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Disentangling invasion processes in a dynamic shipping–boating network

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Abstract

The relative importance of multiple vectors to the initial establishment, spread and population dynamics of invasive species remains poorly understood. This study used molecular methods to clarify the roles of commercial shipping and recreational boating in the invasion by the cosmopolitan tunicate, *Botryllus schlosseri*. We evaluated (i) single vs. multiple introduction scenarios, (ii) the relative importance of shipping and boating to primary introductions, (iii) the interaction between these vectors for spread (i.e. the presence of a shipping-boating network) and (iv) the role of boating in determining population similarity. Tunicates were sampled from 26 populations along the Nova Scotia, Canada, coast that were exposed to either shipping (i.e. ports) or boating (i.e. marinas) activities. A total of 874 individuals (*c.* 30 per population) from five ports and 21 marinas was collected and analysed using both mitochondrial cytochrome *c* oxidase subunit I gene (COI) and 10 nuclear microsatellite markers. The geographical location of multiple hotspot populations indicates that multiple invasions have occurred in Nova Scotia. A loss of genetic diversity from port to marina populations suggests a stronger influence of ships than recreational boats on primary coastal introductions. Population genetic similarity analysis reveals a dependence of marina populations on those that had been previously established in ports. Empirical data on marina connectivity because of boating better explains patterns in population similarities than does natural spread. We conclude that frequent primary introductions arise by ships and that secondary spread occurs gradually thereafter around individual ports, facilitated by recreational boating.

Keywords: population connectivity, population dynamics, primary introduction, spread, tunicate

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Introduction

Human activities have become drivers of global ecosystem change by facilitating the transport and introduction of species outside of their natural ranges, some of which may survive, establish and become invasive (Grosholz 2002). A better understanding of invasion processes is fundamental to describing the structure and function of such populations and mitigating their impacts (Carlton 2003). Only a few studies have investigated post-establishment spread (Estoup *et al.* 2004; Viard *et al.* 2006; Goldstien *et al.* 2010; Bock *et al.* 2011). As successful eradication of invasive species is uncommon, many recent studies have focused on gaining a better understanding of the initial stages of the invasion process to limit the likelihood of new introductions attributed to human vectors (Lodge *et al.* 2006). Successful invasions are generally thought to result from complex interactions between primary

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introductions and secondary spread. Although interactions between multiple human vectors in the invasion process have often been proposed (Floerl *et al.* 2009), empirical examples are largely lacking.

Commercial shipping is considered the most important vector for primary introduction of marine invasive species (International Maritime Organization 2004; Hewitt *et al.* 2009). Following primary introductions, recipient regions (e.g. ports) may, in turn, become donor regions—sources for propagules for spread (Carlton 1996). The likelihood that invaders are transported via a given pathway is dependent on a species' life history traits. For example, species with long planktonic larval stage and a tolerance to a large range of salinities and temperatures, such as the European green crab, *Carcinus maenas*, (Broekhuysen 1936; Eriksson *et al.* 1975; Yamada 2001), or with resistant resting stages, such as dinoflagellate cysts (Casas-Monroy *et al.* 2011), are likely to be introduced via ballast water. When life history traits do not favour introduction by this pathway, rapid expansion of such species' ranges may occur by alternative shipping pathways (e.g. fouling). A classic example is tunicates. The short larval stage in this group of organisms should limit their transport via ballast water and thus their rapid expansion at the global scale is somewhat perplexing (Lambert 2005; Carver *et al.* 2006). Following introduction and establishment, marine species may subsequently spread beyond the boundaries of primary recipient regions through natural (e.g. currents) or anthropogenic (e.g. recreational boats, aquaculture) mechanisms. While the importance of recreational boats for secondary spread of biofouling species has been well recognized (Floerl 2002), recent studies from New Zealand on the solitary tunicate, *Styela clava*, suggest that recreational boats may be a more important vector for primary introductions than was previously assumed (Goldstien *et al.* 2010). However, the relative importance of recreational boats to species spread has been suggested to vary greatly among regions (Minchin *et al.* 2006; Lacoursière-Roussel *et al.* 2012).

Genetic structure may be used to evaluate hypotheses about vectors of introductions and pathways based on a weight of evidence approach (Dupont *et al.* 2009, 2010; Goldstien *et al.* 2010; Bock *et al.* 2011). For example, when species become locally abundant but clustered in a limited number of distinct areas, population connectivity evaluated at different spatial scales may be estimated using genetic markers to provide evidence of vector importance, as has been recently shown for port and marina populations of the invasive tunicate, *S. clava*, in New Zealand (Goldstien *et al.* 2010). Although genetic diversity is expected to decline from native to invasive ranges (Allendorf & Lundquist 2003), propagules from multiple genetically distinct source populations can generate *hotspots* of genetic diversity in areas of primary introductions (Novak & Mack 1993, 2005; Zalewski *et al.* 2010; Tonione *et al.* 2011). Secondary spread may occur in a stepping-stone manner (Reusch *et al.* 2010), resulting in a loss of genetic diversity with distance and/or steps in the invasion (Holland 2000). Thus, comparing genetic diversity among locations within invaded ranges may identify diversity hotspots and infer areas of primary introductions and secondary spread and ultimately help identify vectors that are important at each step of the invasion process.

Tunicates, for which rapid and global invasions have been recorded in recent years, are one of the best taxa to study the effects of anthropogenic vectors on the spread of biofouling species for a number of reasons (Lambert & Lambert 1998). First, the high availability of artificial and off-bottom substrates in ports and marinas (Glasby *et al.* 2007; Tyrrell & Byers 2007) makes these habitats suitable for colonization by invasive tunicates, many of which seem to be largely dependent on disturbed and/or anthropogenic habitat (Lambert 2005). Second, colonial tunicates have a restricted capacity for natural dispersal, limiting their potential for natural secondary spread (Svane & Young 1989; Ayre *et al.* 1997). Third, the enclosed structural environment of ports and marinas may limit natural connectivity among populations (Floerl & Inglis 2003). Frequently visited by human vectors, ports and marinas are therefore considered hubs for species introductions (Carlton & Geller 1993). The spread of tunicate species is thus believed to be a function of connectivity among hub habitats through human-mediated maritime activities.

The colonial tunicate *Botryllus schlosseri* has long been recognized as a model system to study cell biology (Rinkevich 2002; De Tomaso & Weissman 2004). Its large distributional range, high abundance and low capacity for natural dispersal have made it a model for studying dispersal via marine anthropogenic vectors at regional scales (Grosberg 1987; Lopez-Legentil *et al.* 2006). Recent work has shown that this colonial tunicate consists of at least five morphologically cryptic but genetically distinct species (*B. schlosseri* species A–E; Bock *et al.* 2012). Among these, only one, *B. schlosseri*, species A (hereafter referred to as *B. schlosseri*) is invasive and widespread globally, occurring on all continents except Antarctica (Van Name 1945). Its origin is thought to be most likely Europe or the Pacific Ocean (Berrill 1950; Carlton 2005; Lejeusne *et al.* 2011). Molecular data indicate that *B. schlosseri* extends its spatial range through maritime transport (Lopez-Legentil *et al.* 2006), but the importance between multiple potential maritime vectors has not been evaluated empirically. In the past, *B. schlosseri* has only been reported anecdotally on ships (Berrill 1950; Skerman 1960). More recently, the species was

not observed in hull fouling and ballast water surveys of 60 and 77 vessels in Canada, respectively (Humphrey 2008; Sylvester & MacIsaac 2010; Sylvester *et al.* 2011). In contrast, with respect to recreational boats, it has been reported as the dominant tunicate species in eastern Canada (Lacoursière-Roussel *et al.* 2012) and one of the dominant tunicate species in western Canada (Clarke Murray *et al.* 2011) and New Zealand (Lacoursière-Roussel *et al.* 2012). In eastern Canada, despite its first appearance in the early 1900s, *B. schlosseri* has only recently increased remarkably in abundance and is characterized by having a patchy distribution (Carver *et al.* 2006; Sephton *et al.* 2011).

In this study, we use mitochondrial and nuclear markers of *B. schlosseri* to investigate the role of commercial shipping and recreational boating in the invasion process of the species. Specifically, we first use phylogeographic and population genetic diversity of *B. schlosseri* collected in multiple port and marina locations in eastern Canada to evaluate the likelihood of single vs. multiple primary introductions. A single hotspot population—characterized by high genetic diversity and private haplotypes and alleles—will support the hypothesis that an introduction occurred within a single location, whereas multiple distinct hotspots (i.e. distinct geographically structured genetic signatures) will support the hypothesis of multiple introductions across several locations. If population differentiation is not detected, spread from prior introduction(s) may have already occurred among sampling sites and/or multiple introductions from similar genetic pools may have occurred. In the latter case, differentiating between primary introductions and secondary spread will not be possible. The relative location of hotspot populations is used to indicate where primary introductions have probably occurred. This information along with data on shipping and boating networks is used to identify the most likely vectors associated with primary introduction(s) (i.e. commercial ships or recreational boats). The existence of a shipping–boating network is then evaluated based on the genetic similarity between hotspot populations and those established through secondary spread (i.e. non-hotspot populations). Finally, the role of recreational boats in the secondary spread of *B. schlosseri* is further evaluated based on the relationship between population genetic similarity and the strength of boating connectivity between populations.

Materials and methods

Study area and sample collection

Botryllus schlosseri colonies were sampled from five commercial ports (i.e. locations with predominantly commercial shipping activities; $N = 150$ individuals) and 21 marinas (i.e. locations with recreational boating activities; $N = 724$ individuals) on the coast of Nova Scotia, Canada, during 2008–2009 (Table 1). Although we surveyed all eight major ports of the province, *B. schlosseri* was not observed during collector plate and dive surveys (a minimum of 12 diving sites per harbour) in Liverpool (geographic coordinates: 44°02′N, 64°41′W) and Sheet Harbour (44°54′N, 62°30′W). In Halifax Harbour (44°38′N, 63°32′W), only one individual was found on one collector plate, and none were observed in the 24 wharf sites surveyed during diving operations. Consequently, these ports were not assumed to be a potential source of recruits for secondary spread. Port and marina sampling sites were distributed among three distinct regions: Open Coast (ports P1 and P2, marinas M1–M8), Strait of Canso (port P3, marinas M9–M14) and Bras d'Or Lake (port P4, marinas M16–M21; Table 1; Fig. 1). Each region includes one or two port(s) surrounded by marinas. The Open Coast is exposed to the Atlantic Ocean; the Strait of Canso has a rock-filled causeway that limits natural dispersal between the Atlantic and Gulf of St. Lawrence sides of the strait (although recreational boats may cross the Canso Causeway through a canal), and Bras d'Or Lake is a semi-closed saltwater environment that is heavily used by recreational boaters. Two locations, P5 and M15, could not be unambiguously associated with one of the three defined geographical regions and thus were not considered for regional scale analyses.

Sampling was carried out using 10 settlement collectors positioned as far as possible from each other in each port and marina surveyed. All collectors were suspended in the water column from buoys or floating dock structures and included an upside-down 20-cm diameter plastic flowerpot base on top of three 10×10 cm PVC recruitment plates and a rock (if suspended from floating docks) or a buoy (if anchored to the bottom), each separated vertically by at least 10 cm. Prior to sampling, average per cent cover of *B. schlosseri* was estimated for each collector plate. To avoid analysing the same colonies multiple times, single tissue fragments were taken from each collector structure (i.e. plates and rock/buoy) in marinas. However, to increase the sample sizes in ports, multiple individuals were collected from the same plates and the genetic distinctiveness of specimens evaluated prior to downstream analyses using 10 nuclear microsatellite loci. Repeated multilocus genotypes were identified using GENECAP (Wilberg & Dreher 2004) and the probability of identical genotypes arising by chance via sexual reproduction calculated under Hardy–Weinberg expectations and the more conservative scenario of sibling reproduction, following Waits *et al.* (2001). All samples were

Table 1 Locations from which *Botryllus schlosseri* was collected in Nova Scotia, Canada (Fig. 1), with N, sample size; N_h, number of haplotypes; Θ, haplotype diversity; π, nucleotide diversity; N_A, number of alleles; N_P, number of private alleles; A_r, allelic richness; H_E, expected heterozygosity in each location

		mtDNA					Microsatellite				
Site	Location	N	N_h	Haplotypes	Θ	π	N	N_A	N_P	A_r	H_E
Ports											
P1	Yarmouth	29	3	Bs2, HO, HB	0.478	0.0140	29	52	3	3.81	0.5991
P2	Shelburne	9	2	Bs2, HO	0.389	0.0134	10	31	0	—	0.5323
P3	Point Tupper, Port Hawkesbury and Mulgrave*	18	4	Bs2, HO, Bs13, Bs15	0.608	0.0179	16	39	0	3.44	0.5822
P4	Little Narrows	63	1	Bs2	0.000	0.0000	61	39	1	2.66	0.4604
P5	Sydney	30	4	Bs2, HO, Bs4, Bs16	0.524	0.0163	29	46	3	3.36	0.5202
Total		149	7		0.500	0.0123	145	62	7	3.32	0.5388
Marina											
M1	Digby Marina	16	3	Bs2, HO, HA	0.242	0.0086	15	35	0	3.11	0.4714
M2	Lunenburg Yacht Club	40	3	Bs2, HO, Bs14	0.381	0.0093	34	34	0	2.86	0.4783
M3	Mahone Bay Classic Boat Marina	34	4	Bs2, HO, Bs8, Bs14	0.437	0.0106	31	48	2	3.46	0.5480
M4	Oak Island Marina	33	3	Bs2, HO, Bs8	0.402	0.0107	30	35	0	2.89	0.4801
M5	South Shore Marine	30	3	Bs2, HO, Bs14	0.570	0.0175	21	36	0	3.12	0.5175
M6	Chester Yacht Club and The Ripe Loft Restaurant*	36	3	Bs2, HO, Bs14	0.541	0.0178	31	32	1	2.53	0.4302
M7	Hubbards Yacht Club	31	2	Bs2, HO	0.232	0.0080	24	36	0	2.89	0.4793
M8	Shining Waters	29	3	Bs2, HO, HA	0.394	0.0139	22	34	0	2.70	0.3708
M9	Ballantyne's Cove	35	2	Bs2, HO	0.393	0.0135	31	40	0	3.22	0.5351
M10	Cribbon's Point	34	3	Bs2, HO, Bs8	0.169	0.0022	36	42	1	3.08	0.5046
M11	Guysborough Marina	28	2	Bs2, HO	0.254	0.0087	31	40	0	3.09	0.4792
M12	Canso Marina	33	2	Bs2, HO	0.492	0.0169	31	48	1	3.50	0.5310
M13	Petit de Grat Marina	40	2	Bs2, HO	0.385	0.0132	29	42	0	3.35	0.4857
M14	Isle Madame Boat Club	35	2	Bs2, HO	0.292	0.0101	34	39	0	3.08	0.4716
M15	Lennox Passage Yacht Club	37	1	Bs2	0.000	0.0000	19	37	0	2.99	0.4609
M16	St Peters Marina	37	2	Bs2, HB	0.279	0.0037	33	38	0	2.81	0.3776
M17	Barra Strait Marina	41	2	Bs2, Bs8	0.139	0.0003	38	32	0	2.57	0.4420
M18	Baddeck Marine and Bras d'Or Yacht Club*	37	2	Bs2, Bs8	0.054	0.0001	30	36	1	2.76	0.4230
M19	Cape Breton Boat Yard and Inverary resort*	39	1	Bs2	0.000	0.0000	26	27	1	2.36	0.3909
M20	Whycocomagh Harbour	37	2	Bs2, Bs8	0.468	0.0009	31	29	0	2.58	0.3946
M21	Ross Ferry Marina	37	2	Bs2, Bs8	0.054	0.0001	22	31	0	2.64	0.4447
Total		719	6		0.294	0.0079	599	67	7	2.93	0.4627

*Multiple ports or marinas are pooled together when short geographical distances separated the shipping and/or boating traffic.

preserved in 95–100% ethanol prior to genetic analyses. Genomic DNA (gDNA) was extracted from four to six zooids following Elphinstone *et al.* (2003).

DNA amplification, sequencing and genotyping

Mitochondrial DNA. A partial sequence of the COI gene was amplified using the universal primers LCO1490 and HCO2198 (Folmer *et al.* 1994). Because these primers failed to amplify consistently, we designed a species-specific primer (BsCOIR: 5′-GTATTTTATTTTTAGAATT TGGTCAAG-3′), which was used with the universal HCO2198 primer. PCR amplifications were performed in 25 μL reaction volumes, consisting of 1× PCR buffer (with 1.5 mM $MgCl_2$; Genscript), 0.2 mM dNTPs, 0.4 μM of each primer, 0.5 U of *Taq* DNA polymerase (Genscript), and 1 μL (*c.* 50–100 ng) gDNA. Thermal 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. PCR products were purified using the Solid Phase Reversible Immobilization method (Deangelis *et al.* 1995). Sequencing reactions were performed using the HCO2198 primer (Folmer *et al.* 1994), BigDye Terminator 3.1 chemistry, and an ABI 3130XL automated sequencer (Applied Biosystems, Foster City, CA, USA). The BsCOIR primer was used to reverse-sequence and confirms all sequences that contained ambiguous sites. The alignment was constructed and edited using CODONCODE ALIGNER v. 2.0.6 (CodonCode Corporation, Dedham, MA, USA).

Microsatellites. A total of 10 polymorphic microsatellite loci: BS321 (Pancer *et al.* 1994), PB29, PB49, PB41, PBC1

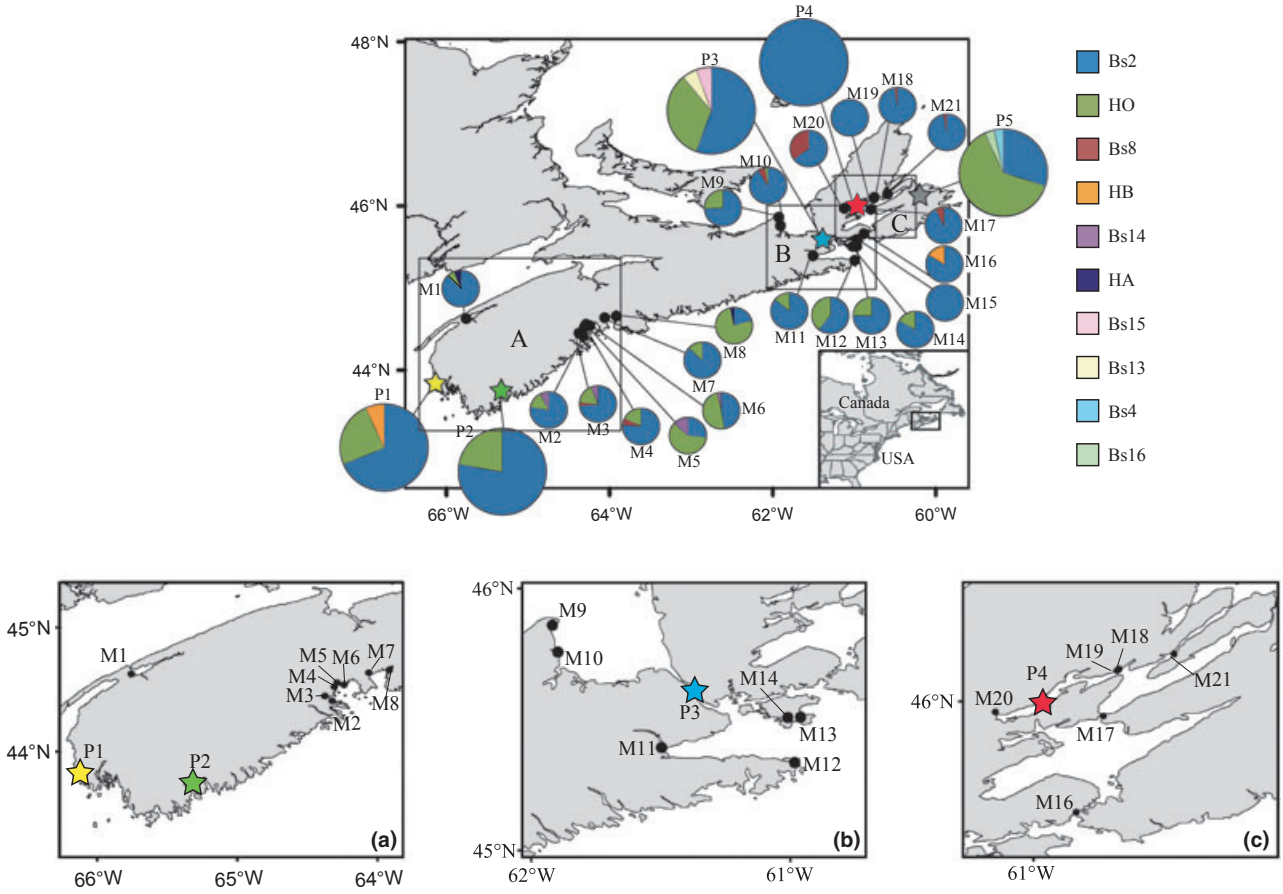

Fig. 1 (a) Site locations and relative frequencies of *Botryllus schlosseri* haplotypes from five ports (P; large circles) and 21 marinas (M; small circles) in Nova Scotia, Canada. Map A shows the Open Coast region, Map B the Strait of Canso region and Map C the Bras d'Or Lake region. Stars represent the port sampling sites.

(Stoner *et al.* 1997), Bsm1, Bsm2, Bsm4, Bsm6 and Bsm9 (Molecular Ecology Resources Primer Development Consortium *et al.* 2010) was examined. PCR amplifications were performed in 10 μL reaction volumes, consisting of 1× PCR buffer with 1.5 mM $MgCl_2$ (Genscript), 0.125 mM of each dNTP, 0.5 μM of each primer, 0.2 U of *Taq* DNA polymerase (Genscript) and 1 μL (*c.* 50–100 ng) gDNA. Forward primers were labelled with one of four fluorophores (6FAM, VIC, NED or PET) according to Schuelke (2000). Thermal cycling parameters 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. Fragment analysis of PCR products was performed on an ABI 3130XL automated sequencer (Applied Biosystems), with GeneScan-500 LIZ internal size standard (Applied Biosystems). Individuals which failed to amplify at four or more loci after two attempts were dropped from the data set, leaving 744 specimens for the microsatellite data analysis. The alleles for each locus were scored manually using GENEMAPPER® v.4.0 (Applied Biosystems).

Data analysis

Within and among population diversity. Mitochondrial DNA: Individual *B. schlosseri* COI haplotypes were identified using COLLAPSE v. 1.2 (Posada 2004) and arranged using a parsimony haplotype network using TCS v. 1.21 (Clement *et al.* 2000). The best-fit model of nucleotide substitution (TrN + I) was selected using MODELTEST v. 3.7 (Posada & Crandall 1998) under the Akaike Information Criterion (AIC; Posada & Buckley 2004). Sequence divergences between haplotypes were corrected using the Tamura and Nei substitution model in MEGA v.4.0 (Tamura *et al.* 2007). Haplotype (Θ) and nucleotide (π) diversities were computed for each sampling location using DNASP v.5 (Rozas *et al.* 2003). Genetic differentiation among populations was examined by computing

population pairwise Φ_{ST} values with 10 000 permutations using ARLEQUIN v. 3.5 software (Excoffier & Lischer 2010). To test for correlation between Rousset's (1997) genetic distance $[\Phi_{ST}/(1 - \Phi_{ST})]$ and geographic distances under the isolation by distance (IBD) model, geneticists often use the Mantel test. This test was shown by Legendre & Fortin (2010) to have lower power than canonical redundancy analysis (RDA). Thus, the two distance matrices were transformed into rectangular matrices using principal coordinate analysis (PCoA) and the relationship between these latter matrices determined using RDA. The canonical relationship (R^2) was tested with 10 000 permutations. The PCoA was computed using the ***pcoa()*** function of the APE package in R (Paradis *et al.* 2011), whereas the RDA test was performed using the ***rda()*** function of the VEGAN package in R (Oksanen *et al.* 2011). Geographic distances were calculated as the minimum along-coast distances between sampling locations estimated using GOOGLE EARTH v.4.3 (beta).

Microsatellites: Distinctiveness of specimens was evaluated using GENECAP (Wilberg & Dreher 2004). Conformance to Hardy–Weinberg equilibrium (HWE) expectations and linkage disequilibrium (LD) was evaluated for each locus and locations using 10 000 permutations in GENEPOP (Raymond & Rousset 1995), with significance levels adjusted for multiple comparisons by sequential Bonferroni corrections (Rice 1989). The total number of alleles (N_A), private alleles (N_P), allelic richness (A_r) and expected heterozygosities (H_E) were calculated using FSTAT v. 2.9.3.2 (Goudet 2002). The approach used by FSTAT to calculate A_r incorporates a rarefaction method (Mousadik & Petit 1996) that compensates for unequal sample sizes.

Genetic differentiation among populations was determined by calculating pairwise F_{ST} values and tested with 10 000 permutations in ARLEQUIN. While *F*-statistics use the population as the unit of comparison, the Bayesian clustering approach implemented in STRUCTURE v.2.3.1 (Pritchard *et al.* 2000) uses the individual as the unit, assessing whether it belongs to one or more genetic clusters (*K*), irrespective of sampling location. For the STRUCTURE analysis, we assessed likelihoods for models with *K* ranging from 1 to 26 (total sites). We used the admixture model, with correlated allele frequencies and no prior population information. For each value of *K*, we carried out five independent Markov Chain Monte Carlo (MCMC) runs, with 10^5 iterations discarded as burn-in followed by an additional 10^6 iterations. We chose the optimal *K* 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, as per Evanno *et al.* (2005). The STRUCTURE results were displayed graphically using DISTRUCT v. 1.1 (Rosenberg 2004). IBD was first evaluated from the fitted curve for Rousset's (1997) genetic distance as a function of geographic distances obtained from a general additive model using the MGCV package in R. Models were compared using the maximum likelihood AIC. The dependence predicted under IBD between Rousset's (1997) genetic distance $[F_{ST}/(1 - F_{ST})]$ and geographic distances was tested by transforming the two distance matrices into rectangular matrices by PCoA and evaluating the relationship between them using RDA. The canonical relationship (R^2) was tested using 10 000 permutations. PCoA and the RDA test were computed using R functions as described earlier.

Human vector and intrapopulation variability

Variation in genetic diversity between populations in ports and marinas was first compared using Student's *t*-test with all regions confounded, and a two-way fixed-effect ANOVA to test for the effect of regions (ports vs. marinas X regions). Genetic diversity was obtained from haplotype, nucleotide diversities, allelic richness and expected heterozygosity. The interdependence between port and marina populations was tested from the relationship between genetic differentiation (estimated by pairwise F_{ST}) and geographic distances for each marina and the nearest port. The relationship between propagule pressure from human vectors and genetic diversity was tested by linear regression of the number of international ship arrivals (natural logarithm-transformed) and the averaged number of boats per marina in 2009. The number of ship arrivals included international merchant and non-merchant vessels reported to Transport Canada and the Canadian Coast Guard (S. Bailey, unpublished). The number of boats in marinas was estimated as the average number of boats counted in each marina during each of three visits (in June, August and October 2009).

Population similarity from boating connectivity

Boating patterns were first obtained from questionnaires asked directly to 37 marina managers randomly distributed among the total of about 50 marinas in Nova Scotia. Questions targeted were as follows: (i) How many berths within the marina? and (ii) How many visitors within the last 5 years? The relationship between Rousset's genetic distance and marina connectivity by recreational boats and geographic proximity was evaluated using partial RDA, as recommended by Legendre & Fortin (2010), after the distance matrices were transformed into rectangular matrices by PCoA. The connectivity between pairs of marinas was estimated by

completing a total of 374 interviews with boat owners from 37 marinas in Nova Scotia between June and October 2009 (the boating season is limited to between May and October). Questions identified which marinas the owners had visited during the 6 months preceding the study. As interviews were carried out only with boaters who were present during at least one of four sporadic visits made to the marinas, there was probably a bias of targeting boats that are frequently occupied. Supplementary details about the questionnaire and specific patterns of boating activities in Nova Scotia are given in Lacoursière-Roussel *et al.* (2012). Connectivity was estimated as the number of trips recorded between pairs of marinas. Trips between marina pairs were assumed to be independent. Because of the great geographical distances separating populations in eastern and western Nova Scotia, the difference between the eastern and western regions was represented by a binary variable in the RDA, whereas the relationships among pairs of sites within each region were represented by MEM spatial eigen functions (Dray *et al.* 2006).

Results

Population diversity and differentiation

Mitochondrial DNA. Partial COI sequences were obtained for 868 *Botryllus schlosseri* individuals, including 149 from ports, and 719 from marinas (Table 1). After alignment and trimming, a final length of 524 bp was used. Ten haplotypes were observed within the data set. Among these, Bs2 and Bs4 have previously been reported from Nova Scotia, Bs8 from Washington (USA), HA and HO from both Nova Scotia and Europe, and HB from Europe (Lopez-Legentil *et al.* 2006; Lejeusne *et al.* 2011). The remaining four haplotypes (Bs13–Bs16; GenBank accession numbers: JN561069–JN561072) are new. Sequence divergence between haplotypes ranged from 0.2% to 4.7%. The statistical parsimony network confirmed the generally high sequence divergence and revealed the existence of 28 different mutational steps between the 10 haplotypes (Fig. 2). Haplotypes Bs4, Bs13, Bs15 and Bs16 were the only private haplotypes and were found within two populations sampled in ports. Haplotype Bs2 was the most common in Nova Scotia (76.38%), and was found in all the populations surveyed (Table 1). The second most common haplotype was HO (18.20%), followed by Bs8 (2.65%), Bs14 (1.15%), HB (0.92%) and HA (0.23%). Moderate genetic structure was observed within the data set, with 25.6% of pairwise Φ_{ST} values remaining significant after sequential Bonferroni correction (Appendix S1, Supporting information). The spatial distribution of haplotypes was marked by a geographic discontinuity. For instance, HA, Bs8 and HB were shared between populations separated by 663, 724 and 1144 km, respectively (Fig. 1; Table 1). Test of canonical R^2 in RDA showed that the IBD model was not significant ($P > 0.1$).

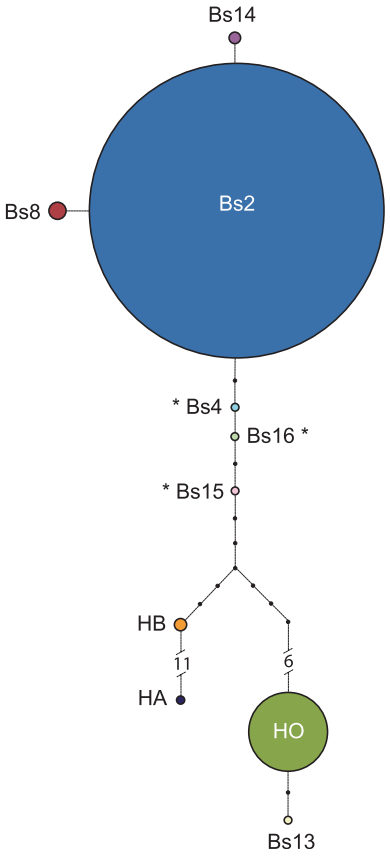

Fig. 2 Parsimony network for mtDNA haplotype of *Botryllus schlosseri* sampled in ports and marinas in Nova Scotia, Canada. Circles show sampled haplotypes, with circle size proportional to haplotype frequency. Haplotype numbers correspond to previous records (see text). Lines represent one mutation and small black dots depict hypothetical haplotypes that are not present in the data set. Asterisks denote private haplotypes sampled in ports. Colour code is as per Fig. 1.

Microsatellites. Genotypes were obtained for 744 *B. schlosseri* individuals, including 145 from ports and 599 from marinas (Table 1). A total of 77 alleles were recovered from among the 10 nuclear microsatellite loci. Clonal genotypes were found in populations that were far apart (Appendix S2, Supporting information) and deviations from HWE were observed at multiple loci and sampling locations. However, there were no systematic deviations from HWE for loci across populations or at all loci within populations (Appendices S2 and S3, Supporting information). No linkage disequilibrium was detected, confirming that each of the 10 microsatellite

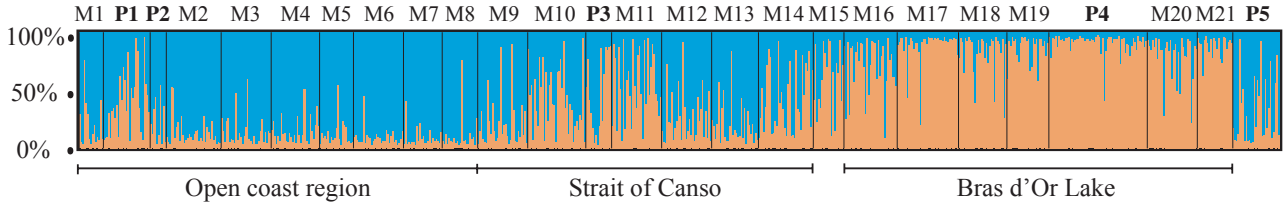

Fig. 3 Bayesian clustering of multilocus *Botryllus schlosseri* genotypes from Nova Scotia, Canada. Two genetic clusters were inferred (represented in blue and orange) by comparing the log-likelihood and the standardized second-order rate of change. Labels (P port; M marina) refer to locations mapped in Fig. 1 and are sorted by geographical order. Box width is proportional to sample size per population.

loci provides independent information. Considerable genetic structure was found, with 65.5% of population pairwise F_{ST} values being statistically significant after sequential Bonferroni correction (Appendix S1, Supporting information). For the Bayesian inference of population structure, the evaluation of both ln $P(X \mid K)$ and ΔK revealed the existence of two main genetic clusters with an irregular admixture pattern across geographical distance (Fig. 3). One of the clusters (i.e. orange cluster in Fig. 3) was found primarily in ports and nearby marinas in the Open Coast and Strait of Canso regions, but was globally more frequent in the Bras d'Or Lake region. Despite a weak but significant linear relationship between Rousset distance and geographical distance ($P < 0.0001$, $r^2 = 0.06$, AIC value = −518.6), the best fit between Rousset's genetic distance and geographical distance was described by a parabolic function ($\Delta = 26.5$; parabola function, $P < 0.0001$, $r^2 = 0.14$, AIC value = −545.1; Fig. 4). This function shows that some

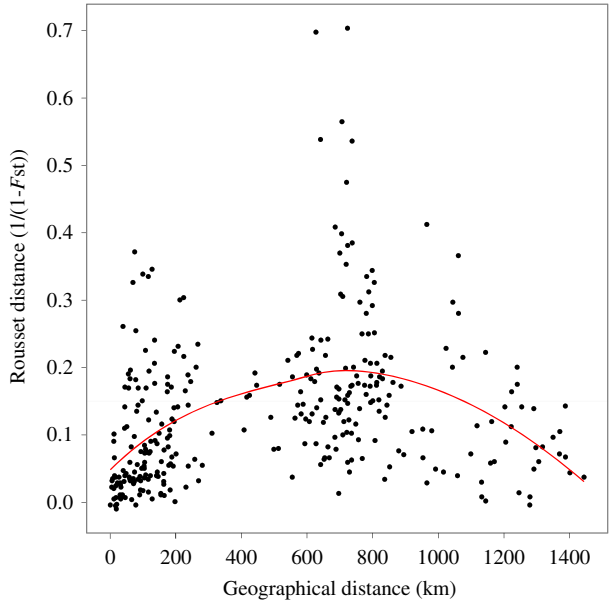

Fig. 4 Best-fitted curve between Rousset distance ($F_{ST}/(1-F_{ST})$) and geographical distance (km) for all populations of *Botryllus schlosseri* sampled in Nova Scotia, Canada.

distant populations had greater genetic similarity than did close populations, thus not supporting the hypothesis of primary introduction followed by natural dispersal. Similarly, the significance test of the canonical R^2 in RDA did not support the IBD hypothesis ($P > 0.1$).

Genetic diversity within and among port and marina populations

Despite a greater sampling effort for marina populations compared to port populations, the total number of haplotypes was greater for port populations (7) than for marina populations (6; Table 1). At the provincial scale, port populations averaged 2.8 COI haplotypes (±1.3; range: 1–4), compared to 2.3 (±0.7; range: 1–4) in marinas. Greater genetic diversity in ports relative to marinas was further supported by estimates of haplotype diversity (0.500 vs. 0.294) and nucleotide diversity (0.0123 vs. 0.0079; Table 1). However, differences of both haplotype and nucleotide diversity were not significantly different between port and marina populations at the provincial scale (*t*-test values, $t = 1.1$, d.f. = 24, $P = 0.27$ and $t = 1.4$, d.f. = 24, $P = 0.18$, respectively). At the regional scale, haplotype diversity and nucleotide diversity were greater in ports than marinas for two regions, Open coast and Strait of Canso, but in the Bras d'Or Lake region the genetic diversity was globally low and only one haplotype was observed (Table 2). However, for haplotype diversity and nucleotide diversity, statistical analyses did not reveal significant difference between ports and marinas once inter-region variation in diversity was accounted for (two-way fixed-effect ANOVA, haplotype diversity: d.f. = 1, $F = 0.37$, $P = 0.55$ and nucleotide diversity: d.f. = 1, $F = 1.42$, $P = 0.25$, respectively).

As the mitochondrial marker, nuclear markers indicated greater variability in populations sampled in ports than marinas. At the provincial scale, port populations averaged 41.4 (±8.0) alleles, compared to 36.7 (±5.4) alleles in marina populations. Allelic richness and expected heterozygosity were significantly higher in ports than marinas when compared with all regions

Table 2 Genetic diversity values for *Botryllus schlosseri* in port (P) and marina (M) locations pooled for each of three main regions in Nova Scotia, Canada (see text). The diversity indices considered are: haplotype diversity (Θ), nucleotide diversity (π), allelic richness (A_r) and expected heterozygosity (H_E)

Region	Port vs. marina	Θ	π	A_r	H_E
Open coast	P	0.434	0.0137	3.81	0.5657
	M	0.400	0.0121	2.95	0.4720
Strait of Canso	P	0.608	0.0179	3.44	0.5822
	M	0.331	0.0108	3.22	0.5012
Bras d'Or Lake	P	0.000	0.0000	2.66	0.4604
	M	0.166	0.0009	2.62	0.4121

confounded (*t*-test values, $t = 2.1$, d.f. = 23, $P = 0.05$ and $t = 2.9$, d.f. = 24, $P = 0.007$, respectively; Table 1), and once inter-region variation in diversity was accounted for (two-way fixed-effect ANOVA, d.f. = 1, $F = 7.58$, $P = 0.014$ and d.f. = 1, $F = 12.36$, $P = 0.002$ respectively; see Table 2 and Fig. 5).

Comparing the pairs formed by each marina and the nearest port, a highly significant linear relationship was detected between the genetic similarity (pairwise F_{ST}) of tunicate populations and geographical distance ($r^2 = 0.66$, $P < 0.001$; Fig. 6). At the provincial scale, despite the low number of ports that gave extremely low power to the tests, a positive linear relationship between genetic diversity and the number of international ship arrivals was significant for haplotype diversity ($r^2 = 0.77$, $P < 0.05$) and there was a similar but not statistically significant trend for nucleotide diversity and expected heterozygosity ($r^2 = 0.67$, $P = 0.09$ and $r^2 = 0.75$, $P = 0.06$, respectively). The relationship between the number of boats in marinas and genetic diversity at the provincial scale was only significant for nucleotide diversity ($r^2 = 0.20$, $P = 0.04$); within regions, the number of boats within marinas was a poor predictor of genetic diversity ($P > 0.1$ in all cases).

Population connectivity from boating activity

The open coast region is the largest region in Nova Scotia ($N = 20$ marinas), averaging 84.4 berths per marina and 66.1 boat visitors per year per marina. Marinas within the Strait of Canso averaged 34.7 berths and 46.9 visitors per year ($N = 7$ marinas). In contrast, Bras d'Or Lake is a small highly visited region, averaging 22.7 berths per marina but 248 visitor per year per marina ($N = 10$ marinas). After excluding boats that did not visit at least two sampled populations, a total of 285 questionnaires were used to test the relationship between boat connectivity among populations and genetic population similarity (test of canonical R^2 in RDA). We obtained a total of 503 marina pair records. At the provincial scale, total variation of the model was 0.72 and population similarity was mainly controlled by the east–west factor ($R^2_{adj} = 0.20$, $P < 0.01$). Both geographical proximity and boat connectivity failed to explain a significant proportion of population genetic similarity ($P > 0.1$, $R^2_{adj} = -0.03$ and $P > 0.1$, $R^2_{adj} = 0.08$, respectively). At the regional scale, the total variance of the model was 0.16, 0.01 and 0.03 for the Open Coast, Strait of Canso and Bras d'Or Lake regions, respectively. Within regions, geographical proximity was also not significant for any region and no trends were apparent except for Bras d'Or Lake (Open Coast: $P = 0.44$, $R^2_{adj} = 0.07$; Strait of Canso: $P = 0.57$, $R^2_{adj} = -0.04$; and Bras d'Or Lake: $P = 0.12$, $R^2_{adj} = 0.67$). Except for the semi-enclosed environment of the Bras d'Or Lake region, the influence of boating connectivity better explained the similarity among populations than did geographic proximity (Open Coast: $P = 0.09$, $R^2_{adj} = 0.65$; Strait of Canso: $P = 0.19$, $R^2_{adj} = 0.48$; and Bras d'Or Lake $P = 0.47$, $R^2_{adj} = 0.38$).

Discussion

Both mitochondrial and nuclear markers provided evidence that multiple cryptic primary introduction events

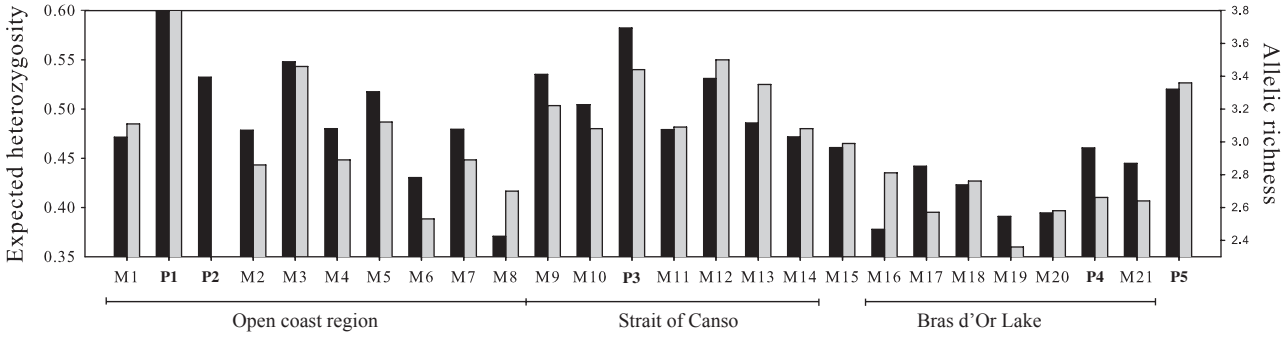

Fig. 5 Expected heterozygosity (black) and allelic richness (grey) in marina (M) and port (P) populations of *Botryllus schlosseri* in Nova Scotia, Canada. Details on sampling locations are given in Fig. 1 and Table 1.

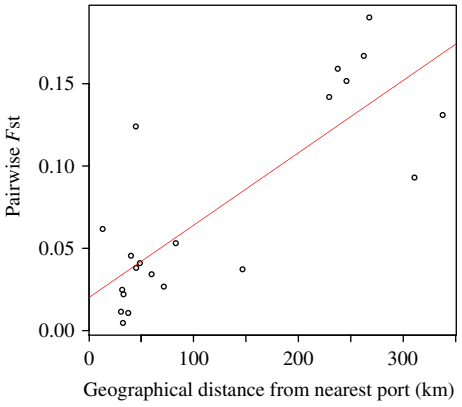

Fig. 6 Plot of pairwise F_{ST} to the geographical distance (km) between marina and nearest port population pairs ($r^2 = 0.66$, $P < 0.001$).

of *Botryllus schlosseri* have occurred in Nova Scotia. This hypothesis is supported by (i) heterogeneity of geographical admixture between Bayesian clusters and (ii) the geographical pattern of several distinct hotspot populations. In the specific case of *B. schlosseri*, high heterozygote deficiency had been globally observed (i.e. United States, Israel, Croatia, New Zealand, France, Portugal and UK; Stoner *et al.* 1997, 2002; Pancer *et al.* 1994; Ben-Shlomo *et al.* 2001; Rinkevich *et al.* 2001; Paz *et al.* 2003) and deviations from HWE have been suggested to be a result of substructured populations and/or non-random matting characteristics, which does not support the hypothesis of multiple introductions (Chakraborty & Jin 1992; Ben-Shlomo *et al.* 2001). Despite a lower sampling effort in ports than in marinas, hotspot populations characterized by high genetic variability and private haplotypes and alleles (>2 private alleles) were only detected in populations exposed to shipping activities (i.e. sites P1, P3, and P5). These three populations were within invaded ports exposed to the highest level of shipping activity in Nova Scotia. P1, P3 and P5 received 178, 453 and 108 international ship arrivals in 2007, respectively, while P2 and P4 received only 56 and 27 arrivals, respectively (S. Bailey, unpublished). This provides evidence that ships were the main vectors of the *B. schlosseri* introductions on the east coast of Canada. More generally, the results also support the importance of propagule pressure associated with shipping activity, suggesting that the greater the shipping activity, the greater the propagule pressure, and the greater the probability of successful establishment occurring in favourable environments (Lockwood *et al.* 2005; Von Holle & Simberloff 2005; Davidson *et al.* 2008).

Few marinas showed genetic characteristics of populations associated with primary introductions. Although most data suggest that primary introduction occurs in ports, the relatively high level of genetic diversity and the presence of private alleles in some marinas (e.g. M3, M16, M18) is also consistent with the scenario of primary introductions occasionally occurring through recreational boating. Marina M3 received 205 visitors per year on average, making it one of the most visited marinas within Nova Scotia. Three other sampled marinas also experienced relatively high levels of boating activity, including M18 (1550 visitors), M16 (727 visitors) and M6 (280 visitors). This again highlights the potential importance of propagule pressure to the introduction of *B. schlosseri*.

Results presented here further suggest that ports act as invasion hubs from which populations undergo secondary spread to nearby marinas. Marina populations typically showed lower genetic diversity relative to port populations. This finding, together with the high similarity between marinas and the nearest port populations, supports the hypothesis of a dependency of marina populations on populations colonized previously within ports. When shipping and boating networks determine population dynamics, weak relationships between the age of introduction and genetic diversity may be found (Goldstien *et al.* 2011). Except for the semi-closed Bras d'Or Lake region, natural spread—evaluated as the correlation between geographical proximity and genetic similarity—did not seem to be important to the spread of *B. schlosseri*, whereas there was a trend for boating connectivity to explain population similarity within regions. Given the observed rapid intra-coastal dispersal (Lambert & Lambert 1998), results from boat fouling surveys (Lacoursière-Roussel *et al.* 2012) and the low natural dispersal of tunicates (Svane & Young 1989; Ayre *et al.* 1997), this suggest that recreational boats are the most likely vector for the spread of *B. schlosseri* among marinas. The other main putative vector for the spread of exotic species in coastal areas, bivalve aquaculture (McKindsey *et al.* 2007), is likely not locally important as aquaculture is not well developed in the study area, culture sites are relatively distant from marinas, stock transfers are limited (about 20 per year in the province, including scientific permits), and stock and associated equipment must be cleaned prior to transfer. Our results suggest that modelling population connectivity because of recreational boats could be improved by integrating trip directionality and seasonal patterns of boat trips. The latter may also alter the risk of spreading biofouling species by altering boating trends and establishment probability because of seasonal variability of environmental conditions and hull biofouling on recreational boats (Lacoursière-Roussel *et al.* 2012).

Sudden and strong dominance by a species may result from Allee effects, biotic interactions, spatial and

temporal environmental heterogeneity (e.g. warming trends; Stachowicz *et al.* 2002), adaptation (Sax & Brown 2000), and/or multiple introductions (Lee 2002; Roman & Darling 2007; Roman 2011). The mechanisms by which genetic diversity shapes invasion success is currently a subject of much debate (Roman & Darling 2007). Multiple introductions may alter invasiveness by sustaining population viability (by overcoming genetic drift and inbreeding effects), respond to selection (new pre-adapted traits) and/or improve adaptation to new environments (Frankham *et al.* 2002; Kolbe *et al.* 2007; Roman & Darling 2007; Facon *et al.* 2008; Roman 2011). Some successful invasions have, however, resulted from single founding events because of the selection of advantageous features (Ross & Keller 1995; Ross *et al.* 1996; Tsutsui *et al.* 2000). The results reported in the present study are consistent with range expansion due to multiple cryptic introductions.

Simple estimates of species abundances in different locations typically suggested insight into the identification of potential vectors, as abundance is logically a function of propagule pressure. However, here we underline that high local abundance of *B. schlosseri* has been found even at very low genetic diversity. For example, in M20, although genetic diversity was low (Table 1), the average cover of *B. schlosseri* on collector plates was 92.7%. At a local scale, high genetic diversity associated with multiple introductions may hence not be essential for successful invasion by the colonial tunicate. Surprisingly, the relationship between dominance (calculated from the average cover of *B. schlosseri* for each location) and allelic richness was significant and negative ($P = 0.03$ and $R^2_{adj} = 0.15$). Similarly, *B. schlosseri* was almost absent from Halifax, one of the main commercial ports in eastern Canada. In Nova Scotia, as the observed genetic diversity was low compared to the native area (i.e. Lopez-Legentil *et al.* (2006) found 16 haplotypes within 181 individuals throughout Europe vs. 10 haplotypes among 868 individuals in the present study), continual maritime transport will probably increase genetic diversity over time. Sustained shipping–boating activities may nevertheless facilitate the introductions of *B. schlosseri* within uninvaded areas and alter its abundance. As the probability of a boat becoming colonized is a function of abundance within source regions (Lacoursière-Roussel *et al.* 2012), a better understanding of anthropogenic effects on population dynamics is fundamental to mitigating the propagation of invasive species.

The probability that multiple vectors interact within the invasion process increases when species traits allow for a range of pathways to result in introductions. Ballast water, hull biofouling, sea chests, ballast tank sediments and sediments associated with anchors, and anchor chains are all potential constituents of a pathway linking multiple shipping activities (Carlton 1985; Eldredge & Carlton 2002; Gollasch 2002; Godwin *et al.* 2004). For *B. schlosseri*, the longer survivorship of buds (bud life was estimated to up to 150 days when unattached and 35 days when attached; Rabinowitz & Rinkevich 2004) suggests that hull biofouling and floating debris and buds in ballast tanks are a more probable pathways of introduction than are larvae in ballast tanks (free-swimming larvae generally survive <36 h and seem better adapted for site selection and settlement than dispersal; Berrill 1950). During an eastern Canadian ship survey, *B. schlosseri* was not observed either in ballast water (Humphrey 2008) or as hull biofouling on ships (Sylvester *et al.* 2011). Further vector studies are hence fundamental to (i) better understanding the complete pathway of dispersal, (ii) develop adequate management strategies and (iii) interpret population structure considering that the level of propagule release may vary greatly among pathways (Voisin *et al.* 2005).

The invasion pattern observed in eastern Canada may also be driven by unknown seasonal effects on population dynamics (Carver *et al.* 2006). Winter may have pronounced effect on genetic variability of biofouling species by altering reproductive patterns (Millar 1971; Grosberg 1988), natural larval dispersal distance (David *et al.* 2010) and survivorship because of low water temperature (Skerman 1958; Ben-Shlomo *et al.* 2001), ice cover and artificial structure removal (e.g. floating docks in marinas are removed each winter). Moreover, in the realm of maritime transport in eastern Canada, although primary introductions from ships may occur during winter, recreational boating activity is virtually absent at this time (October through May). Cold winters may thus increase the importance of the role of shipping relative to that of boating for primary introductions in the region. Decreasing latitudinal genetic diversity has been observed for *B. schlosseri* along European Atlantic Coasts but it had been previously suggested to be a consequence of Pleistocene glaciations (Ben-Shlomo *et al.* 2006). The winter effect on marine fouling populations has not been well investigated and thus cannot be considered further in this case study.

Targeting source location(s), pathway(s) and vector(s) is essential for successful management of invasive species (Grosholz 2002; David & Gollasch 2008). Our results indicate that frequent introductions of *B. schlosseri* arose from commercial ships and that secondary spread occurred gradually thereafter around individual ports via boating. This conclusion is likely to be applicable to invasive dispersal processes that may occur for many other marine biofouling organisms. The

interaction of multiple human vectors may have greatly promoted the global expansion of *B. schlosseri*. The present study shows that even when high dominance may occur at low genetic diversity, interactions among multiple human activities may quickly promote species dominance at large scale. Such a gene flow demonstrates that the interaction among human vectors may sustain population connectivity and lead to successful invasion.

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This study forms part of A.L.R's Ph.D. project on the effect of maritime activities on biodiversity, population connectivity and invasion success. C.W.M. studies human - coastal ecology issues with an emphasis on exotic species and aquaculture-environment interactions. D.G.B. and M.E.C. are molecular ecologist investigating the evolutionary consequences of aquatic invasions. F.G. is a theoretical ecologist interested in non-linear and spatial dynamics of coastal ecosystems. P.L. is a quantitative ecologists who develops methods of spatial analysis for numerical ecology; he also writes computer software. P.G. is a molecular ecologist studying the impacts of local dynamics and individual behaviours on the evolution of ecosystems.

Data accessibility

COI sequences: Genbank accessions JN561069–JN561072.

Microsatellite data, COI data by population and by individual, boating connectivity by population: DRYAD entry doi:10.5061/dryad.n3v10.

Supporting information

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

Appendix S1 Pairwise ΦST (above diagonal) and *F*ST (below diagonal) comparisons between *B. schlosseri* populations obtained for the mitochondrial COI marker (ΦST) and 10 nuclear microsatellite loci (*F*ST).

Appendix S2 Clonal genotypes observed in the dataset.

Appendix S3 Genetic diversity at 10 microsatellite loci for 21 sites of *Botryllus schlosseri*.

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