring of population genetics (García-Navas et al., 2015; Osborne, Carson, & Turner, 2012)

may further elucidate the properties of an invasive species (Jason Kennington, Hevroy, & Johnson, 2012) and the roles of their genetic background in dictating the invasiveness potential of alien species introduced into new territories (Wellband, Pettitt-Wade, Fisk, & Heath, 2017). Reem, Douek, Katzir and Rinkevich (2013a) and Karahan, Douek, Paz and Rinkevich (2016) were the first to study the long-term genetic structures (13 and 12 years, respectively) of *B. schlosseri* in Santa Cruz and Moss Landing, two sites in California, USA, separated by just 20km of shoreline, revealing two disparate population genetic structures. The Santa Cruz population (Reem et al., 2013a) is relatively isolated and under high genetic drift, further characterized by high mutation rates. The Moss Landing population (Karahan et al., 2016) is affected by episodic freshwater floods, and subsequent recovery.

These long-term studies on *Botryllus schlosseri* populations (Karahan et al., 2016; Reem et al., 2013a) detailed the population genetic parameters of two populations already established for several decades. It is therefore of great interest to compare their results with lately established *B. schlosseri* populations, like those of the US north Pacific coast (Puget Sound, Washington), where in the late 1980s this species was missing or just introduced. We studied genetics parameters of four *B. schlosseri* populations residing in marinas in the Seattle WA area, separated by up to a 124 km coastline, during a period of up to 19 years. Our aim was primarily to get an overall regional scale perspective of possible genetic structure fluctuations in recently introduced populations of an invasive species.

Materials and methods

Sampling sites

Puget Sound (Fig. 1) is part of the Salish Sea, an estuarine system in Washington State, USA, linked to Pacific Ocean via three connections to the Strait Juan de Fuca. Four marinas were studied, three (Edmonds, Shilshole and Des Moines marinas) located in the central basin, and Shelton Yacht Club, located in the southern basin. With 36,000 recreational boats, approximately 24,000 actively cruising, the Salish Sea is a famous destination for recreational boating (City of Des Moines Marina, http://www.desmoinesmarina.com/uploads/7/2/2/4/72248139/city_of

has ˜1500 mooring slips, and is the biggest marina considered in this study. The City of Des Moines Marina has anywhere between 400 - 800 boats moored, with a constant flow of 10-30 daily visitors (Tara Reilly, Office Specialist of City of Des Moines Marina, pers. comm.). Edmonds Marina has 668 mooring slips and receives around 3100 visiting boats per year. The remote Shelton Yacht Club offers just 109 mooring slips, and is surrounded by extensive shellfish aquaculture.

Sampling procedures, DNA extraction and microsatellite typing

Botryllus schlosseri samples were collected every two years during the period 1999 to 2007, and in 2013 and 2018 (7 collection sessions; Suppl. Table A.1). Colonies, or colony fragments separated by at least one meter, were removed using single edge-razor blades and put into separate 1.5ml vials. The DNA extraction followed the protocol in Paz, Douek, Mo, Goren and Rinkevich (2003).

Eight *B. schlosseri* microsatellites, BS-2, BS-8, BS-9 (Abdoullaye et al., 2010), BS-811 (Pancer, Gershon, & Rinkevich, 1994), PB-29, PB-41, PB-49 and PBC-1 (Stoner, Quattro, & Weissman, 1997) were amplified by polymerase chain reaction (PCR) using specific primers under the following conditions: 95°C, 4min; 35x [94°C, 60s; 48-56°C, 60s; 72°C, 60s], 72°C, 45min; 10°C. The PCR-mix (10µl) containing 5µl 2x *Taq* PCR MasterMix (KT201, Tiangen Biotech Beijing.), 3.9µl DDW, 0.1µl primer mix and 1µl DNA. The PCR products were run on 1.5% agar in gel electrophoresis to determine amplification success. When unsuccessful, PCR was repeated several times using deviant protocols. 1µl of PCR product mix of four primers were added to 0.4µl LIZ 500 size standard and 8.6µl formamide per well and were analyzed using the ABI-PRISM 310 sequencer. The length of the microsatellite alleles was evaluated using GeneMapper 5.0 (Applied Biosystems) software

package. Where in doubt, peak sizes were shared and discussed with other researchers and amplifications were repeated when necessary.

Data analysis

The analysis considered four geographic scales. On the local scale, each site (Des Moines, Edmonds, Shilshole and Shelton), was analyzed separately. On the regional scale, all four sites were simultaneously compared. On the US west coast scale, the results on the Puget Sound area were compared with two southern sites in central California: Santa Cruz (Reem et al., 2013a) and Moss Landing (Karahan et al., 2016). Finally, allelic patterns from worldwide sites were compared on an international scale.

The alleles were binned using AutoBin v 0.9 (Guichoux et al., 2011) and checked for scoring errors using Micro-Checker v. 2.2.3 (Van Oosterhout, Hutchinson, Wills, & Shipley, 2004). The frequency of null alleles was calculated in FreeNA using the EM algorithm (Chapuis & Estoup, 2007). Null alleles might bias the outcome of the analysis, therefore pairwise population differentiation (Fst) was calculated with and without the excluding null alleles (ENA) correction in FreeNA using 50,000 bootstrap iterations. A two-tailed t-test was used to compare these two Fst estimates.

Allele frequencies, private alleles (for each location, for each sampling year and for the entire list of samples), observed and expected heterozygosity, population differentiation (Dest), population structure (Fst) and the inbreeding coefficient (Fis) (both using the G-statistic approach), Nei genetic distances and Mantel tests were calculated in GenAlEx v.6.51b2 (Peakall & Smouse, 2012). The allelic richness was calculated in FSTAT (Goudet, 2003) using Hurlbert's (1971) rarefaction approach as modified by Petit, Mousadik and Ponst (1998), while HP-Rare (Kalinowski, 2005) was used to calculate private allelic richness. The significance of the deviance in heterozygosity was tested using the GENEPOP online version (http://www.genepop.curtin.edu.au). Bayesian Analysis of Population Structure BAPS (Corander, Waldmann, Marttinen, & Sillanpää, 2004) and STRUCTURE v.2.1 (Pritchard, Stephens, & Donnelly, 2000) were used for the Bayesian structure analysis, and BAPS to determine the gene flow between different populations. In BAPS, we used the clustering groups of individual option for the analysis and in STRUCTURE, the length of the Burn-in period was set to 100,000 and the number of Markov-chain-Monte-Carlo repetitions to 10^6. The number of clusters, K , was allowed to range between one and ten and five iterations for each K were carried out. The optimal K was chosen using the Structure harvester (Earl & vonHoldt, 2012) and the different runs aligned using CLUMPP (Jakobsson & Rosenberg, 2007). This step was repeated three times to evaluate if the number of clusters is consistent. Population clustering should be used with caution due to the different assumptions of the programs (Sinai et al., 2019), therefore NetStruct was used as a third method for analyzing the population structure using the network theory (Greenbaum, Templeton, & Bar-David, 2015). After an initial run, the threshold was set between 0.2 and 0.4 and 999 permutations were carried out. The genetic drift (Fs') was calculated in TempoFS (Jorde & Ryman, 2007). Pairwise Fst, a measure of population structure that is commonly used for showing population differentiation, was calculated in Arlequin (Excoffier, Laval, & Schneider, 2005).

Results

Altogether, 533 tissue samples were collected (521 analyzed) within a period of 19 years, between 1999 and 2018 in four locations at the Puget Sound area, WA, USA. Detailed allele frequencies of all eight microsatellite loci are shown in Suppl. Table A.2. A two-tailed t-test between the Fst values (without and with ENA correction for null alleles) was non-significant (p=0.463), indicating that null alleles do not influence the results. The inbreeding coefficient (Fis) was highly significant for every population.

Local scale

Des Moines marina

In total, 190 tissue samples from seven sampling dates (years 1999-2018; Suppl. Table A.3) were analyzed. All eight microsatellite loci were polymorphic, summing up to 71 alleles. About one third of total alleles (23/71), appeared in only a single sampling period, eight private alleles were found in year 2018, three in each of the sampling years 1999, 2003 and 2013, and two in each of the sampling years 2001, 2005 and 2007. Null alleles were detected in all loci, with high occurrence (> 0.1) in PB-41 (0.284), BS-811 (0.255), PB-49 (0.229), BS-8 (0.18), PB-29 (0.175) and BS-9 (0.126). The genetic diversity indices are summarized in Table 1 and reveal fluctuating allelic richness and significant heterozygote deficiency, suggesting that the populations are not in Hardy-Weinberg equilibrium (HWE). The highest peak in allelic richness was in 2018, followed by another peak in 2003, both of which coinciding with the peaks in private allelic richness.

The Mantel test showed no significant correlation between Nei's genetic distance and the time between the different sampling periods (Suppl. Table A.4). The Des Moines populations were overall significantly ($p=0.001$) differentiated (Table 2). Interestingly, the populations from 1999-2007 exceeded the 2013-2018 population's Dest with 0.064 ($p=0.001$) and 0.024 ($p=0.062$), respectively. The estimated genetic drift (Fs') was further stronger between 1999-2007 (0.076) than between 2013 and 2018 (0.048). Pairwise population differentiation (Fst) values showed that only the pairs 2005/2013, 2005/2018 and 2007/2013 were not significantly different (Suppl. Table A.5A). This lack of similarities between the years contradicts the BAPS analysis, which revealed only a single cluster. Yet, two clusters were elucidated by STRUCTURE (Fig. 2A). In 1999 and 2001, cluster 1 (red in Fig. 2A) was dominant, whereas the later years were more mixed, with some slight dominance of cluster 2 (yellow in Fig. 2A) in 2007 and 2013.

Shilshole marina

In total, 152 tissue samples were analyzed from Shilshole marina. Collections were performed seven times during 1999 -2018, but year 1999 was not included in the analysis as only a single colony was observed (not collected). It is also noted that year 2005 sampling yielded with just 13 samples, despite increased collection efforts. All microsatellites were polymorphic, representing however, only a single BS-8 allele in five of the sampling periods (2001, 2003, 2005, 2007 and 2018), and just one allele of BS-9 in 2005. A total of 63 microsatellite alleles were found, with 15 private alleles, occurring just in a single sampling period (Suppl. Table A.3). Four private alleles occurred in 2018, followed by three in years 2013 and 2007, two in years 2005 and 2003 and a single private allele in 2001. Null alleles were frequent (>0.1) in BS-811 (0.302), PB-41 (0.270), PB-49 (0.141), PB-29 (0.127), PBC-1 (0.126) and BS-9 (0.101). The summarized genetic indices (Table 1) reveal a gradual decline in gene diversity (He) and that the populations were not under the HWE. The highest value of allelic richness was reached in 2005, just slightly higher than in the two preceding sampling years, whereas the peak in private allelic richness was reached in 2007. Nei's genetic distance and the time between the sampling periods are significantly correlated (Suppl. Table A.4). Each run in STRUCTURE resulted in four clusters (Fig. 2C). Cluster 1 (blue in Fig. 2C) was more dominant in 2001 and 2003, cluster 2 (pink) revealed same levels across all years, and clusters 3 (yellow) and 4 (red) were more dominant as from 2005. BAPS suggested two clusters, years 2001-2003 and 2005-2018, a construction which is in line with the STRUCTURE analysis. Pairwise Fst values suggest that samplings from years 2001 and 2003, are significantly different from years 2007, 2013 and 2018. However, also 2007 and 2013 samplings are significantly different ($p<0.05$) (Suppl. Table A.5B). These Fst values further validate the clustering of 2001 with 2003. There was a weak but significant overall differentiation of the populations (Dest) and a slight genetic drift (Fs') in Shilshole (Table 2). Interestingly, Fs' was in an order of magnitude lower in the period of 2001 and 2003 (0.004) compared to 2013-2018 (0.039). The lower genetic drift between years 2001 and 2003 is congruent with the clustering of these two periods.

Edmonds marina

Four sampling periods have yielded 101 tissue samples. In an additional sampling session (year 1999), no colony was found on the marina's hard bottom shallow substrates. All microsatellite loci were polymorphic, yet, only a single allele was found in 2003 and 2018 in locus BS-9. Out of the 55 alleles (Suppl. Table A.3), 17 were private alleles, two private alleles in year 2003, and 5 private alleles in each of the other sampling years. Null alleles were frequent in PB-41 (0.351), BS-811 (0.279), PB-29 (0.172), BS-8 (0.166) and PB-49 (0.108). The genetic indices (Table 1) reveal an increase in allelic richness and gene diversity from 2003 to 2005, with a peak in allelic richness and private allelic richness in 2005. There was no correlation between Nei's genetic distance and the time between the sampling periods (Suppl. Table A.4). Pairwise Fst values suggest a significant difference between years 2003/2005, 2003/2018 and 2005/2018 ($p<0.05$) (Suppl. Table A.5C). The weak Dest was significant ($p=0.006$) and the Fs' moderate (Table 2). BAPS suggested two clusters, one for year 2003 and the second for 2005-2018. Nevertheless, the inconsistent number of K suggested by STRUCTURE proposes a very weak population structure. Two clusters were finally assigned due to constant, but not always tall Delta K peaks at $K = 2$ (Fig. 2D). The BAPS results were confirmed by STRUCTURE, with year 2003 being assigned in a single cluster, whereas the other three sampling dates failing to group into a specific cluster.

Shelton marina

Shelton's marina was visited only during three sessions, yielding 78 tissue samples. All microsatellite loci were polymorphic, although in 1999, loci BS-8, BS-9 and PB-41, in 2003 locus BS-9 and in 2018 locus BS-8 were represented by just a single allele. Forty-six alleles were recorded, whereof 19 were private, e.g., found in a single sampling date (Suppl. Table A.3). Most private alleles occurred in 2003 (n=9), followed by 2018 (n=7) and 1999 (n=3). Null alleles were frequent in four loci (PB-41, PB-49, BS-811 and PB-29), ranging from 0.220 to 0.268. The genetic indices (Table 1) reveal peaks of allelic richness and private allelic richness in 2003. Nei's genetic distance was not correlated to the time passed between the sampling events (Suppl. Table A.4). STRUCTURE suggested three clusters (Fig. 2B), however, no meaningful trend could be detected. BAPS, on the other hand, grouped the years 1999 and 2003 as one cluster, and 2018 as the second. The different populations were weakly ($p=0.014$) separated according to Dest (Table 2), and the Fs' was moderate. Pairwise Fst showed a significant differentiation between 1999/2018 and 2003/2018 (Suppl. Table A.5D), which is in line with the clusters suggested by BAPS.

Regional scale - Puget Sound

Overall analysis

At the regional scale, 104 alleles were recorded during the 19-years sampling period. Nineteen private alleles were found in Des Moines (26.8% of local alleles), eight (17.4%) in Shelton, nine (14.5%) in Shilshole and eight (14.5%) in Edmonds. Analyses for the three most frequent alleles per locus (Fig. 3) revealed just a single allele in loci BS-8 and BS-9 (181bp and 194bp, respectively) dominant every year in each location, while loci BS-811, PB-29 and PBC-1 showed variable allele frequencies. In Edmonds, the third most frequent allele in locus BS-811 was highly frequent in 2003 and 2018, but absent in the years between. Only eight alleles were present in every sampled population, alleles 186bp and 189bp in BS-2, alleles 152bp and 156bp in PB-29, and alleles 199bp, 202bp, 205bp and 210bp in PBC-1. Some alleles reflect site specific distributions. For example, allele 178bp in microsatellite BS-2 was presented in all Shilshole and Shelton populations, but was absent from Des Moines and Edmonds. In contrast, allele 192bp in microsatellite BS-8 was present every sampling period in Des Moines and Edmonds, but completely absent from Shilshole and Shelton.

The Des Moines populations were almost twice as much differentiated (Dest) compared to the other three marinas (Table 2), a result further supported by the population structure Fst, revealing the highest values for Des Moines. For both indices, the differences among the four locations (Dest and Fst) were higher in the earlier period (1999-2007) than between 2013 and 2018. For the inbreeding (Fis) index, only Shelton

revealed remarkable lower values. The overall genetic drift values were similar in Des Moines, Edmonds and Shelton, and less than half in Shilshole, while for allelic richness the highest numbers were assigned to Des Moines (Table 1), followed by Shilshole, Edmonds and Shelton. Des Moines also showed the highest He, also a measure of the evenness of the allelic frequencies, similar to the number of effective alleles (Brown & Weir, 1983). These high levels were followed by Edmonds, Shilshole and Shelton.

Analyzing all 20 populations simultaneously resulted in four BAPS individual clusters, where each location was assigned to a separate cluster (Suppl. Fig. A.1). STRUCTURE, on the other hand, suggested two clusters (Fig. 4A). The Des Moines samples were mainly assigned to cluster 1 (yellow in Fig. 4A), whereas Shilshole samples belonged primarily to cluster 2 (red in Fig. 4A), with Shelton's and Edmonds' individuals being mixed. Netstruct suggested three highly significant clusters (Fig. 4B), with Des Moines belonging mainly to cluster one (blue in Fig. 4B), Shilshole to cluster two (red in Fig. 4B) and Shelton to cluster three (green in Fig. 4B), whereas Edmonds seems to be well-mixed.

Gene flow between sites for the whole period studied was somewhat restricted (Suppl. Fig. A.2), as Shelton received gene flow just from Shilshole, and thus emerged as the most isolated site, with only 0.02 of the genetic material being received from other locations, compared to 0.06 in Edmonds and Shilshole and 0.07 in Des Moines. The strongest gene flow was from Shilshole to Edmonds (0.031), followed by Des Moines to Shilshole (0.023). Edmonds and Shelton were not connected at all, while there was a unidirectional connection from Shelton to Des Moines (0.022) and a bidirectional exchange with Shilshole. Comparing only the sites without yearly divisions shows highly significant pairwise Fst for all pairs (Suppl. Table A.5E).

Yearly analyses

Des Moines and Shilshole exhibited in most sampling dates a similar number of private alleles and generally the highest numbers (Table 3). Yet, in 2005, Edmonds had more private alleles than Des Moines and Shilshole combined. Interestingly, Shelton had much fewer private alleles than Des Moines in 1999 and all other sites in 2018. On a yearly basis, pairwise Fst between years for the whole Puget Sound area shows that all combinations except 2001/2003, 2003/2005 and 2007/2013 are significantly differentiated (Suppl. Table A.5F).

U.S. west coast scale

The analyses included the Puget Sound sites, and two Californian sites, Santa Cruz (Reem et al., 2013a) and Moss Landing (Karahan et al., 2016), involving the five shared microsatellite loci (BS-811, PB-29, PB-41, PB-49, PBC-1) used for analyses in these locations.

Overall

Allelic richness and expected heterozygosity in the Puget Sound populations were lower than in the two Californian populations (Table 4). Despite similar allelic richness and expected heterozygosity, Moss Landing and Santa Cruz populations were clustered to two different groups (orange and red in Fig. 5A) in STRUCTURE, and all four Puget Sound populations formed a third cluster (yellow in Fig. 5A). BAPS created three distinct clusters when considering the four Puget Sound sites as a single location, and no gene flow between any of the sites was discernible (Suppl. Fig. A.3). Three highly significant clusters were selected using Netstruct for the west coast analysis (Fig. 5B). Strikingly, the Santa Cruz populations clustered together to a single entity, whereas the Moss Landing populations were mostly associated with the remote Seattle populations, showing minimal similarities to the close Santa Cruz cluster.

Separate years

During four sampling years (1999, 2001, 2005, 2007) DNA samples were taken in at least a single Californian and two Puget Sound sites. Analyses on each year separately, revealed only a single genetic connection

between California and Puget Sound (year 2007), a substantial gene flow (0.039) from Des Moines to Moss Landing, whereas Santa Cruz, Shilshole and Edmonds remained isolated (Fig. 6).

Global scale

For the analyses, we used 10 worldwide sites (Table 4) and five shared microsatellite loci (BS-811, PB-29, PB-41, PB-49, PBC-1). Locus BS-811 had the highest number of alleles in all locations (20-65; Table 4). When considering the total number of individuals analyzed, Puget Sound emerged as the region with the lowest number of alleles per individual on either locus and on the combined loci. This puts the whole Mediterranean area (Reem et al., 2017), Moss Landing (Karahan et al., 2016) and South America (Rachel Ben-Shlomo et al., 2010) as containing the highest numbers of allele per 100 colonies (N=57-66). The number of alleles in the Puget Sound was notoriously low (N=16), primarily on locus PBC-1, presenting just 61.5% of alleles as compared to the second lowest site, Moss Landing. The European and Mediterranean locations (without Scandinavia) showed the highest number of alleles in PB-29, PB-41 and PBC-1, and were also leading among the other two loci. The average expected heterozygosity among Puget Sound populations was by far the lowest across the globe, suggesting a limited evenness of the allele frequencies. Furthermore, the allelic richness was among the lowest in Puget Sound, whereas the highest values were observed in Israel and Santa Cruz.

The analysis on the frequent alleles (>0.1 in at least a single population; Suppl. Table A.6) revealed some disparities between worldwide sites. For example, all the frequent alleles on locus PB-29 in the U.S. west coast are under 160bp, while in the other regions at least one allele was >160bp. In the most polymorphic locus BS-811, most of the European, South American and New Zealand frequent alleles are <260bp in contrast to the North American (<300+bp). Locus PB-41 did not display different patterns among locations, except for Israel (Paz et al., 2003). While in most sites the majority of frequent alleles was below 180bp, most alleles in the Israeli populations were >180bp. For locus PB-49, allele sizes >240bp were just found in Santa Cruz (Reem et al., 2013a), the US east coast (Stoner, Ben-Shlomo, Rinkevich, & Weissman, 2002) and the Mediterranean Sea (Reem et al., 2017), while in Israel (Paz et al., 2003), Scandinavia (Reem, Mohanty, Katzir, & Rinkevich, 2013b) and South America (Rachel Ben-Shlomo et al., 2010), the majority of the frequent allele sizes was <220bp. Locus PBC-1 contained alleles >210bp everywhere except in the Puget Sound (this study), South America (Ben-Shlomo et al., 2010) and New Zealand (Ben-Shlomo et al., 2001). In the Mediterranean Sea (Reem et al., 2017), the Atlantic coasts of Europe (Ben-Shlomo, Paz, & Rinkevich, 2006) and in South America (Ben-Shlomo et al., 2010), the alleles are shorter, <190bp. In New Zealand (Ben-Shlomo et al., 2001), most PBC-1 alleles were between 190 and 200 bp, while in the Puget Sound (this study), Moss Landing (Karahan et al., 2016) and Israel (Paz et al., 2003), sizes of 201-210bp were most commonly observed.

Discussion

Botryllus schlosseri is a Mediterranean sea/European Atlantic shallow water (down to 200 m depth) marine invertebrate (Berrill, 1950; López-Legentil, Turon, & Planes, 2006; Paz et al., 2003; Reem et al., 2017) that has become a cosmopolitan alien species on man-made submerged hard bottom substrates, inhabiting primarily marinas and harbors all over the northern and southern hemispheres' temperate zones (Berrill, 1950; Lambert, 2001, Ben-Shlomo et al., 2001, 2010, Reem et al. 2013a,b, 2017). This species is also known as a fouling pest species tagged with economic costs in aquaculture (Arens et al., 2011; Carver et al., 2006). Traits like fast adaptation to man-made environmental conditions (Lambert & Lambert, 2003; Lambert, 2001) and enhanced mutation rates that increase genetic variability in newly established populations (Reem et al., 2013a) further contribute to *B. schlosseri's* invasiveness success in a way that upon the establishment of pioneering colonies, this species quickly spreads to become one of the common species in the hard bottom invertebrate consortia (Lambert & Lambert, 1998; Lambert & Lambert, 2003; Locke, Hanson, MacNair, & Smith, 2009; Martin, LeGresley, Thorpe, & McCurdy, 2011). Many of the *Botryllus schlosseri* populations

beyond the Mediterranean/European Atlantic coasts are established for more than five decades (e.g. in Africa [Millar, 1955] and South America [Orensanz et al., 2002]) and up to a century and more, as in the US east coast (Gould, 1841), California (Van Name, 1945) and New Zealand (Van Name, 1945). Puget Sound populations are probably among the youngest regional populations of this invasive species. Despite the early mentioning of *B. schlosseri*in a naval shipyard in the Seattle area (US Navy, 1951), this species has not been recorded in civil marinas for several decades. Indeed, the partial absence in our 1999 survey may indicate a very recent establishment and coincides with Cohen et al. (1998), who found *B. schlosseri* in Des Moines and Shelton, but not in Edmonds. Thus, the Puget Sound provides a unique opportunity to study relatively recent (ca. three decades) established *B. schlosseri* populations.

The results of the present study reveal significantly differentiated populations on a spatial and temporal scale. Moderate genetic drift (ranging from 0.027 to 0.071) within the populations and limited gene flow (up to 0.031) between locations were also noticed. Population clustering showed that the Des Moines and Shilshole populations are assigned to different clusters, while the Edmonds and Shelton populations represent a mix of the clusters. The low number of microsatellite alleles found in the Puget Sound populations compared to other sites worldwide, was remarkable.

The absence of *B. schlosseri* in two surveys conducted in Edmonds marina in 1998 (Cohen et al., 1998) and 1999 (this study), put this marina as a candidate for the most recent established site (out of the four studied marinas) for the *B. schlosseri* Puget Sound populations. Four years later (2003 sampling session), the allelic indices of this presumed newly established population significantly differed from those of the subsequent sampling period (two years later). In contrast, the Shilshole populations showed fewer fluctuations in the allelic indices between the sampling points, despite only a single colony found in 1999. This indication of a stable population is further supported by genetic drift, which is much lower in Shilshole compared to the other three sites. The genetic indices showed diverse behaviors in the studied marinas. While the allelic richness (AR) and gene diversity (He) were highly fluctuating in Des Moines, Edmonds and Shelton, these two indices were slowly declining over time in the Shilshole marina. The two northernmost marinas, Edmonds and Shilshole, showed a peak in AR during the 2005 sampling period, while in Des Moines and Shelton, the highest numbers were observed in 2018 and 2003, respectively. Only in Shilshole, the peaks of He (year 2003) and private allelic richness (year 2007), did not coincide with the AR. The differences in the genetic indices propose that even close populations exhibit distinct genetic patterns. Yet, Shelton had by far the lowest values in genetic indices, all indicating its remoteness.

Heterozygote deficiency and significant inbreeding coefficients (Fis) found in all Puget Sound populations are in line with previous studies on the U.S. west coast (Karahan et al., 2016; Reem et al., 2013a; Stoner et al., 2002) and worldwide populations (Ben-Shlomo et al., 2010; Lacoursière-Roussel et al., 2012; Paz et al., 2003; Reem et al., 2017; Reem et al., 2013b). Temporal fluctuations in allelic richness seem to be characteristic of *B. schlosseri* populations (this study; Karahan et al., 2016; Reem et al., 2013a) and differ from long-term trends in isolated populations of other organisms, where a decreasing (García-Navas et al., 2015) or increasing (Jason Kennington et al., 2012) allelic richness was observed.

On the U.S. west coast scale, the four studied Puget Sound populations are clustered together, despite more than 120 km separating the two furthest populations. In contrast, the Moss Landing (Karahan et al., 2016) and Santa Cruz (Reem et al., 2013a) populations in California were always assigned in separate clusters, despite them being just 20 km apart. This attests that, despite significant Fst and Dest values, and different AR peak timings, the Puget Sound populations on the west coast level remain genetically close. It is further of interest to find that Moss Landing populations are genetically closer to the Puget Sound populations than to the Santa Cruz populations, as revealed by the Netstruct clusters and the gene flow charts, a result supported by Reem et al. (2013a) assumption that Santa Cruz populations are isolated. Additionally, null alleles were frequent in both, Puget Sound and Moss Landing (Karahan et al., 2016), but not in Santa Cruz populations (Reem et al., 2013a). Year 2007 was marked with the highest gene flow between the Puget Sound and Moss Landing, a year following a severe flooding occurring in Moss Landing, where *B. schlosseri* was temporarily eradicated from that marina (Karahan et al., 2016). Thus, genetic flow originating from the

Puget Sound might have its footprint in the recolonization of the new population in Moss Landing marina.

On a global comparison, the Puget Sound's *B. schlosseri*populations present one of the lowest allelic richness and gene diversity. These low numbers, even when compared to remote populations such as New Zealand (Ben-Shlomo et al., 2001) and South America (Ben-Shlomo et al., 2010), further illustrate the late establishment of the Puget Sound populations, and can be explained by the limited time for the accumulation of mutations and inflow of genetic material from far away populations. Further, *B. schlosseri* microsatellite loci exhibit disparate repertoires of allele sizes and allele richness in different populations, worldwide. While BS-811 has numerous alleles in all locations, other loci, like PBC-1 reveal fewer alleles in regions invaded recently by *B. schlosseri* colonies. Also, the amplification success of *B. schlosseri* microsatellites shows local differences, even when following the same protocols. In the Puget Sound populations, the amplification success of BS-8, BS-9 and PB-41 was relatively low (up to 30 % missing data), whereas the amplification of those loci in samples from the Israeli coast resulted in no failures (S. Tamir, pers. Comm.). Moreover, the native European populations (Reem et al., 2017; Reem & Rinkevich, 2014) revealed more alleles per any specific microsatellite locus, further supporting the Mediterranean/eastern Atlantic origin notion for *B. schlosseri* .

Studying newly established *B. schlosseri* populations for a period of 19 years in the Puget Sound revealed highly fluctuating patterns in genetic indices such as allelic richness (AR), gene diversity (He), inbreeding coefficients (Fis) and population structures (Fst), showing a significant deviation from the Hardy-Weinberg equilibrium in all populations. Due to these fluctuations, no temporal trend could be observed, and it is suggested, that despite remarkable variations between the different sites, the Puget Sound populations remain isolated and still closely related. A comparison on a worldwide level revealed a considerably lower number of alleles in the Puget Sound populations, supporting the recent introduction hypothesis.

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Data accessibility

Microsatellite data available in Dryad (doi will follow once available) and supplementary material.

Author Contributions

BR conceived the study and collected the samples. JZ carried the laboratory work, assisted by ER and JD, all authors contributed to data analyses, JZ and BR wrote the article, all authors revised advanced versions and approved submission.

Tables

Table 1: Indices of population genetics for the Puget Sound marinas. Combined values for all eight loci. S.E.: standard error. N: mean number of samples; NA: mean number of alleles; AR: allelic richness; PAR: private allelic richness; Ho: observed heterozygosity; He: expected heterozygosity; Fis: Inbreeding coefficient, calculations of average per locus. *: Ho significantly ($p<0.05$) differs from He.

Des Moines							
Year	N	NA	AR	PAR	Ho	He	Fis
1999	29.625	5.250	3.829	0.200	0.376*	0.544	0.303
S.E.	0.596	1.306			0.073	0.080	0.116
2001	23.625	4.375	3.724	0.180	0.230*	0.521	0.596
S.E.	0.498	0.865			0.075	0.091	0.114
2003	22.250	5.250	4.266	0.300	0.277*	0.578	0.533
S.E.	0.818	1.176			0.060	0.078	0.087
2005	26.375	4.625	3.820	0.200	0.258*	0.544	0.619
S.E.	1.194	1.017			0.096	0.095	0.147
2007	23.625	4.500	3.489	0.170	0.282*	0.479	0.514
S.E.	1.603	1.150			0.080	0.088	0.120
2013	23.375	5.125	3.864	0.230	0.340*	0.530	0.377
S.E.	0.460	1.217			0.064	0.076	0.086
2018	24.625	5.500	4.417	0.360	0.332*	0.589	0.443
S.E.	1.179	1.254			0.074	0.064	0.102
TOTAL	24.786	4.946	3.916	0.191	0.299	0.541	0.484
	0.470	0.414			0.028	0.030	0.043
Shilshole							
Year	N	NA	AR	PAR	Ho	He	Fis
2001	24.250	4.625	3.889	0.24	0.243*	0.516	0.525
S.E.	1.031	0.981			0.071	0.106	0.096
2003	22.250	4.875	3.825	0.230	0.328*	0.527	0.388
S.E.	0.773	1.246			0.097	0.097	0.128
2005	11.625	4.500	4.031	0.290	0.252*	0.473	0.451
S.E.	0.596	1.165			0.076	0.113	0.122
2007	27.250	5.000	3.798	0.310	0.264*	0.461	0.503
S.E.	0.996	1.254			0.098	0.104	0.129
2013	27.125	4.875	3.566	0.240	0.226*	0.397	0.470
S.E.	0.766	1.156			0.078	0.111	0.138
2018	27.375	5.000	3.612	0.260	0.289*	0.419	0.320
S.E.	0.944	1.195			0.085	0.109	0.128
TOTAL	23.313	4.813	3.787	0.262	0.267	0.467	0.443
	0.876	0.452			0.033	0.042	0.049
Edmonds							
Year	N	NA	AR	PAR	Ho	He	Fis
2003	16.125	3.500	3.149	0.270	0.263*	0.466	0.371

Des Moines							
S.E.	1.008	0.732			0.085	0.102	0.143
2005	24.500	5.250	4.014	0.460	0.344*	0.574	0.345
S.E.	0.866	1.398			0.077	0.092	0.110
2007	23.000	4.250	3.539	0.280	0.268*	0.518	0.469
S.E.	0.926	1.161			0.097	0.087	0.142
2018	26.875	4.875	3.796	0.370	0.276*	0.536	0.501
S.E.	0.953	1.217			0.080	0.090	0.110
TOTAL	22.625	4.469	3.624	0.345	0.288	0.524	0.420
	0.846	0.561			0.041	0.045	0.061
Shelton							
Year	N	NA	AR	PAR	Ho	He	Fis
1999	18.000	3.125	2.693	0.310	0.236*	0.362	0.355
S.E.	0.926	0.875			0.093	0.110	0.144
2003	23.375	4.250	3.437	0.690	0.201*	0.470	0.585
S.E.	2.044	0.648			0.074	0.090	0.123
2018	26.750	4.125	3.104	0.540	0.293*	0.434	0.318
S.E.	1.221	0.972			0.105	0.097	0.162
TOTAL	22.708	3.833	3.078	0.513	0.243	0.422	0.426
	1.107	0.477			0.051	0.056	0.082
Grand Total	23.600	4.644	3.693	0.278	0.279	0.497	0.449

Table 2: Changes in the Puget Sound *Botryllus schlosseri*population genetics between early and late sampling periods (1999-2007 vs. 2013-2018). *Dest* : Measure of population differentiation;*Fst* : Coefficient for population structure measuring the ratio of gene flow to genetic drift; *Fis* : Inbreeding coefficient;*Fs'* : genetic drift. * p<0.05; ** p[?]0.001. Edmonds and Shelton are included for the total period comparisons.

		Des Moines	Shilshole	Edmonds	Shelton
Dest	Total	0.053** ± 0.030	0.023** ± 0.019	0.027* ± 0.016	0.021* ± 0.014
	99-07	0.064** ± 0.042	0.007 ± 0.016	0.029* ± 0.015	0.003 ± 0.017
	13-18	0.024 ± 0.013	0.007 ± 0.007		
Fst	Total	0.041**	0.030**	0.023**	0.030**
	99-07	0.051**	0.015*	0.025*	0.008
	13-18	0.018*	0.010		
Fis	Total	0.416**	0.406**	0.409**	0.356**
	99-07	0.428**	0.430**	0.395**	0.418**
	13-18	0.385**	0.358**		
Fs'	Total	0.071	0.027	0.069	0.069
	99-07	0.076	0.024	0.08	0.050
	13-18	0.048	0.039		

Table 3: Private alleles (PA) obtained in the yearly analyses. # Samples: Total number of samples per site; Total PA: sum of private alleles across all loci in a specific year; PA/sample: sum of private alleles / total number of samples per site; DM: Des Moines; SH: Shilshole; E: Edmonds; SN: Shelton.

SITE	# SAMPLES	1999	2001	2003	2005	2007	2013	2018	Total PA	PA/Sample
DM	190	23	11	10	5	7	13	14	83	0.44
SH	152	-	13	9	5	10	11	8	56	0.37

SITE	# SAMPLES	1999	2001	2003	2005	2007	2013	2018	Total PA	PA/Sample
E	101	-	-	3	11	6	-	6	26	0.26
SN	78	6	-	7	-	-	-	3	16	0.21
	Total PA	29	24	29	21	23	24	31	181	
	Total samples	48	48	84	63	74	51	106		
	PA/Sample	**0.60**	**0.50**	**0.35**	**0.33**	**0.31**	**0.47**	**0.29**		

Table 4: A summary for five microsatellite loci in 10 assigned populations worldwide. *Area:* Location of the study; *N:*Number of studied samples; *BS-811, PB-29, PB-41, PB-49, PBC-1:*number of alleles found in each locus (number alleles/N*100);*Total:* number of alleles over all loci; *NA/100 specimens:*total number alleles per 100 specimens; *AR:* allelic richness;*He:* expected heterozygosity; Sources: 1: current study; 2: Karahan et al. (2016); 3: Reem et al. (2013a); 4: Stoner et al. (2002); 5: Reem et al. (2017); 6: Paz et al. (2003); 7: Ben-Shlomo et al. (2006); 8: Reem et al. (2013b); 9: Ben-Shlomo et al. (2010); 10: Ben-Shlomo et al. (2001).

Region	Area	N	BS-811	PB-29	PB-41	PB-49	PBC-1	Total	NA/100
N. America	(1) Puget Sound	521	43 (8.3)	5 (1.0)	12 (2.3)	13 (2.5)	8 (1.5)	81	16
	(2) Moss Landing	141	36 (25.5)	5 (3.5)	13 (9.2)	13 (9.2)	13 (9.2)	80	57
	(3) Santa Cruz	278	56 (20.1)	6 (2.2)	11 (4.0)	32 (11.5)	22 (7.9)	127	46
	(4) US East Coast	99	32 (32.3)	4 (4.0)	3 (3.0)	6 (6.1)	-	45	45
Europe/Israel	(5) Mediterranean	288	65 (22.6)	12 (4.2)	27 (9.4)	37 (12.8)	41 (14.2)	182	63
	(6) Israel (Mediterranean)	323	36 (11.1)	14 (4.3)	23 (7.1)	25 (7.7)	31 (9.6)	129	40
	(7) Atlantic Europe	360	45 (12.5)	11 (3.1)	25 (6.9)	22 (6.1)	-	103	29
	(8) Scandinavia	319	49 (15.4)	7 (2.2)	12 (3.8)	20 (6.3)	20 (6.3)	108	34
Others	(9) South America	130	32 (24.6)	5 (3.8)	11 (8.5)	23 (17.7)	15 (11.5)	86	66
	(10) New Zealand	195	20 (10.3)	4 (2.1)	8 (4.1)	8 (4.1)	15 (7.7)	55	28

Figures

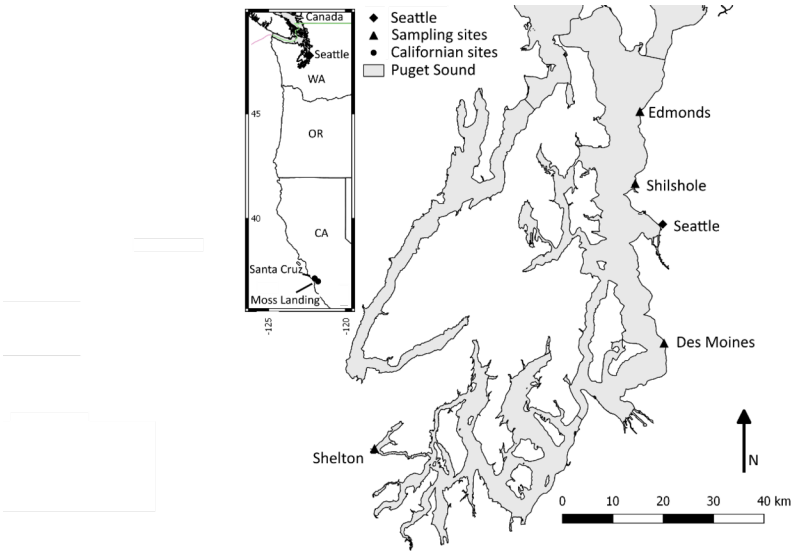

Figure 1: Puget Sound collection sites and the two Californian reference sites on the USA west coast map.

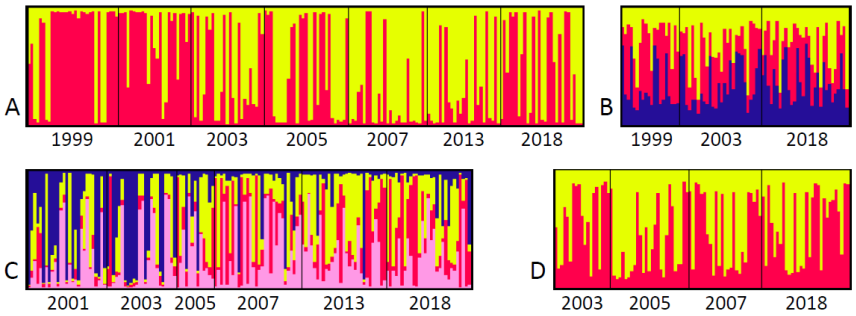

Figure 2: Clusters obtained from STRUCTURE, visualized with Ghostscript. A: Des Moines (K=2); B: Shelton (K=3); C: Shilshole (K=4); D: Edmonds (K=2).

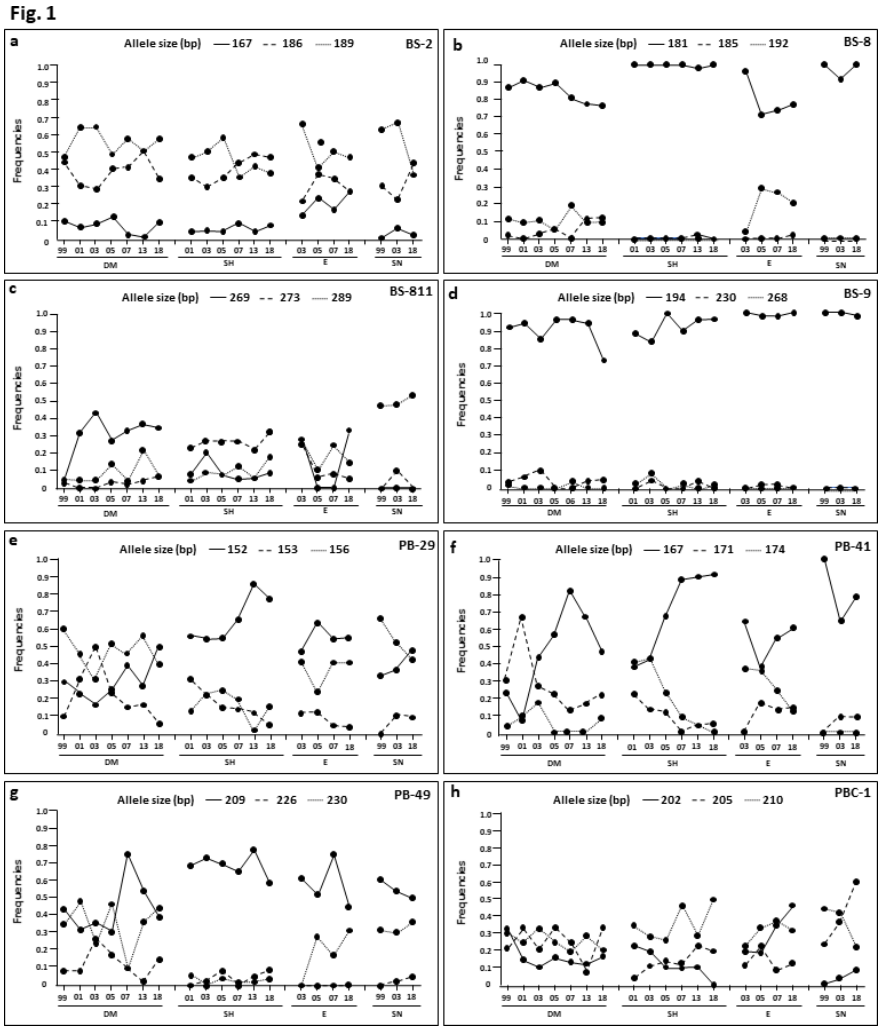

Figure 3: Frequencies of the three most frequent alleles for each locus.

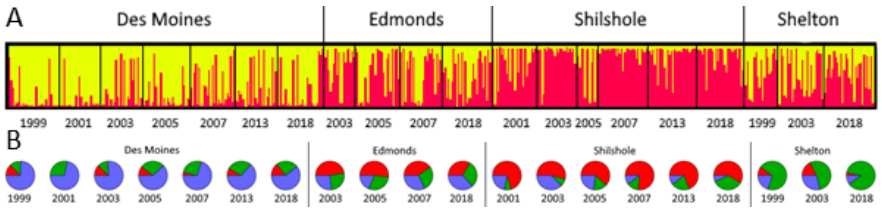

Figure 4: Clusters of Puget Sound populations obtained for all locations and years simultaneously. A: STRUCTURE (K=2), visualized with Ghostscript; B: NetStruct with threshold 0.256 and modularity 0.3 (K=3)

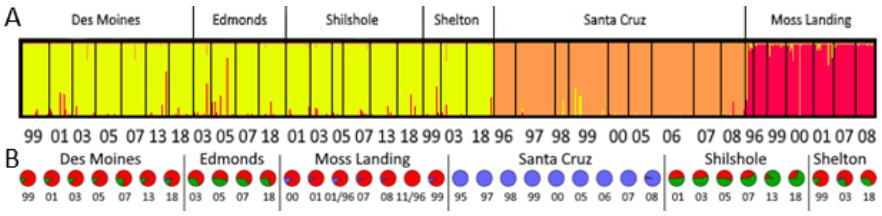

Figure 5: Clusters obtained for the US West Coast populations. A: STRUCTURE analysis, visualized with Ghostscript; B: Netstruct analysis with threshold 0.25 and modularity 0.34 (K=3); Numbers show the year of collection (01/96 and 11/96 for January and November 1996, respectively).

Figure 6: Possible gene flow patterns computed by BAPS between Puget Sound sites and California populations in 2007. Cluster 1: Des Moines; Cluster 2: Shilshole/Edmonds; Cluster 3: Moss Landing; Cluster 4: Santa Cruz.