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Looking at both sides of the invasion: patterns of colonization in the violet tunicate *Botrylloides violaceus*

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Abstract

Understanding the ecological and evolutionary forces that shape the genetic structure of invasive populations and facilitate their expansion across a large spectrum of environments is critical for the prediction of spread and management of ongoing invasions. Here, we study the dynamics of postestablishment colonization in the colonial ascidian *Botrylloides violaceus*, a notorious marine invader. After its initial introduction from the Northwest Pacific, *B. violaceus* spread rapidly along the Pacific and Atlantic coasts of North America, impacting both aquaculture facilities and natural ecosystems. We compare genetic diversity and patterns of gene flow among 25 populations (N = 679) from the West and East coasts, and evaluate the contribution of sexual vs. asexual reproduction to this species' invasion success using data from the mitochondrial cytochrome *c* oxidase subunit I (COI) gene and 13 nuclear polymorphic microsatellite loci. Our results reveal contrasting patterns of spread in the coastal waters of North America. While the West coast was colonized by noncontiguous (long-distance) dispersal, the East coast invasion appears to have occurred through contiguous (stepping-stone) spread. Molecular data further indicate that although dispersal in colonial ascidians is predominantly achieved through sexually produced propagules, aquaculture practices such as high-pressure washing can facilitate fragmentation and potentially exacerbate infestations and spread *via* asexual propagules. The results presented here suggest that caution should be used against the general assumption that all invasions, even within a single species, exhibit similar patterns of colonization, as highly contrasting dynamics may transpire in different invaded ranges.

Keywords: clonal genotypes, colonial ascidian, invasive species, isolation by distance, phylogeography, population genetics

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Introduction

During the past 500 years, the rate of biological invasions has increased to an unprecedented level as a result of human activities (Ricciardi 2007). As a result, nonindigenous species (NIS) are now significantly affecting the ecological and economic integrity of terrestrial and aquatic habitats around the world (e.g. Lejeusne *et al.* 2010). In the face of this growing biodiversity threat, there is an urgent need to understand the complex ecological and evolutionary factors that facilitate establishment and subsequent spread of NIS. Traditionally, invasion biology studies have focussed heavily on the historical context by investigating the origin of NIS and searching for preadapted characteristics of native populations that promote invasiveness (e.g. Tsutsui *et al.* 2000; Cristescu *et al.* 2001). Recently, attention shifted to the invaded ranges, with the goal of capturing both stochasticity of the invasion (e.g. number and frequency of propagules introduced) as well as ecological and/or genetic changes that invasive species experience after being introduced to a new environment (e.g. Kolbe *et al.* 2004; Brown & Stepien 2009; Rollins *et al.* 2009; Zhan *et al.* 2010). Despite growing interest to identify factors that determine invasion success, we still

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have a limited understanding of how patterns of colonization vary across the large spectrum of environments typically encountered by widespread invaders. Such information is essential for recognizing how dispersal vectors and life history traits contribute to the spread of invasive species, for understanding the evolutionary forces that drive invasion success, and ultimately for developing effective management strategies.

Evidence that postestablishment dispersal varies not only between but also within species has started to accumulate with the recent expansion in the geographical scale of NIS genetic surveys (e.g. Voisin *et al.* 2005; Darling & Folino-Rorem 2009; Ramakrishnan *et al.* 2010). Collectively, these studies suggest that fine-scale population genetic analyses performed at multiple spatial scales or settings might be the key for understanding how region-specific attributes can drive within-species invasion patterns. In this context, studies of widespread invaders such as colonial ascidians provide excellent opportunities for exploring how invasion history, vector availability and life history traits influence colonization dynamics in different areas of introduction.

Botrylloides violaceus (Stolidobranchia, Styelidae), also known as the violet tunicate, is an invasive colonial ascidian, commonly recognized as a biofouling nuisance species. In natural ecosystems, it has been shown to overgrow and outcompete indigenous species, at times becoming dominant in subtidal benthic communities (Berman *et al.* 1992). In aquaculture facilities, it smothers target species, limits food availability and covers available substrate making harvesting difficult (Carver *et al.* 2006). *B. violaceus* is considered native to the Northwest Pacific, most likely Japan (Saito *et al.* 1981). Outside of its native range, it occurs on both coasts of North America (Carver *et al.* 2006) as well as the coasts of Australia, Italy, the United Kingdom, Ireland and the Netherlands (Zaniolo *et al.* 1998; Gittenberger 2007; Minchin 2007; Perez-Portela *et al.* 2009). The first nonindigenous population of *B. violaceus* is thought to have established on the coast of California (Lambert & Lambert 2003). However, because of confusion with the congener *B. diegensis*, the date of the first published report remains uncertain and can be either 1945 (collection made in 1939; Van Name 1945; unconfirmed) or the 1970s (Fay & Johnson 1971; confirmed). At present, *B. violaceus* occupies much of the West coast, achieving high abundance from Ensenada, Mexico to Alaska (Lambert & Sanamyan 2001; Lambert & Lambert 2003). The establishment of *B. violaceus* on the East coast is equally obscure, also because of taxonomic confusion. It is thought that the first populations were established sometime between 1973 and 1978 on the coasts of Massachusetts and Maine (Dr. J.T. Carlton, personal communication). Since then, the East coast *B. violaceus* invasion spread rapidly south to Connecticut and north to Prince Edward Island (Carver *et al.* 2006) and Newfoundland (Callahan *et al.* 2010). Multiple vectors are considered responsible for the dispersal of *B. violaceus* along the West and East coasts. Coastal vessel hull fouling and the movement of aquaculture shellfish stocks are considered the primary vectors (Dijkstra *et al.* 2007; Dr. J.T. Carlton, personal communication), although ballast water discharge (Dijkstra *et al.* 2007), rafting of fragmented colony parts (Carver *et al.* 2006) and spread as epibionts on large crustaceans (Bernier *et al.* 2009) have also been suggested to facilitate dispersal of the species.

Biological characteristics linked to the high invasiveness of *B. violaceus* include the ability to engage in both sexual and asexual reproduction. In particular, asexual budding of individual zooids often leads to the formation of large mats, which commonly fragment and regenerate, leading to an increased local abundance and dispersal potential (Carver *et al.* 2006). *B. violaceus* is highly effective at reattaching when dislodged from substrates (Bullard *et al.* 2007), thus the common aquaculture management technique of high-pressure washing is largely ineffective and may, in fact, lead to an increase in colony fragmentation and subsequent infestation. Although the distribution, impacts and ecology of *B. violaceus* have been extensively studied, a comprehensive examination of genetic patterns associated with this species' rapid range expansion is still lacking.

Here we use mitochondrial COI sequences and 13 polymorphic nuclear microsatellite loci to characterize the genetic structure of 25 introduced populations of *B. violaceus* sampled along the West and East coasts of North America. The primary goals of this study were to: (i) investigate the genetic diversity of introduced populations on the West and East coasts; (ii) compare the patterns of gene flow within and between coasts; and (iii) evaluate the relative contribution of sexual and asexual reproduction to this species' rapid population expansion. Characterization of genetic structure in established populations further allowed us to make inferences on the colonization history of *B. violaceus* in the two invaded ranges in North America.

Materials and methods

Sampling and DNA extraction

We sampled 679 colonies of *B. violaceus* from 25 North American locations: 344 (50.7%) colonies from the West coast and 335 (49.3%) from the East coast (Fig. 1; Table 1). A fine-scale sampling scheme was employed for the northern part of the current distribution range where rapid spread of *B. violaceus* is challenging

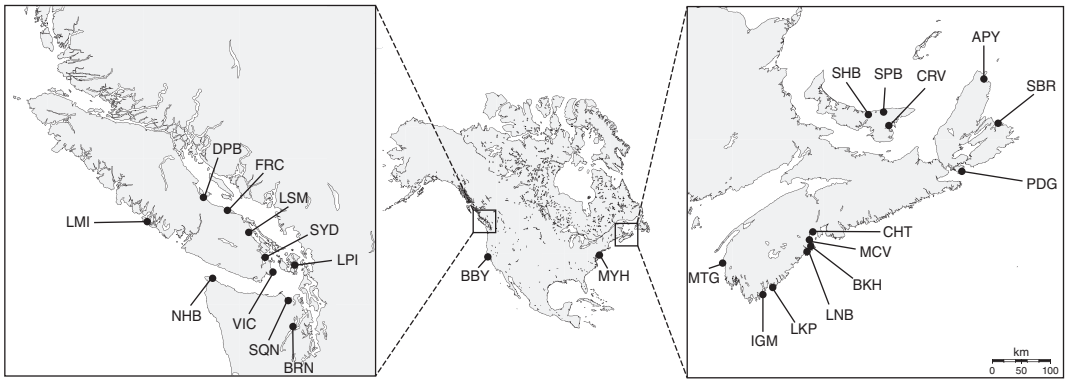

Fig. 1 Sampling locations for the violet tunicate *Botrylloides violaceus* on the West and East coasts of North America, with locality names defined in Table 1. Inset maps show the focal sampling regions on each coast.

Table 1 Locations of *Botrylloides violaceus* sampling and genetic diversity indices for mitochondrial and microsatellite markers with N_C, sample size including clonal genotypes; N, sample size after removal of clones; N_h, number of haplotypes; h, haplotype diversity; π, nucleotide diversity; N_A, number of alleles; N_{AP}, number of private alleles; A, allelic richness; H_O, mean observed heterozygosity; H_E, mean expected heterozygosity

		mtDNA						Microsatellite						
Location	ID	N_C	N	N_h	Haplotype codes	h	π	N_C	N	N_A	N_{AP}	A	H_O	H_E
West Coast														
Deep Bay, BC	DPB	22	18	3	Bv1; Bv2; Bv10	0.386	0.007	19	15	53	2	3.3	0.568	0.576
French Creek, BC	FRC	24	21	2	Bv1; Bv2	0.095	0.001	17	14	51	1	3.3	0.571	0.569
Lemmens Inlet, BC	LMI	48	37	4	Bv1; Bv2; Bv3; Bv11	0.417	0.004	53	42	90	3	4.0	0.511	0.612
Ladysmith, BC	LSM	26	21	4	Bv1; Bv2; Bv8; Bv9	0.610	0.010	20	15	49	0	3.2	0.429	0.601
Sydney, BC	SYD	32	31	1	Bv1	0.000	0.000	32	31	61	1	3.4	0.434	0.554
Victoria, BC	VIC	31	30	1	Bv1	0.000	0.000	32	31	68	2	3.7	0.562	0.643
Lopez Island, WA	LPI	30	30	3	Bv1; Bv2; Bv3	0.522	0.008	30	30	77	2	3.8	0.472	0.596
Neah Bay, WA	NHB	30	30	3	Bv1; Bv2; Bv3	0.503	0.008	30	30	89	1	4.4	0.545	0.704
Sequin, WA	SQN	30	30	1	Bv1	0.000	0.000	30	30	83	1	4.0	0.553	0.641
Brinnon, WA	BRN	30	30	3	Bv1; Bv3; Bv9	0.549	0.009	28	28	75	5	4.0	0.517	0.621
Bodega Bay, CA	BBY	35	35	2	Bv1; Bv8	0.363	0.001	34	34	86	4	3.9	0.487	0.604
Total		338	313	7		0.313	0.005	325	300	155	22	3.7	0.514	0.611
East Coast														
St. Peter's Bay, PEI	SPB	38	33	1	Bv1	0.000	0.000	44	39	57	0	3.3	0.468	0.561
Savage Harbour, PEI	SHB	45	43	1	Bv1	0.000	0.000	50	50	62	0	3.5	0.613	0.610
Cardigan River, PEI	CRV	42	38	1	Bv1	0.000	0.000	45	41	64	1	3.5	0.531	0.600
Aspy Bay, NS	APY	29	29	2	Bv1; Bv3	0.133	0.002	29	29	57	0	3.4	0.545	0.601
South Bar, NS	SBR	30	29	2	Bv1; Bv3	0.133	0.002	28	27	59	1	3.5	0.484	0.599
Petit de Grat, NS	PDG	23	23	2	Bv1; Bv3	0.166	0.002	24	24	60	0	3.5	0.567	0.604
Chester, NS	CHT	12	12	1	Bv1	0.000	0.000	13	13	49	0	3.2	0.518	0.561
Martin's Cove, NS	MCV	15	15	1	Bv1	0.000	0.000	17	17	51	0	3.4	0.526	0.621
Black Harbour, NS	BKH	13	12	1	Bv1	0.000	0.000	16	15	55	3	3.6	0.554	0.652
Lunenburg, NS	LNB	20	20	2	Bv1; Bv3	0.100	0.002	22	22	67	1	3.9	0.515	0.662
Methegan, NS	MTG	7	7	1	Bv1	0.000	0.000	7	7	39	0	3.0	0.478	0.576
Lockeport, NS	LKP	8	8	1	Bv1	0.000	0.000	8	8	54	1	3.8	0.461	0.612
Ingomar, NS	IGM	7	7	1	Bv1	0.000	0.000	10	10	51	0	3.5	0.472	0.579
Mystic Harbor, CT	MYH	17	15	1	Bv1	0.000	0.000	20	18	52	0	3.2	0.532	0.559
Total		306	291	2		0.038	0.001	333	320	110	7	3.4	0.518	0.600

aquaculture operations. Sampling was undertaken by SCUBA diving and/or by excising colonies from submerged ropes and buoys in harbours, marinas and infested aquaculture facilities. To prevent re-sampling of the same colonies, all specimens were taken at least 1 m apart from one another. Samples were preserved in

95% ethanol at −20 °C prior to genetic analyses. Genomic DNA (gDNA) was extracted from four to six zooids using the protocol of Elphinstone *et al.* (2003).

Mitochondrial DNA amplification and sequencing

A fragment of the COI gene was initially amplified using the universal primer pair LCO1490 and HCO2198 (Folmer *et al.* 1994). Because these primers failed to amplify consistently, species-specific primers (BvCOIF: 5′-TTTGTATTTTATTTTTAGGGTTTGG-3′ and BvCOIR: 5′-TCAAAATAAATGTTGATAAAGTACAGG-3′), which amplify a 659-bp fragment, were designed and used. The 25 μL PCR volume consisted of 1 μL (approximately 50–100 ng) gDNA, 1× PCR buffer with 1.5 mM $MgCl_2$ (Genscript), 0.2 mM dNTPs, 0.4 μM of each primer and 0.5 U of *Taq* DNA Polymerase (Genscript). PCR cycling parameters consisted of an initial denaturation step at 94 °C for 5 min, followed by 35 amplification cycles (94 °C for 30 s, 50 °C for 30 s, 72 °C for 45 s), and a final elongation step at 72 °C for 5 min. Sequencing reactions were performed using the reverse primer (BvCOIR), BigDye Terminator 3.1 chemistry and an ABI 3130XL automated sequencer (Applied Biosystems, Foster City, CA, USA). The forward primer (BvCOIF) was used to confirm all sequences that contained ambiguous sites.

Microsatellite genotyping

All the 679 *B. violaceus* samples – representing 25 populations – were genotyped for 13 polymorphic microsatellite markers (Bvm2, Bvm4 - 9, Bvm12 - 13, Bvm15 - 18; Molecular Ecology Resources Primer Development Consortium *et al.* 2010). PCR cocktails (10 μL) contained 50 ng of gDNA, 1× PCR buffer with 1.5 mM $MgCl_2$ (Genscript), 0.125 mM of each dNTP, 0.5 μM of each primer and 0.2 U of *Taq* DNA Polymerase (Genscript). Forward primers were labelled with one of four fluorophores (6FAM, VIC, NED or PET) according to Schuelke (2000). The cycling PCR profile consisted of an initial denaturation at 95 °C for 3 min, 10 cycles of 35 s at 95 °C, 35 s at an initial annealing temperature of 60 °C that decreased by 1 °C in each of 10 cycles, and 45 s at 72 °C followed by 35 cycles of 35 s at 95 °C, 35 s at 52 °C, 45 s at 72 °C, and a final extension for 10 min at 72 °C. Amplified fragments were separated using an ABI 3130XL automated sequencer (Applied Biosystems, Foster City, CA, USA), with GeneScan™-500 LIZ™ (Applied Biosystems) internal size standard. The alleles were scored using GeneMapper® software v.4.0 (Applied Biosystems). To confirm genotyping accuracy, 3% of the samples, chosen at random, were rerun.

Mitochondrial DNA analysis

Sequence data were aligned and edited using CodonCode Aligner v. 2.0.6 (CodonCode Corporation, Dedham, MA, USA). DnaSP v.5 (Rozas *et al.* 2003) was used to identify individual *B. violaceus* haplotypes, calculate the number of haplotypes (N_h), haplotype diversity (h) and nucleotide diversity (π), and to test whether the sequences evolved under neutrality according to Tajima's D statistic. Neighbor-joining (NJ) and maximum-likelihood (ML) phylogenetic analyses were conducted in PAUP* v.4b10 (Swofford 2001) and PHYML v. 2.4.4 (Guindon & Gascuel 2003) respectively. To determine the best-fit nucleotide substitution model, we used ModelTest v.3.7 (Posada & Crandall 1998) under the Akaike Information Criterion (AIC). Phylogenetic reconstructions were rooted using the congeneric species *B. fuscus* (GenBank accession number: GQ365690). Relationships among the COI haplotypes were further examined using a statistical parsimony haplotype network generated at the 95% connection limit with TCS v.1.21 (Clement *et al.* 2000).

Population pairwise Φ_{ST} was calculated with 10 000 permutations in ARLEQUIN v.3.1 (Excoffier *et al.* 2005) using the Tamura & Nei (TrN) substitution model. To assess genetic differentiation among sampling sites, we conducted a hierarchical analysis of molecular variance (AMOVA; Excoffier *et al.* 1992) in ARLEQUIN. Molecular variance was partitioned into three levels: between coasts, among populations within coasts and within populations. Isolation by distance (IBD) within the West and East coasts was further examined by testing the correlation between genetic distance $[\Phi_{ST}/(1 - \Phi_{ST})]$ and geographical distances using a Mantel test with 10 000 permutations implemented in GENEPOP v. 3.4 (Raymond & Rousset 1995). Geographical distances were calculated as the minimum coastline distances between adjacent sampling locations using GOOGLE EARTH v.4.3 (beta).

Microsatellite DNA analysis

The number of repeated multilocus genotypes was calculated using GENECAP software (Wilberg & Dreher 2004). For all putative clones, we estimated the probability of identical genotypes arising by chance *via* sexual reproduction. We computed the lower bound of this probability, P_{HW} under Hardy–Weinberg expectations and the more conservative upper bound P_{sib}, under strict sibling reproduction, as recommended by Waits *et al.* (2001). Calculations for P_{HW} and P_{sib} consider the observed allele frequencies in the population within which clones were identified (Wilberg & Dreher 2004).

Microsatellite data were checked for departures from Hardy–Weinberg equilibrium (HWE) using 10 000 permutations in GENEPOP, with levels of significance adjusted by sequential Bonferroni corrections (Rice 1989). The total number of alleles (N_A), allelic richness (A), mean observed and expected heterozygosities (H_O and H_E), and the inbreeding coefficient (F_{IS}) were calculated using FSTAT v.2.9.3.2 (Goudet 2002; updated from Goudet 1995). FSTAT incorporates a rarefaction method (Mousadik & Petit 1996) that compensates for unequal sample sizes to calculate A. The degree of genetic differentiation between pairs of populations was assessed using pairwise F_{ST} values computed with 10 000 permutations in ARLEQUIN. Additionally, population structure was determined by conducting a three-dimensional factorial correspondence analysis in GENETIX v. 4.05 (Belkhir *et al.* 2004) and by the Bayesian clustering approach implemented in STRUCTURE v. 2.3.1 (Pritchard *et al.* 2000). For the STRUCTURE analysis, for each value of K (population clusters), we carried out five independent Markov Chain Monte Carlo (MCMC) runs with 10^5 generations discarded as burn-in followed by an additional 10^6 generations. The simulated K values ranged from 1 to 25 (total sites) when pooling individuals from all localities and from 1 to 10 and 1 to 15 when using individuals previously grouped into a single cluster based on the initial global analysis. The optimal number of clusters was estimated by comparing the log-likelihood of the data given the number of clusters [ln $P(X|K)$] (Pritchard *et al.* 2000) and by examining the standardized second-order rate change of ln $P(X|K)$, ΔK (Evanno *et al.* 2005).

Contemporary gene flow was assessed by individual-based assignment using GENECLASS v. 2.0 (Piry *et al.* 2004). We used the partially Bayesian method of Rannala & Mountain (1997); this method is preferred when not all possible source populations have been sampled (Berry *et al.* 2004). Genotype assignments were determined by assessing probabilities through 10 000 MCMC simulations (Paetkau *et al.* 2004). The sample with the highest probability of assignment was considered the most likely source for the assigned genotype. Individuals not assigned to any population with a probability of $P > 0.05$ were assumed to be from an unsampled location. To test for a pattern of IBD, we used a Mantel procedure with 10 000 permutations implemented in GENEPOP to assess the dependence between Rousset's (1997) genetic distance [$F_{ST}/(1-F_{ST})$] and geographic distances. A hierarchical AMOVA was performed in ARLEQUIN based on microsatellite genetic distances between populations, and partitioning variance between the West and East coasts, among populations within coasts and within populations.

Results

Mitochondrial DNA analyses

The final 558 bp COI alignment contained a total of 27 polymorphic sites (nine parsimony-informative), with 26 synonymous substitutions and one nonsynonymous substitution corresponding to a valine - isoleucine change. Within all 604 sequences, we identified only seven distinct haplotypes: Bv1 - Bv3 (GenBank accession numbers GQ365691 – GQ365693) and Bv8 - Bv11 (GenBank accession numbers GU946476 – GU946479). Most haplotypes were shared between two or more sampling locations. Only haplotypes Bv10 and Bv11 were restricted to the Deep Bay (DPB) and Lemmens Inlet (LMI) populations respectively (Appendix S1). The most abundant haplotype on the West coast was Bv1 (66.8%) followed by Bv3 (20.2%) and Bv2 (6.2%). On the East coast, 98.6% of colonies sampled shared haplotype Bv1 and only 1.4% haplotype Bv3 (Table 1). Haplotype diversities were typically much higher in West coast (range: 0–0.610; mean 0.313) than in East coast (range: 0–0.166; mean 0.038; Table 1) populations. Tajima's D statistic was not significant ($D = 1.076$; $P > 0.10$) for the entire data set, suggesting that selection was not acting on this locus and that a neutral model of evolution cannot be rejected.

The neighbour-joining and maximum likelihood phylogenetic analyses revealed that all North American *B. violaceus* haplotypes correspond to two phylogroups (Appendix S2). This finding was confirmed by the 95% parsimony haplotype network, where two groups of haplotypes separated by nine substitution steps were identified (Fig. 2). One group included the dominant Bv1 haplotype, shared between the two coasts, and Bv8, restricted to the West coast. The second group consisted of four haplotypes that occur on the West coast (Bv2, Bv9, Bv10 and Bv11), and one (Bv3) detected on both coasts (Fig. 2).

Pairwise Φ_{ST} values indicated genetic structure exists within the West coast, with 43.6% of comparisons significant after Bonferroni corrections (Appendix S3). The Lemmens Inlet (LMI) and Bodega Bay (BBY) populations were differentiated from most populations sampled on the West coast, with Φ_{ST} values ranging from 0.12 to 0.84. By contrast, all pairwise Φ_{ST} values between East Coast populations were low and not significant. Within the entire data set, the highest pairwise Φ_{ST} values (0.86) were attained between West and East coast populations. Hierarchical AMOVA based on mitochondrial data revealed that most of the genetic variance was found within sampling sites (48.46%). Significant partitioning occurred among populations within each coast and between the West and East

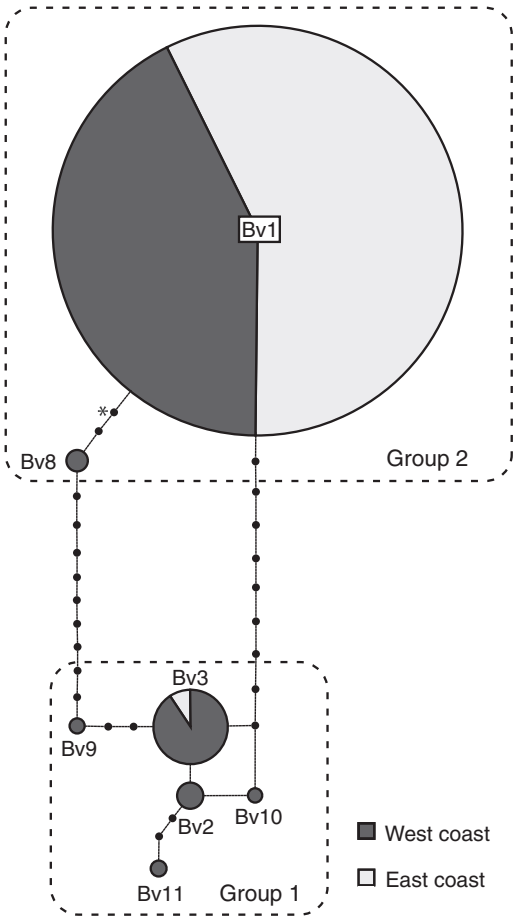

Fig. 2 Statistical parsimony network of *Botrylloides violaceus* cytochrome *c* oxidase subunit I (COI) haplotypes. Circle size is proportional to haplotype frequency, and small black dots indicate unsampled haplotypes inferred from the data. The nonsynonymous substitution is indicated by an asterisk. Groups 1 and 2 correspond to clades well supported by the neighbour-joining and maximum likelihood phylogenetic analyses (Appendix S2).

coasts, accounting for 33.30% and 18.24% of the variation respectively ($P < 0.05$; Table 2). Mantel tests failed to reveal relationships between genetic distance and coastline distances between populations on both the West coast ($r^2 = 0.012$, $P = 0.619$) and East coast ($r^2 = 0.002$, $P = 0.338$).

Microsatellite DNA analyses

We identified 27 multilocus repeated genotypes across all populations (C1 – C27; Appendix S4). Most putative clones were restricted to single populations; only genotype C4 was shared between Deep Bay (DPB) and Ladysmith (LSM). The repeated genotypes were encountered between one and seven times depending on the location (Appendix S4). The probability of separate occurrences of the same genotype arising *via* sexual recombination under Hardy–Weinberg assumptions (P_{HW}) was extremely low, ranging from 5.34×10^{-6} to 1.93×10^{-12} (Appendix S4). In addition, the more conservative estimate (P_{sib}) remained low and nonsignificant ($P < 0.05$), suggesting that repeated genotypes resulted from resampling of fragmented colonies. Data analysis was performed with and without clones, producing comparable results. We present results obtained after removing clonal genotypes from the data set.

A total of 620 *B. violaceus* colonies were analysed at 13 microsatellite loci. In total, we identified 169 alleles across North America, of which 155 (91.2%; mean 12 alleles/locus) were detected in West coast populations, and 110 (65%; mean 8.5 alleles/locus) in East coast samples (Appendix S5). The number of private alleles also differed between the West coast (22 private alleles) and the East coast (seven private alleles; Table 1). The allelic richness and expected heterozygosity varied from 3.2 to 4.4 (mean 3.7) and 0.554 to 0.704 (mean 0.611), respectively, on the West coast, and from 3.0 to 3.9 (mean 3.4) and 0.559 to 0.662 (mean 0.600), respectively, on the East coast. West coast populations sampled at Lemmens Inlet (LMI), Neah Bay (NHB) and Sequin (SQN) had the highest genetic diversity. Conversely, the lowest variation was identified on the East coast at Chester (CHT), Methegan (MTG) and Mystic Harbor (MYH; Table 1). While most loci conformed to HWE, 35 of 325 cases exhibited significant deviations after sequential Bonferroni corrections (Appendix S6). However, no systematic deviations were observed for loci across all populations or at all loci within populations. Microsatellite data showed highly significant genetic differentiation after Bonferroni correction between most pairs of samples (Appendix S7), with the exception of one pair of sites on the West coast (DPB - LSM) and five more on the East coast (APY - SPB; CRV - SHB; BKH - MCV; BKH - LNB; LNB - MCV).

The two different approaches used to identify population structure, factorial correspondence analysis and Bayesian clustering, provided a largely concordant picture. Three-dimensional factorial correspondence analysis (3D-FCA) illustrated high genetic distances between geographically proximate West coast populations (Fig. 3). However, for the East coast, neighbouring samples generally grouped together. Component 1 explained 27.25% of genetic variance and nearly perfectly separated West from East coast populations (Fig. 3). The only exception was BBY, which clustered with East coast populations. The Bayesian algorithm implemented in STRUCTURE indicated that all *B. violaceus* individuals could be assigned to two main genetic clusters ($K = 2$). This partitioning was supported by the evaluation of both ln $P(X|K)$ and ΔK.

Table 2 Analysis of molecular variance (AMOVA) results on *B. violaceus* mtDNA and microsatellite data for East vs. West coast population grouping. All fixation indices are statistically significant

Source of variation	d.f.	Variance components	% variation	Fixation indices	*P* value
mtDNA					
Between coasts	1	0.295 Va	18.25	F_{CT}: 0.182	0.0332
Among populations within coasts	23	0.538 Vb	33.30	F_{SC}: 0.407	0.0000
Within populations	579	0.782 Vc	48.46	F_{ST}: 0.515	0.0000
Microsatellite					
Between coasts	1	0.122 Va	6.42	F_{CT}: 0.064	0.0000
Among populations within coasts	23	0.208 Vb	10.93	F_{SC}: 0.117	0.0000
Within populations	1215	1.570 Vc	82.65	F_{ST}: 0.173	0.0000

The likelihood of the data was the lowest for $K = 1$ and the largest difference of successive likelihoods was between $K = 1$ and $K = 2$. After $K = 2$, the likelihood of the data plateaued and standard deviations increased (Appendix S8). When ln $P(X|K)$ only marginally increases above a certain value of K, the smallest value of K before the plateau (here $K = 2$) is considered the best model (Pritchard *et al.* 2007). The estimation of ΔK as per Evanno *et al.* (2005) also showed a clear pattern for $K = 2$ (Appendix S8), confirming that this is the most parsimonious model for the global data set. Two genetic clusters, hereafter S1 and S2, corresponded remarkably well with the West vs. East coast geographic partitioning of our data (Fig. 4a). For West coast populations, membership coefficients (Q) to cluster S1 averaged 92% with the exception of BBY, which averaged only 18%. For East coast populations, Q to S2 averaged 93% (Fig. 4a). When clustering analysis was performed separately for S1 and S2, two genetic clusters were identified for each data subset. The substructure within S1 revealed the distinctiveness of proximate populations such as Victoria (VIC)—Sydney (SYD) or Sequin (SQN)—Brinnon (BRN; Fig. 4b). Within S2, the partition in two clusters revealed that individual genotypes assign to different clusters in the northern and the southern parts of the East coast, with assignment ratios following a gradual transition along the north–south axis (Fig. 4c). In addition, southern locations (MYH—CHT) appeared to be most similar to BBY.

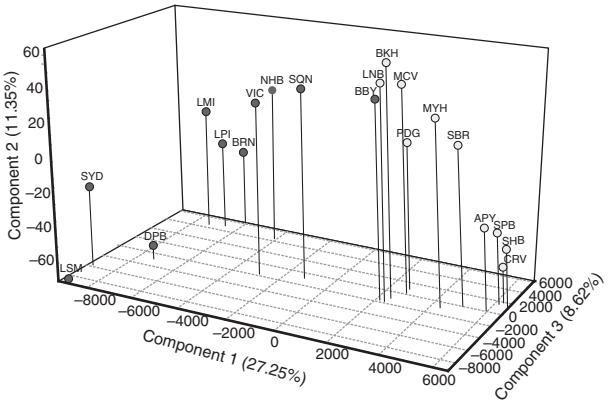

Fig. 3 Three-dimensional factorial correspondence analysis (3D-FCA) of *Botrylloides violaceus* microsatellite data showing clustering between North American West coast (dark grey) and East coast (light grey) sites. Bodega Bay population (BBY) clusters tightly in the analysis with East coast populations. Sampling sites with <15 individuals ($N < 15$) were not considered for this analysis.

The analysis for detection of migrants showed high overall assignment success, approximately 99.7% (603 of 605 individuals were definitively classified at $P > 0.05$). On the West coast, the inferred migration events appeared to be distributed throughout the sampling region, and only one population (BRN) displayed 100% self-assignment (Table 3). On the East coast, the majority of putative migrant genotypes (32/38) were restricted to the northern part of the sampling region (SPB; SHB; CRV and APY). Outside this area, there was limited indication of migration (Table 3). Ten individuals were identified as potential inter-coastal dispersers. The inferred migration events were highly directional, from the West to the East coast (Table 3). The pattern of dispersal on the West coast did not reflect a correlation between genetic differentiation and geographical distances (Fig. 5a). The Mantel test remained nonsignificant after excluding the genetically distinct BBY population from analysis (not shown). Conversely, for East coast populations, the Mantel test suggested highly significant (Fig. 5b). A strong correlation between genetic IBD and geographic distance was supported at the large (1706 km) and smaller (504 km) scales for East coast populations. Hierarchical AMOVA of microsatellite data revealed that most variation was attributed within sampling sites (82.65%). Variation within coasts (10.93%) and between coasts (6.42%) was also statistically significant (Table 2).

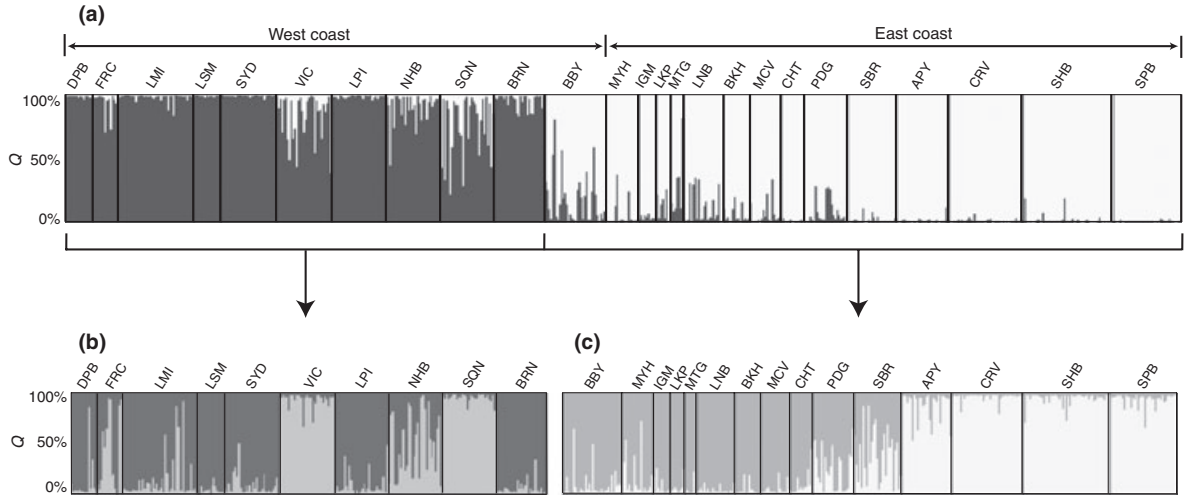

Fig. 4 Bayesian clustering of *Botrylloides violaceus* genotypes performed in STRUCTURE for all samples (a) and each of the two main genetic clusters (b, c). Each individual is represented by a thin vertical line, which is partitioned into $K = 2$ segments representing the individual's estimated proportional membership (Q). Sampling sites are separated by black lines. For (a) dark grey corresponds to cluster S1 and light grey to cluster S2. Different shades of grey are used to represent two genetic clusters in each data subset.

Discussion

Contrasting patterns of genetic structure and regional spread on the West and East coasts

Overall, our genetic diversity estimates for *B. violaceus* in North America are lower than those reported for other invasive ascidians. For example, mitochondrial genetic diversity estimates computed for 25 invasive populations (seven haplotypes, mean h of 0.176) were much lower than those reported for the solitary ascidian *Microcosmus squamiger* (30 haplotypes, mean h of 0.712 estimated in nine invasive populations sampled worldwide; Rius *et al*. 2008) and *Ciona intestinalis* spB (46 haplotypes, mean h of 0.727 estimated in 14 populations sampled worldwide; Zhan *et al.* 2010). Moreover, our microsatellite gene diversity estimates (mean H_E of 0.606, computed using 13 microsatellites) were lower than those identified for the closely related colonial species, *Botryllus schlosseri,* in North America (mean H_E of 0.845, computed over seven invasive populations using four microsatellites; Stoner *et al.* 2002) or *Ciona intestinalis* spB in North America (mean H_E of 0.820 computed over six populations using eight microsatellites; Zhan *et al.* 2010), and were comparable to those reported for the solitary ascidian *Styela clava* in North America (mean H_E of 0.536, computed over four invasive localities using six microsatellites; Dupont *et al.* 2010).

Despite the overall low level of genetic diversity, we observed strong geographic partitioning of genetic variance between North American *B. violaceus* samples, with West coast populations exhibiting higher levels of genetic diversity than those on the East coast (Table 1). This pattern was supported by the mitochondrial number of haplotypes (7 vs. 2), mean haplotype diversity (0.313 vs. 0.038) and nucleotide diversity (0.005 vs. 0.001). This difference in genetic diversity was consistent with that observed for microsatellite markers, mostly in the total number of alleles (155 vs. 110) and private alleles (22 vs. 7) sampled. The genetic partitioning between the two coasts might reflect bottleneck events associated with the introduction of this species to North America. As such, the *B. violaceus* invasion could have proceeded in a 'stepping stone' fashion, with East coast populations being seeded from West coast stocks. As expected, the bottleneck signature on the East Coast is more apparent in the mitochondrial genome, which is more sensitive to demographic population fluctuations than the nuclear genome, because of its smaller effective population size, more rapid extinction of lineages, and lack of recombination (Avise 2000).

Overall, the pattern of regional spread and resultant population genetic structure differed sharply in the two main sampling regions analysed, as revealed by microsatellite genetic variation. On the West coast, almost all pairwise F_{ST} values were high and significant (Appendix S7). Moreover, factorial correspondence analysis (Fig. 3) revealed high genetic divergence between most sampling locales. On the other hand, increased levels of genetic connectivity were detected between few distant sites such as DPB—LSM, situated 90 km apart (F_{ST} = 0.03; Appendix S7; see also Table 3), suggestive of long distance (likely human-mediated) dispersal. Consistent with this observation, there was no indication of

Table 3 Results of assignment test, with source populations listed by column and recipient populations by row. Populations on the West coast are separated by a box in the upper left corner. Individuals assigned to the sampling site where they were collected are indicated in bold along the diagonal. Populations with sample size of <10 individuals ($N < 10$) were not included. Locality names are defined in Table 1.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1. DPB	**14**	1																					
2. FRC		**13**		1																			
3. LMI			**38**				1	1		2													
4. LSM	6			**9**																			
5. SYD	2			2	**24**					2													
6. VIC	1					**29**		1															
7. LPI	2						**26**			2													
8. NHB								**29**	1														
9. SQN								2	**28**														
10. BRN										**28**													
11. BBY								1			**32**					1							
12. SPB												**33**	3	3									
13. SHB								1				1	**43**	3	2								
14. CRV												4	1	**36**									
15. APY												9	6		**14**								
16. SBR												1				**26**							
17. PDG																	**22**		1				
18. CHT																		**13**					
19. MCV																			**17**				
20. BKH																				**14**	1		
21. LNB								2			2								2		**16**		
22. IGM											1											**9**	
23. MYH											3												**15**

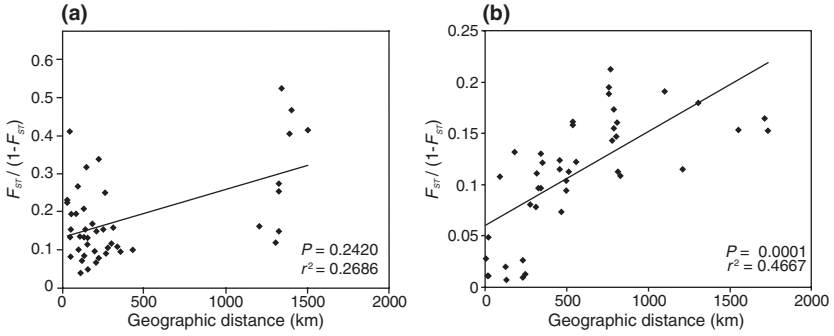

Fig. 5 Results of isolation by distance (IBD) analysis performed using microsatellite data for West coast (a) and East coast (b) *Botrylloides violaceus* populations. Coefficient of determination (r^2) and significance of correlation (P) are indicated for both tests.

IBD for West coast populations ($r^2 = 0.2686$, $P = 0.2420$; Fig. 5a). The pattern of genetic structure observed on the West coast may reflect the combined effects of low levels of natural dispersal coupled with long distance spread (most likely human-mediated) between key locations. Similarly complex patterns of connectivity have been reported in other invasive taxa, such as the solitary ascidian *Styela clava*, the anemone *Nematostella vectensis* and the hydrozoan *Cordylophora caspia*, that possess limited natural dispersal capability and spread mainly *via* anthropogenic vectors (Darling & Folino-Rorem 2009; Darling *et al.* 2009; Dupont *et al.* 2009).

On the East coast, genetic differentiation augments with increasing spatial separation between sites (Figs 3 and 4c, Appendix S7). High genetic similarity was detected between adjacent sites (i.e. 1.5 km apart) such as MCV—BKH ($F_{ST} = 0.01$) or LNB—BKH ($F_{ST} = 0.02$; Appendix S7), while high genetic divergence was

observed between more spatially separated sites (i.e. 150–250 km apart) such as CRV—SBR (F_{ST} = 0.12) or PDG—SBR (F_{ST} = 0.16; Appendix S7). Also, STRUCTURE analysis revealed that East coast genotypes were assigned to different clusters in the northern and the southern parts of the sampling region, with assignment ratios following a gradual transition along the north–south axis (Fig. 4c). Consequently, a highly significant pattern of IBD was detected for East coast populations (r^2 = 0.4667; P < 0.001; Fig. 5b). This finding was surprising considering that strong associations between the two metrics are typically considered indicative of systems under migration–drift equilibrium (Hutchinson & Templeton 1999). However, historical evidence suggests that East coast *B. violaceus* is unlikely to have achieved such equilibrium. The species is thought to have been introduced in the 1970s on the coasts of New England (Dr. J.T. Carlton, personal communication), and was not reported in Nova Scotia until approximately 30 years later, in 2001 (Carver *et al.* 2006). Therefore, most populations analysed here likely represent relatively recent introductions, and the assumption of equilibrium is unreasonable. The strong IBD pattern is most likely a result of contiguous stepping-stone spread. Historical records also indicate that *B. violaceus* spreads gradually along the East coast, facilitated by human-mediated vectors (Carver *et al.* 2006; Dijkstra *et al.* 2007; Locke *et al.* 2009). Still, we did not observe a cline in microsatellite genetic variation along the coast. The bottleneck signature expected at the periphery of the invasion front was most likely obscured as a result of gene flow between sites in the northern part of the East coast (discussed below).

Evidence presented here indicates that natural dispersal is not a major contributor to ascidian spread on either coast. Most populations show high levels of genetic differentiation, reflecting a general restriction to natural spread (Table 3; Appendix S7). In addition, the observed regional invasion patterns do not appear to be associated with the dynamics of marine currents. Most sites on the West coast were located in the Strait of Georgia, which is characterized by currents of low intensity (LeBlond 1983) and thus not likely to drive long distance dispersal. Likewise, on the East coast, the southern flowing Nova Scotia current (Hannah *et al.* 2001) appears to be at odds with observational records indicating the invasion spread northward on the coast (Dijkstra *et al.* 2007; Locke *et al.* 2009).

Therefore, the divergent patterns of spread observed between the two coasts appear to be influenced at least partially by differences in anthropogenic vector dynamics. More specifically, evidence for long distance dispersal was more frequently observed throughout the sampling region on the West coast (Table 3; Fig. 5). Although evidence for human-mediated 'jump' dispersal was also detected on the East coast, these events appear to be more spatially restricted. Low F_{ST} values (0.01–0.02; Appendix S7) were detected between four northern sites separated by 15–200 km (SPB, SHB, CRV and APY; Fig. 1), which also share the majority of identified East coast migrant genotypes (32/38; Table 3). Previous studies of other prominent aquatic invaders, such as the zebra mussel (*Dreissena polymorpha*), have shown that patterns of long distance dispersal can be correlated with the spatial and temporal variation of human-mediated vectors such as recreational vessels (Bossenbroek *et al.* 2007). Indeed, a possible explanation for our findings might be that the number of recreational vessels currently registered in British Columbia is significantly higher than in Nova Scotia (Dr. T. Therriault, personal communication) providing more opportunities for long distance spread. Moreover, ice formations during winter in Nova Scotia significantly limit vessel traffic between sites, whereas in British Columbia, shipping occurs year round, providing more opportunities for the dispersal of *B. violaceus* propagules *via* hull fouling or ballast water transfer. Apart from vessel-mediated vectors, aquaculture is also considered a primary means of introduction and spread of *B. violaceus* (Dijkstra *et al.* 2007). This vector could be responsible for the long distance dispersal events observed in northern range areas on the East coast. Previous studies have suggested that transfer of living material and equipment between mussel growers in the region is a major contributor to the spread of invasive tunicates including *B. violaceus* (Locke *et al.* 2009).

Contribution of vegetative proliferation to the spread of B. violaceus

Theory predicts that asexual reproduction can substantially facilitate invasion success, especially during the introduction stage, when population size is small (Sakai *et al.* 2001). Although successful colonization by clonal lineages has been demonstrated in a number of widespread invaders (e.g. *Daphnia pulex* in Africa, Mergeay *et al.* 2006), in other cases, asexual reproduction has a more limited role in postestablishment spread (e.g. *Cordylophora caspia* in North America, Darling & Folino-Rorem 2009). Dispersal of colonial ascidians can occur either through sexual propagules (larvae and adult colonies) or asexually derived fragments. As the larval stage is very short, often lasting only minutes to a few hours (Lambert & Lambert 2003), and settled adults have limited mobility (Lambert 2005), the generation and spread of colony fragments have often been cited as a potentially important mechanism of secondary spread (Lambert 2005; Carver *et al.* 2006). Additionally,

a recent study demonstrated that three of the most notorious invasive colonial ascidians (*Botrylloides violaceus*, *Botryllus schlosseri* and *Didemnum vexillum*) can easily reattach to substrata after fragmentation under laboratory conditions (Bullard *et al*. 2007).

Our genetic analyses of 25 established populations suggest that although dispersal of *B. violaceus* fragments is limited, it can be prevalent near infested aquaculture facilities. Our sampling design allowed us to investigate the extent of fragment dispersal mostly at regional (i.e. along approximately 500 km of coastline on the West and East coasts; Fig. 1) and finer scales (i.e. within sampling locations). At the regional scale, the importance of fragment dispersal was limited. Only genotype C4 was sampled independently at Deep Bay and Ladysmith, locations situated approximately 90 km apart (Appendix S4). As these two populations were collected from local marinas, hull fouling is the most likely vector that could have facilitated exchange of colonies between sites. Ships heavily fouled with *B. violaceus* have been observed in other locations such as Prince Edward Island (Locke *et al*. 2009) and Newfoundland (Callahan *et al*. 2010), and are a primary vector responsible for the spread of colonial ascidians (Dijkstra *et al*. 2007).

The majority of multilocus genotypes (26/27) were restricted at smaller spatial scales, within populations, where each clone was encountered between one and seven times (Appendix S4). The highest number of clones (7) was identified for the LMI population on the West coast, which was sampled in an infested aquaculture facility. The same pattern was observed on the East coast, where most clones were detected in aquaculture sites such as Cardigan River (CRV; 4 clones) and Saint Peter's Bay (SPB; 3 clones). This pattern may be a consequence of the removal of *B. violaceus* colonies (and other fouling organisms) from contaminated aquaculture gear using high-pressure seawater, a common practice that may in fact facilitate fragmentation. An increase in local infestations and spread through surviving asexual propagules may, therefore, be expected if this management strategy is used to counteract problems associated with colonial ascidian invaders in aquaculture facilities.

On the invasion history of B. violaceus *in North America*

The observed distribution of genetic variation between the West and East coasts allows us to formulate several conclusions regarding the invasion history of *B. violaceus* in North America. On the West coast, California and Northwest populations are highly genetically diferentiated (Fig. 4a, Appendices S3 and S7). Two competing scenarios are compatible with this strong genetic structure: multiple, independent introductions from genetically diferentiated populations from the native range, or *in situ*, gradual divergence following a single colonization. Overall, historical records appear to support the scenario of multiple West coast introductions. Although the first confirmed North American report of the species is attributed to southern California in the early 1970s (Fay & Johnson 1971), *B. violaceus* was recorded at about the same time in surveys conducted approximately 1500 km north at Puget Sound (Dr. G. Lambert, personal communication). Alternatively, the limited natural dispersal capacity of *B. violaceus* could provide ample opportunities for *in situ* differentiation following a single introduction, driven by genetic drift. This scenario is less likely, given that similar genetic differentiation did not evolve between the California and East coast samples (also founded in the 1970s, Dr. J.T. Carlton, personal communication) which, on the contrary, show a high degree of genetic resemblance (see below).

On the East coast, all sampled populations were genetically similar to Bodega Bay (Figs 3 and 4a). This finding suggests that the East coast invasion was founded either from California or from a native population that is genetically similar to Bodega Bay. Although the assignment test indicated that contemporary transport of *B. violaceus* propagules from the West to the East coast might still occur (Table 3), this possibility needs to be investigated further by studies undertaking a broader geographic coverage of samples, including a detailed, comprehensive coverage of the native range. Results presented here should serve as a stimulus for future research aiming to resolve this issue and clarify the colonization history of this globally invasive species.

Conclusions

Our results provide strong evidence that the invasion of *B. violaceus* in North America was linked to highly contrasting patterns of postestablishment spread within the two sampling regions analysed. As similar patterns may very well have been shaped in other invasive species, we highlight the necessity of considering multiple invaded spatial ranges in genetic surveys of NIS. Evidence presented here indicates that colony fragmentation and regeneration may have a limited contribution to the regional dispersal of colonial ascidians. However, in aquaculture facilities, the treatment of fouled equipment with high-pressure seawater, unless performed on land, may inadvertently lead to an increase in local infestations and should be avoided.

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This study forms part of D.G.B's M.Sc. project on the ecological and evolutionary genetics of invasive ascidians in the Cristescu laboratory. A.Z. is interested in conservation and evolutionary genetics of aquatic animals. C.L. studies the ecological and genetic consequences of disturbances such as climate change or biological invasions in aquatic invertebrates. H.J.M. studies vectors of biological invasions. M.E.C. investigates the evolutionary causes and consequences of aquatic invasions with special emphasis on the genetics of habitat transitions.

Supporting information

Additional supporting information may be found in the online version of this article.

Appendix S1 Occurrence of *Botrylloides violaceus* mtDNA COI haplotypes in 25 North American locations,with GenBank accession numbers and frequency.

Appendix S2 Neighbour-joining phylogenetic reconstruction of *Botrylloides violaceus* cytochrome *c* oxidase subunit I (COI) haplotypes. Numbers at phylogenetic nodes indicate the neighbour-joining and maximum-likelihood bootstrap support with 1000 replicates. The number of samples possessing each haplotype is presented in brackets.

Appendix S3 Pairwise Φ_{ST} comparisons for *Botrylloides violaceus* populations using the mitochondrial COI marker. *Significant ($P < 0.05$); ** remains significant after sequential Bonferroni correction (Rice 1989). Populations on the West coast are separated by a box in the upper left corner.

Appendix S4 Clonal genotypes observed in the data set with N, number of times the genotype appears in the data set; N_{gen}, number of genotypes in the population of origin; P_{sib}, probability of identity considering strict sibs reproduction; P_{HW}, probability of identity under Hardy–Weinberg equilibrium. Clonal genotypes shared between locations are indicated with an asterisk.

Appendix S5 Microsatellite allele frequency for *Botrylloides violaceus* from 25 locations in the North American invaded range.

Appendix S6 Genetic diversity at 13 microsatellite loci for 23 sites of the violet tunicate, *Botrylloides violaceus*. N, sample size; N_A, number of alleles; A, allele richness; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS} value; P_{HW}, exact P-value for Hardy–Weinberg equilibrium. Significant departures from equilibrium after sequential Bonferroni correction are indicated in bold.

Appendix S7 Pairwise F_{ST} comparisons for *Botrylloides violaceus* populations using 13 microsatellite markers. *Significant ($P < 0.05$); ** remains significant after sequential Bonferroni correction (Rice 1989). Populations on the West coast are separated by a box in the upper left corner. Populations with a sample size of less than 15 individuals ($N < 15$) were not included in this analysis.

Appendix S8 The log probability of the data, LnP ($X \mid K$) ± SD, and the rate of change in the probability between successive runs ($\triangle K$), as a function of K for the 25 *Botrylloides violaceus* populations.

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