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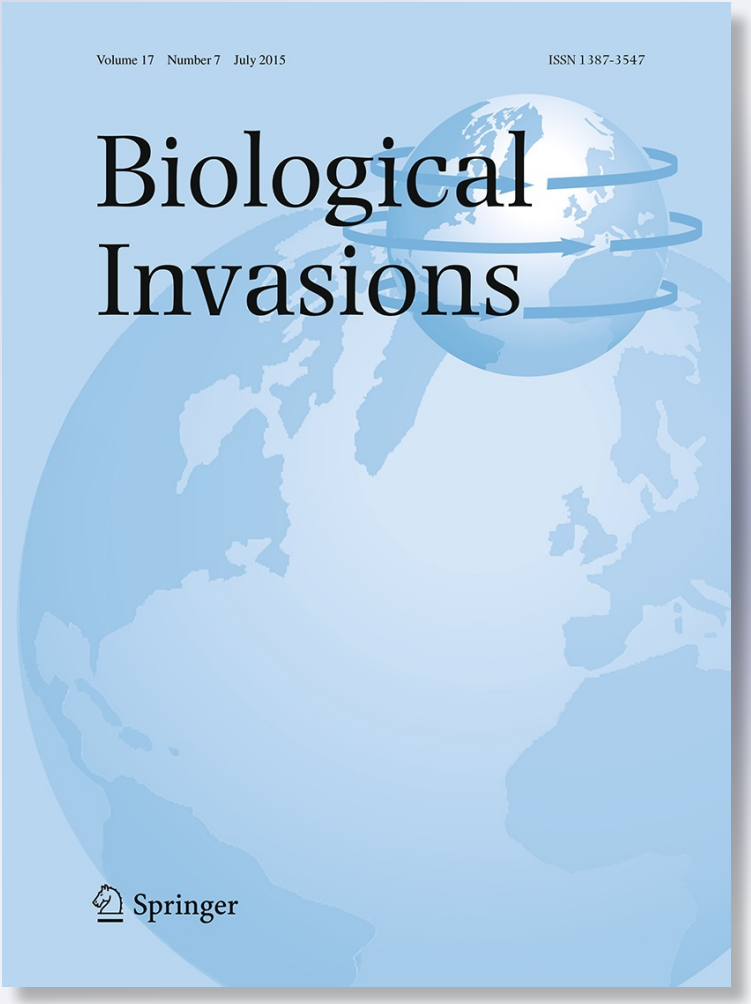

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ORIGINAL PAPER

Aquaculture and the spread of introduced mussel genes in British Columbia

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Abstract Aquaculture can promote the introduction of non-indigenous species (NIS) into wild marine environments. In addition, NIS aquaculture escapees may hybridize with closely-related native species introducing foreign alleles to their gene pool. To quantify the influence of mussel aquaculture on the native community in British Columbia we sampled mussels from fourteen locations on Vancouver Island. There are two native species in this region, *M. trossulus* and *M. californianus*, and two farmed NIS, *Mytilus edulis* and *M. galloprovincialis*, both originally from Europe. DNA was extracted from mussel tissue and the mitochondrial cytochrome c oxydase subunit 1 (*COI*) gene was sequenced. One nuclear locus that exhibits different alleles for *M. edulis*, *M. galloprovincialis* and *M. trossulus* (*Glu-5′*) was also characterized, using PCR, in order to identify heterozygotes. We found the proportion of NIS introgression depended primarily on farm density. Other habitat traits such as the degree of exposure to the open sea and, to a minor extent, salinity, contributed significantly to explain the distribution of introgressed individuals. Different habitat preference of NIS and native species, and marine currents, provide additional explanations for the distribution of alien and native species along Vancouver Island coasts. As a whole, our results suggest that native *M. trossulus* populations are more introgressed by *M. galloprovincialis* genes in open habitats.

Keywords Non-indigenous species · Introgression · *Mytilus* · Mussel farming · Habitat preference · Genetic markers

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Introduction

The introduction of non-indigenous species (NIS), for example in farming, aquaculture, pest control, stocking for hunting/fishing, ship ballast and fouling, and even as pets, encompasses the risk of some individuals escaping and the species becoming invasive. Such introductions are considered to be one of the most important environmental issues today (e.g. Grosholz 2002; Elliott et al. 2008; Lowe et al. 2012; Methratta et al. 2013). In aquatic habitats most NIS are crustaceans and molluscs and their introduction in a region is strongly associated with trade patterns, as a consequence of ship fouling and with aquaculture (e.g. Naylor et al. 2000; Molnar et al. 2008; Lowe et al. 2012). Although aquaculture operations are supposed to be placed in isolated areas and attempt to contain the

farmed individuals, it is almost impossible to prevent escapes of gametes, larvae and/or adults (Heath et al. 1995). Commercial fish and shellfish farming have thus led to the intentional and/or accidental introduction of alien aquatic species in many parts of the world (Hindar et al. 1991; Carlton 1992; Carriker 1992; Consuegra et al. 2011).

Not all aquaculture escapees become invasive because domestic individuals are not always able to adapt to wild habitats (e.g. Hindar et al. 1991; Fleming and Gross 1993; Blanchfield et al. 2009; Danancher and Garcia-Vazquez 2011). However, there are many that do, and through a diversity of mechanisms, some disrupt or even displace native species (e.g. Hindar et al. 1991; Huxel 1999; Molnar et al. 2008). Invasives might out compete local species, especially in disturbed environments (e.g. Didham et al. 2007). Another form of disruption is hybridization, leading to the introduction of foreign DNA/genes into local populations, a process known as introgression. Introgressive hybridization following introduction of NIS has been considered a risk for native species where they are rare (Rhymer and Simberloff 1996), but in other cases, for example in interspecific hybridization and introgression between wild and farmed south European salmonids (Castillo et al. 2008; Horreo et al. 2014), the populations incorporate heterospecific genomes without extinction. The extent of genomic introgression will depend mainly on the degree of domestication of cultivated stocks (how different they are from wild populations—this is not always equal to the degree of domestication), and the quality and abundance of native populations (Danancher and Garcia-Vazquez 2011). The trace of introgressions may last for a long time, modifying the gene pool of natives and causing unpredictable effects in the long term (e.g. Mallet 2005; Fitzpatrick et al. 2009; Lamaze et al. 2012).

Taking into account the present scenario of increasing aquaculture activities, it is crucial to understand the conditions that favour invader expansion and introgression into native gene pools. This knowledge would help to adopt precautionary approaches when establishing new aquaculture operations, and to establish scientifically-informed mitigation measures to help native species and populations to recover from invasions. Although it is not easy to identify factors that enhance invasions in complex environments, some interrelated processes that mediate invasion processes have been discovered including environmental changes in donor and recipient regions, invasion opportunity windows and dispersal vectors (reviewed in Carlton 1996). NIS take advantage of anthropogenic and/or disturbed habitats (e.g. Suchanek 1981; Linde et al. 2008; Shield et al. 2008). Although invasions depend on resource availability in the recipient habitat (Stachowicz et al. 2002), human factors such as country wealth and human population density are the only statistically significant predictors of biological invasions in the majority of models, even when analyzed jointly with climate, geography, and land features (Pysek et al. 2010). A closer examination of areas where the invaders are currently in expansion is necessary in order to identify those factors that promote or restrict the invader's adaptation in a region. Such knowledge could be applied for mitigation or prevention of further invasions, taking into account the environmental grain and dispersal capacity of potential invaders. For instance, new farms (if any) should be preferentially placed in areas surrounded by habitat hostile to invaders, while locations where potential escapees can easily settle should be strictly avoided. Similarly, containment measures, eradication programs, pre- and post-border controls can be better planned if the conditions that are unfavorable for invaders are known; for example, adverse habitat types could be mimicked for corridors when invasives are too abundant for envisaging eradication.

Many examples of introgression of aliens escaped from farms into native gene pools, like those cited above, have been provided from fish. There are also some examples of NIS shellfish introduced via farming that are producing changes in the host environment and modifying the native fauna composition (e.g. Couceiro et al. 2012). Dispersal of farm escapees can be favoured by different factors such as marine currents or fouling (Carlton 1992; Geller et al. 1994; Gilg and Hilbish 2003). One case of successful shellfish NIS expanding in the new environment occurs on the North American Pacific coast and has the mussels of the genus *Mytilus* as protagonists.

Mytilus is one of the most thoroughly studied genera of marine molluscs (e.g. Kenchington et al. 1995; Shields et al. 2010; Thomsen et al. 2013). These mussels are important components of the intertidal community in rocky shores (e.g. Suchanek 1985; Koehn 1991). Within this genus, the *M edulis* complex contains three species (*Mytilus trossulus* Gould, *M. galloprovincialis* Lamarck and *M. edulis* Linnaeus), which are morphologically quite similar and often difficult to identify

visually (Heath et al. 1995). They are however genetically different and can be distinguished employing different molecular markers, such as allozymes (McDonald et al. 1991), nuclear 18S rDNA (Kenchington et al. 1995), genes coding for adhesive foot proteins (e.g. Inoue et al. 1995; Rawson et al. 1996), intron-spanning primers (Bierne et al. 2002), microsatellites (e.g. Lallias et al. 2009), and SNP (e.g. Zbawicka et al. 2012). *M. galloprovincialis* has been included in the list of 100 of the world's worst invasive alien species by the Invasive Species Specialist Group of the International Union for the Conservation of Nature (Lowe et al. 2000, available at http://www.issg.org/database/species/reference_files/100English.pdf Accessed July 2014). This species is strongly differentiated in its native area between Atlantic and Mediterranean waters, and its introductions worldwide occurred often from Mediterranean populations except in South Africa (Daguin and Borsa 2000).

Some *Mytilus* species are commercially important and are cultured worldwide, for example *Mytilus galloprovincialis*, which is farmed in Asia, America, Europe and Africa (www.fao.org). In the Pacific coast of North America, there are two native species, *M trossulus* and *M. californianus* Conrad (Morris et al. 1980; McDonald et al. 1991; Santaclara et al. 2006). Phylogenetically, *M. californianus* is the sister group to the *M. edulis* complex (Kenchington et al. 1995; Distel 2000; Martinez-Lage et al. 2002). The NIS *M. edulis* L. and *M. galloprovincialis* L., both native to Europe (Quesada et al. 1998; Hilbish et al. 2000; www.fao.org), have been intensely farmed on North American Pacific coasts since the beginning of the twentieth century (Hilbish et al. 2010). They have established feral populations there (Heath et al. 1995; Rawson and Hilbish 1995; Santaclara et al. 2006; Shields et al. 2010). *Mytilus galloprovincialis* is expanding its distribution and has been declared an invasive species (e.g. Braby and Somero 2006). In southern California it appears to have largely replaced the native *M. trossulus* (Geller 1999).

Hybridization among species in the *M edulis* complex is frequent due to their related ancestry and shared habitat (e.g. Gosling 1992; Riginos and Cunningham 2005; Shields et al. 2010). Sympatric species always exhibit hybridization in this complex and interbreeding extent varies both by geographic region and taxa (Toro et al. 2004; Riginos and Cunningham 2005; Braby and Somero 2006). Although there are isolation mechanisms between these species (Bierne et al. 2006), the hybrids are able to produce backcrosses (Beaumont et al. 1993; Hilbish et al. 2002; Wood et al. 2003). Natural hybrid zones are well known in Atlantic European (e.g. Bierne et al. 2002, 2003) and American waters (e.g. Comesaña et al. 1999). Hybridization between species of this complex has been described on the North American Pacific coast (e.g. Heath et al. 1995; Saavedra et al. 1996; Suchaneck et al. 1997; Rawson et al. 1999), as well as hybrid zones involving native and cultured species (Elliott et al. 2008; Shields et al. 2010).

Given the known hybridization and introgression between *Mytilus* species, and the current expansive trend of *M. galloprovincialis* in the North American Pacific coast, this area seems to be a good case study for investigating environmental factors that may promote aquaculture-derived invasions. The present study is focused on Vancouver Island (VI) (British Columbia, Canada; Fig. 1), where mussel farms are concentrated in some locations whereas other zones are almost free of mussel aquaculture (www.dfo-mpo.gc.ca/index-eng.htm). Mussels were sampled from rocky shores in areas that varied in exposure to open sea, waves, type of substrate, algae coverage, farm density and also other anthropogenic factors such as distance to ports and substrate modifications. We used species-specific molecular markers to identify NIS introgression. Our hypothesis was that the level of NIS introgression will depend on farming density, but can be modified by other environmental factors. The contribution of these environmental variables to NIS spread via introgression will be tested. The final objective was to identify main factors that, alone or in combination, may favour NIS introgression. This knowledge could be further applied to NIS control and containment measures.

Materials and methods

Ecological and geographical setting of the study area

Mussels of the genus *Mytilus* are highly fecund spawners and their larvae can survive in the water for up to 2 months (Suchanek 1985; Bayne 1965). They exhibit a high dispersal capacity (range estimated for realized dispersal capacity between 30 and

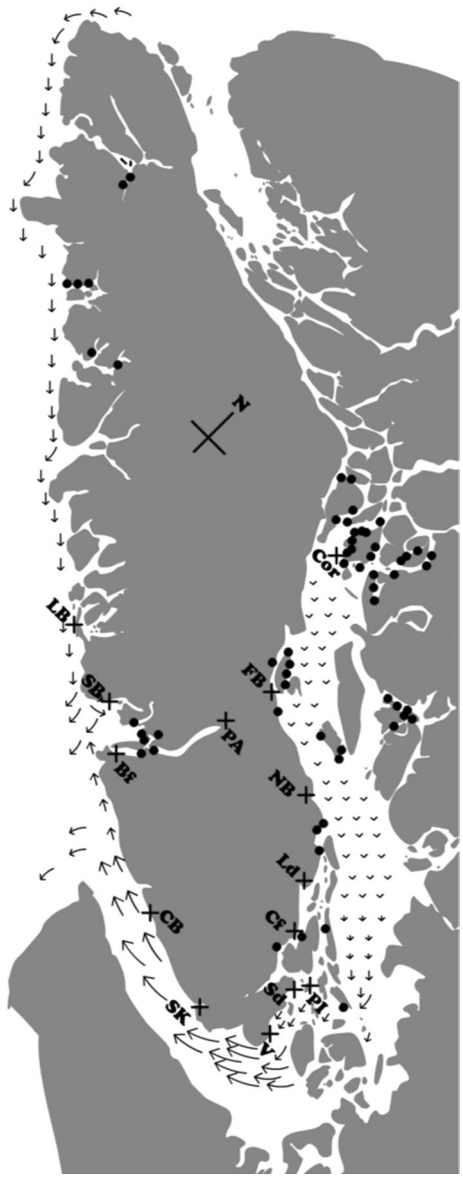

Fig. 1 Map of Vancouver Island including the sampling locations (*crosses*), aquaculture farms (*dots*) and prevailing marine surface currents (*arrows*; arrow sizes are indicative of current intensity). Location names: *V* Victoria, *Bf* Bamfield, *Sd* Sidney, *CB* China Beach, *Sk* Sooke, *Cf* Crofton, *Ld* Ladysmith, *NB* Nanoose Bay, *FB* Fanny Bay, *SB* Salmon Beach, *LB* Long Beach, *PA* Port Alberni, *PI* Portland Island, *Cor* Cortes Island

60 km, >100 km in some cases, although dispersal capacity is much higher; McQuaid and Phillips 2000; Gilg and Hilbish 2003; Addison et al. 2008). Accordingly, extensive dispersal and panmixia among coastal populations is expected for this genus (Shields et al. 2010). However, VI may be an exception for the reasons explained below.

Vancouver Island is separated from the British Columbia mainland by the Strait of Georgia (Fig. 1), which is a long (220 km), narrow (18–55 km) and deep (156–420 m) fjord estuary (www.nwfsc.noaa.gov/publications/techmemos/tm44/environment.htm) with small islands, peninsulas and shallow areas which restrict water flow (Herlinveaux and Tully 1961; Masson and Cummins 2004). Although wide water gyres occur all year round, they are stronger in summer (Masson and Cummins 2004) coincident with the mussel spawning season and may act to restrict larval dispersal (e.g. Toro et al. 2004). The northeast part of VI has an assemblage of small islands and peninsulas with many tidal rapids that may limit dispersal and gene flow isolating the northern region from the rest of VI (Heath et al. 1995; Shields et al. 2010). The West coast of VI is open to the Pacific Ocean and thus exposed to strong North-South marine currents near the shelf (Fig. 1). Therefore, the convoluted hydrology and topology surrounding VI suggests panmixia may not occur (Shields et al. 2010). The marine currents around the island depicted in Fig. 1 were obtained from the Department of Fisheries and Oceans Canada (www.dfo-mpo.gc.ca/index-eng.htm, Accessed January 2014), the NOAA Ocean Surface Current Simulator (OSCURS) (las.pfeg.noaa.gov/oscurs; Accessed January 2014), and the Institute of Ocean Sciences of Canada (www.pac.dfo-mpo.gc.ca/science/facilitiesinstallations/ios-ism/index-eng.htm, Accessed January 2014) (Davenne and Masson 2001). The flood tide runs around the north and south ends of the island (from the Pacific side) and then meets at approximately the middle of the Strait on the east side.

The four species studied here are common in intertidal marine environments (Koehn 1991; McDonald et al. 1991). *Mytilus californianus* can be easily identified *de visu* (Fig. 2) because they exhibit strong radial ribs (Conrad 1837), is phylogenetically very distant to the other three species of the genus and it is not known, and is very unlikely, to hybridize with them. As mentioned above, *M. trossulus* and the farmed *M. edulis* and *M. galloprovincialis* can hybridize in this area, and hybrids and introgressed individuals are not morphologically distinguishable from the parental species, thus genetic analysis is necessary for species identification (Rawson et al. 1999; Shield et al. 2010).

From the existing reports, in British Columbia *M. galloprovincialis* has been the mussel species most intensely farmed, followed by *M. edulis* in lower quantity (Heath et al. 1995; Hilbish 1999; Anderson et al. 2002). Farmers produce mainly these two imported species, and a few farms cultivate *M. trossulus.* The industry is based on hatchery-produced supply of seed, mainly imported from the United States. Triploid seed has been recently developed, but production locations are not identified in the sources consulted, principally the British Columbia Shellfish Grower's Association (www.bcsga.ca, Accessed July 2014). Mussel farm locations and the species farmed in the sampling areas considered were obtained from the records available at the Centre for Shellfish Research at VI University, the British Columbia Shellfish Grower's Association and the Canada Department of Fisheries and Oceans.

Sample collection and preparation

A total of 700 adult *Mytilus* sp. individuals from 14 sites (Fig. 1; 50 samples per point) on VI were collected from rocky beaches of the intertidal zone (natural intertidal populations) between March and June of 2012. Sampling locations that varied with respect to mussel farm density, exposure to waves, algae coverage, substrate type (from natural to artificial such as stone or the most artificial concrete walls), distance to seaports, distance to freshwater and degree of sheltering from the open sea, were deliberately selected (Table 1). These environmental factors were chosen because in addition to aquaculture, salinity, exposure, type of substrate and maritime traffic are known to be keys for shaping *Mytilus* communities (e.g. Riginos and Cunningham 2005; Robinson et al. 2005; Elliott et al. 2008; Dias et al. 2009; Shields et al. 2010). The sheltered sites experienced limited water flow and higher summer water temperatures, relative to the exposed sites (Heath et al. 1995). Sea ports with a harbour and regular ship traffic (ferry lines, commercial fish landing, big touristic ports) were considered. In addition, small boats travel all around the island and may stop at any shelter. These were not considered here.

At each location, samples were obtained from the intertidal transect, covering an area of 300–2,000 m^2 depending on mussel abundance and habitat homogeneity. Shell measurements (length, width, and height) were obtained with callipers (±0.1 mm). Valves were opened and the samples stored in 95 % ethanol until genetic analyses were performed. The ethanol was changed twice on consecutive days to improve tissue preservation.

Genetic analyses

Total genomic DNA was extracted from a piece of 50 mg (approx.) of mussel foot tissue, with 1 mm

Fig. 2 Photographs of mussels sampled. *Mytilus trossulus* (*top left*), *M. galloprovincialis* (*top right*), and *M. californianus* (*bottom*)

Table 1 Characteristics of the points sampled

Site	Lat	Long	Subs.	Dist port	Algae (%)	Wave exposure	Farms	Farmed species	Shelter	Freshwater	Length	Width	Height
V	48.411038	−123.299998	4	1.1	80	4	0	0	0	0	23.33	12.77	10.54
Bf	48.830218	−125.136837	3	0	90	3	7	Mg, Me	2	0	27.49	14.80	10.36
Sd	48.685879	−123.400997	2	0.34	30	1	2	Mg, Me	0	0	22.48	12.57	10.50
CB	48.430226	−124.088539	2	30	10	5	0	0	0	1	79.76	35.68	27.72
SK	48.365445	−123.714823	4	0	50	1	0	0	0	0	32.96	18.13	12.80
Cf	48.855334	−123.616738	3	1.6	90	1	5	Mg, Me	0	0	29.03	16.27	11.44
Ld	49.037256	−123.747480	3	6.8	90	1	5	Mg, Me	2	0.9	32.98	18.47	13.73
NB	49.276779	−124.120329	4	2.3	10	2	8	Mg, Me, Mt	0	1	28.00	15.16	11.35
FB	49.544327	−124.863123	4	2.9	60	2	14	Mg, Me, Mt	0	0	33.83	17.81	13.97
SB	48.953473	−125.438088	2	9.3	50	5	7	Mg, Me	0	0.53	46.24	25.28	20.61
LB	48.999087	−125.658151	1	18.7	50	5	0	0	0	0.95	76.82	35.55	36.57
PA	49.170853	−124.829612	5	6.8	90	1	0	0	2	1	33.34	16.02	12.35
PI	48.735007	−123.365201	1	4.2	20	2	2	Mg, Me	2	0	33.79	17.13	14.10
Cor	50.105662	−125.053353	3	0.05	70	3	21	Mg, Me, Mt	1	0.5	25.14	13.40	11.05

Sampling sites: Victoria (V), Bamfield (Bf), Sidney (Sd), China Beach CB), Sooke (SK), Crofton (Cf), Ladysmith (Ld), Nanoose Bay (NB), Fanny Bay (FB), Salmon Beach (SB), Long Beach (LB), Port Alberni (PA), Portland Island (PI) and Cortes Island (Cor); latitude and longitude; substrate type from 1 (natural) to 5 (totally artificial); distance to the nearest marine port (in Km); % algae coverage; wave exposure from 1 (none) to 5 (very exposed); number of farms in the surrounding coast (50 km); species farmed near the sampling location: *Mytilus galloprovincialis* as Mg, *M. edulis* as Me, *M. trossulus* as Mt; sheltering from marine currents (2, sheltered in a gulf or bay; 1, semi-exposed; 0, open waters); distance to freshwater (lake or river mouth, in kilometers); mean mussel shell measures, in mm

silica beads and a PrepMan^{TM} Ultra sample preparation reagent (Applied Biosystems) following the manufacturer's instructions. DNA was stored at −2 °C.

Introgression of NIS genomes (mainly *M. galloprovincialis*) into the local gene pool was measured employing two loci, one mitochondrial and one nuclear. The mitochondrial locus considered was the cytochrome c oxidase subunit I gene (*COI*), which is the main mitochondrial DNA Barcode for animals (e.g. Waugh 2007). It was amplified with the primers COI-H and COI-L (Folmer et al. 1994) and the PCR conditions described therein. For preliminary species assignation sequences were compared with the NCBI GenBank database employing the BLAST application (http://blast.ncbi.nlm.nih.gov/), using a threshold of 98 % identity, above the 97–97.4 % commonly accepted for this gene in Barcoding projects (e.g. Meyer and Paulay 2005).

The nuclear locus was *Glu-5′*, which encodes a mussel polyphenolic adhesive protein. Two PCR reactions employing different primer pairs that anneal within that gene were done to double-check the heterozygote or homozygote status of the samples. For the primers JH-5 and JH-54 (Rawson et al. 1996), the expected amplification products are one or two fragments 350/380 base pairs (bp) long for *M. edulis*, one 240 bp long fragment for *M. trossulus*, and one or two fragments of 300/500 bp for *M. galloprovincialis*. The primers Me-15 and Me-16 (Inoue et al. 1995) yield amplification fragments 180 bp long for *M. edulis*, 168 bp long for *M. trossulus* and 126 bp long for *M. galloprovincialis*. PCR amplifications were carried out in a mixture of 17 µl containing 0.5 µl of DNA template, 7.88 µl of distilled water, 4 µl of buffer containing 7 mM of $MgCl_2$ (Promega), 1.5 µl of dNTP (2.5 mM, Invitrogen), 0.75 µl of each pair of primers (0.2 mM) and 0.12 µl of Taq Polymerase (Promega, 5 U/ml). The two PCRs were done separately on an Eppendorf Mastercycler ep gradient S PCR machine. Amplification protocols consisted of an initial denaturation at 94 °C for 3 min, 30 cycles of 94 °C for 20 s, 48 °C for 20 s and 72 °C for 45 s, and a final extension at 72 °C for 15 min for the primers JH-5 and JH-4. For the primers Me-15 and Me-16 the protocol consisted of an initial denaturation at 95 °C for 5 min, 30 cycles of 94 °C for 30 s, 52 °C for 30 s and 70 °C for 90 s, and a final extension at 70 °C for 15 min. A negative control, no template DNA, was included in all PCR assays. Amplified products were directly resolved in an 3 % agarose gel stained with SYBR Safe DNA stain (Invitrogen) by comparison with a 50 bp DNA ladder (New England BioLabs). Only the individuals that yielded consistent results for the two PCRs were considered for the statistical analysis.

Phylogenetic analysis

Final species identification based on mitochondrial sequences was done from phylogenetic assignation. Sequences identified as different mussels (*M. edulis, M. californianus, M. galloprovincialis* and *M. trossulus*; Me, Mc, Mg and Mt respectively) from BLAST-based methodology were aligned using the BioEdit program (Hall 1999) together with reference sequences of each mussel species retrieved from GenBank (Me: KF369153, Mc: GQ902216, Mg: GQ480284, Mt: GU570484) (www.ncbi.nlm.nih.gov/genbank/).

Phylogenetic analyses were conducted using MEGA version 6 (Tamura et al. 2013). This software was used to construct a phylogenetic tree based on the aligned sequences. The phylogenetic trees containing the reference and mussels problem sequences were reconstructed using this software by the methods of Neighbor-Joining with the following settings: Tamura Nei model (1993) and uniform rates. Robustness of the Neighbor-Joining tree topology was assessed using 1,000 bootstrap replicates.

Statistical analysis

The environmental variation of the study sites was analyzed employing a principal component analysis (PCA) with the software PAST (Hammer et al. 2001), with Correlation option and 0.7 Jolliffe's cut. The contribution of the site characteristics to the total variance was estimated, as well as the correlation between sampling site characteristics, the proportion of heterozygotes and the introgression. Introgression was measured as the genetic contribution of *M. galloprovincialis* (G) to the mussel gene pool of a population (sampling site). For the mitochondrial locus each individual contributes with one variant (mtG is the introgressed gene). For the *Glu 5′* locus, homozygotes TT provide zero NIS genes, heterozygotes GT provide one, and homozygotes GG two genes. We have used non-parametric statistics

(Kendall's Tau correlation tests) given the non-parametric nature of many data (farm density, wave exposure and others). When correlation values >0.8 were obtained between two parameters, one of those was eliminated from the PCA to avoid auto-correlation biases.

Having thus established an independent (non-correlated) environmental dataset, the PC1 (the principal component with greatest contribution to the total variance) components was used as the dependent variable in a Generalized Linear Model, with AIC as a guide for best fit model, for testing directly for the contribution of the different environmental variables. In addition the genetic data (introgression) were used as dependent variables in a multivariate multiple linear analysis to test for the relative contribution of each environmental variable to such introgression with another test.

Comparisons between populations (global, pairwise and among groups of populations) for differences in allele (G versus T for *Glu*-5′) and haplotype (mtG amd mtT) frequencies were done employing the software ARLEQUIN suite version 3.5.1.3 (Excoffier and Lischer 2010), with 10,000 permutations. The AMOVA structure tested categorized the populations by farm density in three groups: one group of populations with 0–2 farms (Port Alberni, Portland Island, Sidney, Sooke, Victoria), another with >2 to 8 farms (Bamfield, Crofton, Ladysmith, Nanoose Bay, Salmon Beach) and another with the two sites with highest farm density (Cortes Island and Fanny Bay, with 21 and 14 farms respectively). The estimates of population differentiation were based on conventional F-statistics, for differences in haplotype frequencies in the case of mitochondrial DNA. Exact tests for pairwise genetic differentiation were also made.

Results

Genetic estimate of NIS introgression on Vancouver Island

COI sequences were obtained from 543 individuals of the *M edulis* complex (25–50 per population). The different haplotypes found were submitted to GenBank and are available under the accession numbers KC732781–KC732805. Matches to reference GenBank sequences with more than 98 % nucleotide identity were found, although in a few cases the same highest identity was found for more than one species. The COI sequences retrieved were of female type (F-mitochondrial DNA) in all cases, as expected because we extracted DNA from the mussel foot and male mitochondrial DNA is only found in the gonads of males (e.g. Zouros et al. 1994; Quesada et al. 1998). The tree reconstructed from these F-haplotypes, sequences obtained from our *Mytilus californianus* samples for species confirmation, and references from GenBank (Fig. 3 shows an example using only 10 problem sequences for clarity), exhibited a strong phylogenetic signal that confirmed preliminary BLAST species assignation and served to solve ambiguous cases. Most of the *COI* sequences obtained in our survey were *M. trossulus* (530, 97.61 %) followed by *M. galloprovincialis* (11 individuals, 2.03 %) and two *M. edulis* (0.37 %). The species composition was different among sites, with *M. californianus* concentrated in only three sites (Long beach, China Beach and Salmon Beach; Table 2). NIS sequences appeared from five locations with a higher frequency in Fanny Bay (Table 2).

For the species-specific nuclear locus, with the Me-15 and Me-16 primers we found fragments of 168 and 126 nucleotides typical of *M. trossulus* and *M. galloprovincialis* respectively. Individuals exhibiting a pattern with the fragments of two species simultaneously were classified as heterozygotes (Fig. 4a). For the JH-4 and JH-5 primers, fragments of 300 and 240 nucleotides typical of *M. galloprovincialis* and *M. trossulus* respectively were retrieved, many of them also in heterozygosis (Fig. 4b). In total we found 517 (95.2 %), 26 (4.6 %) and one (0.2 %) individuals with TT, GT and GG genotypes respectively.

Considering the two loci together (mitochondrial and nuclear loci), in our survey we found a total of 41 NIS genes (13 mitochondrial and 28 nuclear genes) in the samples of the *M. edulis* complex, corresponding to 2.52 % global introgression. These introgressed genes were unequally distributed in the locations considered (Table 2). Some areas like Cortes Island, Fanny Bay and Crofton exhibited higher NIS introgression, while samples from four sites (Port Alberni, Victoria, Bamfield and Ladysmith) contained exclusively genes of native *M. trossulus* (Table 2).

Aquaculture seems to promote NIS introgression in our study area. For populations from sites with different densities of blue mussel farms, the AMOVA

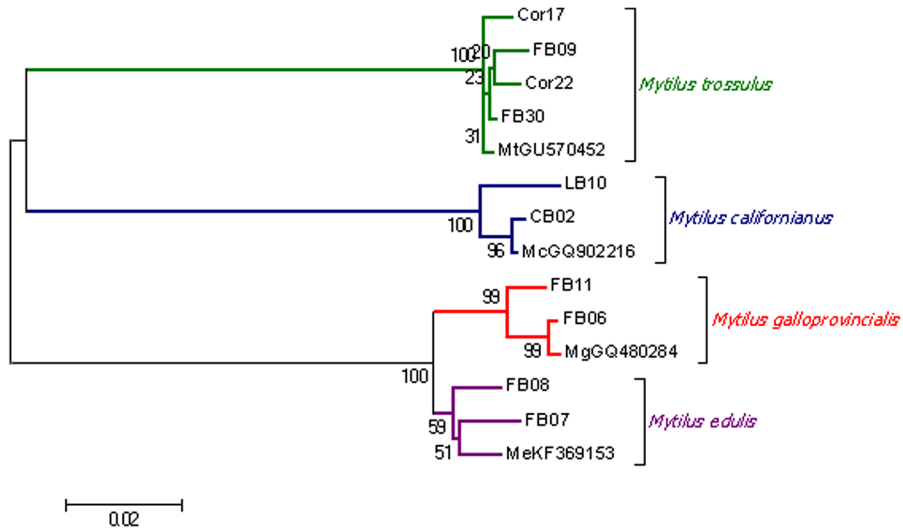

Fig. 3 Neighbor-Joining tree reconstructed based on reference and nine problem COI gene mussel sequences. Bootstrap values are marked on the branches, in percentage. For problem sequences *CB*, *Cor*, *FB* and *LB* correspond to China Beach, Cortes Island, Fanny Bay and Long Beach respectively, followed by the sample number. *Mc*, *Me*, *Mg* and Mt are acronyms of *Mytilus californianus*, *M. edulis*, M. galloprovincialis and *M. trossulus* respectively, indicating the species at the beginning of GenBank reference sequences

Table 2 Genetic identification of *Mytilus* samples in each site

Site	N	COI sequences				*Glu-5′* genotypes		
		Mc	Mt	Mg	Me	TT	GT	GG
V	46	0	100	0	0	100	0	0
Bf	45	0	100	0	0	100	0	0
Sd	46	0	100	0	0	97.83	2.17	0
CB	49	97.96	2.04	0	0	100	0	0
SK	49	0	100	0	0	97.96	2.04	0
Cf	49	0	97.96	2.04	0	85.72	14.28	0
Ld	50	0	100	0	0	100	0	0
NB	44	0	95.45	4.55	100	95.45	4.55	0
FB	49	0	83.67	12.24	5.08	79.59	20.41	0
SB	50	50	50	0	0	96	4	0
LB	50	100	0	0	0	–	–	–
PA	47	0	100	0	0	100	0	0
PI	46	0	97.83	2.17	0	97.83	2.17	0
Cor	47	0	95.74	4.26	0	91.49	6.38	2.13

Percentage of *Mytilus trossulus* (Mt), *M. californianus* (Mc), *M. edulis* (Me) and *M. galloprovincialis* (Mg) cytochrome oxidase I (COI) sequences. For Mg and Mt, percentage of each genotype (TT, GT and GG are homozygote Mt, heterozygote MgMt and homozygote Mg respectively) for the nuclear locus *Glu-5′*. Victoria (V), Bamfield (Bf), Sidney (Sd), China Beach CB), Sooke (SK), Crofton (Cf), Ladysmith (Ld), Nanoose Bay (NB), Fanny Bay (FB), Salmon Beach (SB), Long Beach (LB), Port Alberni (PA), Portland Island (PI) and Cortes Island (Cor). Sample size: n

based on mitochondrial DNA (Table 3a) revealed significant variation among groups (*P* value for variance among groups Va = 0.018 ± 0.004), indicating the influence of farms in the introgression of blue mussel alleles. This result was confirmed with the *Glu-5′* genotypes (Table 3b), which also provided

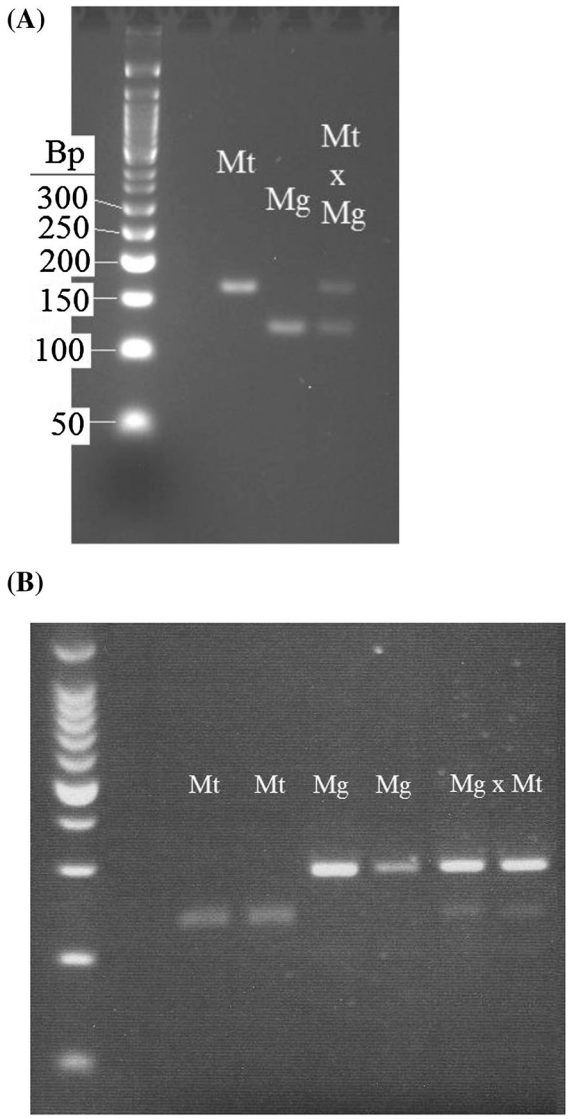

Fig. 4 Agarose gel (2%) stained with ethidium bromide containing PCR amplification products for the *Glu-5′* locus obtained with the primers Me-15 and Me-16 (**a**) and JH-4 and JH-5 (**b**). Mt: *Mytilus trossulus*; Mg: *M. galloprovincialis*; MgxMt: *M. galloprovincialis* x *M. trossulus* heterozygote. A 100 bp ladder (Promega) was added as a DNA fragment size marker in the *first lane*

significant differences among groups (P value for Va = 0.031 ± 0.006). The groups did not exhibit significant internal heterogeneity (P value for variance among populations within groups or Vb were not significant). NIS introgression was 0.57, 2.03 and 8.68 % for sample groups from areas with low, medium and high farm density respectively. Pairwise exact tests revealed significant differences between the populations with no introgression and Cortes Island, Fanny Bay and Nanoose Bay for mitochondrial DNA, and between Fanny Bay, Cortes Island and Crofton and the other populations for *Glu-5′* (data not shown).

Environmental data and introgression

Introgression and heterozygotes were correlated with r = 0.933. We retained only introgression for further analysis. None of the other variables were correlated with r > 0.7, thus the complete environmental set was considered. In the PCA four components accounted for almost 80 % of the total variance (Table 4). The main environmental factors contributing to these components (marked in bold on Table 4) were introgression and degree of sheltering in PC1 and farms followed by distance to freshwater in PC2. A plot of the contribution of all the variables to PC1 and PC2 is represented in Fig. 5.

The Generalized Linear Model identified only two factors with significant ($P < 0.05$) contribution to introgression: farms ($y = 0.333x + 0.570$, $G = 48.346$, $P = 3.572E{-}12$ for slope $\neq 0$) and sheltering ($y = -1.530x + 3.686$, $G = 24.002$, $P = 9.624E{-}07$ for slope $\neq 0$), with normal distribution and the identity link as the best fit model. For the multivariate multiple linear analysis with introgression as the dependent variable we obtained multiple r = 0.964, $r^2 = 0.929$, and r^2 adjacent = 0.807. It was significant with F = 7.556, $P = 0.034$. Three environmental factors exhibited significant slopes (Table 5): farm density and distance to ports with positive slope, and distance to freshwater with negative slope. These results seem to discard ports as a factor promoting introgression (high distance to ports would be associated with introgression, perhaps because farms that are the source of NIS are not located within ports), and suggest farms and exposure to open waters (identified from the Generalized Linear Model; introgression with a significant negative slope on the degree of sheltering), and proximity to freshwater as environmental factors contributing significantly to NIS introgression. According to the results found in the models above, introgression exhibited significant tau-correlation values with farm density and sheltering ($\tau = 0.517$, $P = 0.019$ and $\tau = -0.455$, $P = 0.039$ respectively).

Table 3 Analysis of molecular variance (AMOVA) comparing the three groups of populations with low (up to 2 farms in the surrounding area), medium (2–8) and high (>8) density of mussel farming for NIS introgression measured from the mitochondrial COI gene (A) and the nuclear *Glu-5′* locus (B). Degrees of freedom: *df*

Source of variation	*df*	Sum of squares	Variance	% Variation	*P* value
A					
Among groups	2	0.284	0.00067	2.65	0.01760 ± 0.00392
Among populations within groups	9	0.240	0.00004	0.18	0.15347 ± 0.01256
Within populations	531	13.115	0.02470	97.17	0.00098 ± 0.00098
Total	542	13.639	0.02542		
B					
Among groups	2	0.448	0.00115	5.22	0.03910 ± 0.00631
Among populations within groups	9	0.240	0.00013	0.59	0.23754 ± 0.01349
Within populations	531	11.047	0.02080	94.19	0.03715 ± 0.00840
Total	542	11.735	0.02209		

Table 4 Results of the principal component analysis for environmental factors of the sampling sites, indicating the variance explained by each component (in percent) and the relative effect of each factor within each component

	Component 1	Component 2	Component 3	Component 4
Variance (%)	26.6	20.2	17.9	13.4
Substrate	−0.157	0.219	**−0.679**	0.244
Distance to Port	**−0.329**	0.418	0.414	0.187
Algae coverage	−0.256	0.183	−0.430	**−0.608**
Wave exposure	0.206	0.264	0.385	−0.349
Farm density	0.358	**0.517**	−0.052	−0.254
Shelter	**−0.489**	0.046	0.127	−0.348
Freshwater	−0.398	**0.490**	0.034	0.387
Introgression	**0.489**	0.402	−0.117	0.089

The highest values within each component are marked in bold

For the native *Mytilus californianus*, sampled only from three sites, its frequency was negatively associated to anthropogenic subtrates ($\tau = -0.539$, $P = 0.007$) and positively with distance to ports ($\tau = 0.601$, $P = 0.003$) and wave exposure ($\tau = 0.639$, $P = 0.001$), likely preferring natural and open habitats. These values should be taken with caution because the species frequency was 0 in most sites. In addition, the distance to ports was positively correlated with mussel size ($\tau = 0.633$, $P = 0.001$), as might be expected (better mussel growth in cleaner sites).

Discussion

Our study shows that molecular techniques may help to identify and confirm the source of alien genes introgressed into native mussels, in this study likely due to interactions between aquaculture facilities and natural mussel populations. The results reveal a significant association between mussel farms and NIS introgression. Based on one nuclear and one mitochondrial locus we found between 0.6 and 8.7 % of individuals carrying NIS genes within the *M edulis* complex. These results support the view of VI containing hybrid zones for mussels as proposed by Shields et al. (2008, 2010). It seems that there is some resistance to the invasion since heterozygote proportion was not high except in locations near aquaculture farms. Two decades ago, Heath et al. (1995) reported hybridization between native *M. trossulus* and introduced *M. galloprovincialis* and *M. edulis*. We found *M. edulis* genes in very low frequencies. Since European populations are introgressed by *edulis*

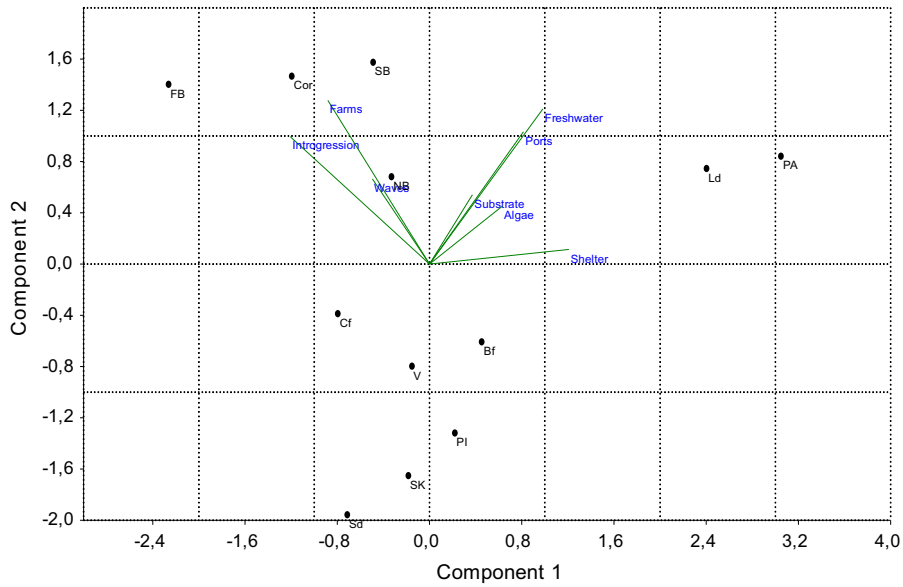

Fig. 5 *Plot* of the principal components PC1 and PC2, with the relative contribution of each factor proportional to diagonal length. Population names: *V* Victoria, *Bf* Bamfield, *Sd* Sidney, *CB* China Beach, *Sk* Sooke, *Cf* Crofton, *Ld* Ladysmith, *NB* Nanoose Bay, *FB* Fanny Bay, *SB* Salmon Beach, *LB* Long Beach, *PA* Port Alberni, *PI* Portland Island, *Cor* Cortes Island

Table 5 Multivariate multiple linear analysis with Introgression as the dependent variable

	Slope	Standard error	t	p
Constant	−0.80599	1.9783	−0.40741	0.70457
Substrate	1.2859	0.60605	2.1217	0.10115
Ports	**0.8858**	**0.21443**	**4.1309**	**0.014484**
Algae	−0.024738	0.020317	−1.2176	0.2903
Waves	−0.88422	0.37594	−2.352	0.078345
Farms	**0.50321**	**0.081029**	**6.2103**	**0.0034208**
Shelter	−1.0343	0.59487	−1.7386	0.15709
Freshwater	**−6.4759**	**1.7714**	**−3.6558**	**0.02166**

Slopes for each environmental factor with their estimated standard errors, results of t-tests and associated *P*-values. Factors with significant slopes are marked in bold

mtDNA haplotypes (Quesada et al. 1998), a likely explanation could be that the few *M. edulis* haplotypes observed are consistent with an introduction of *M. galloprovincialis* only. *M. edulis* has been farmed to a lesser extent than *M. galloprovincialis* in British Columbia (Anderson et al. 2002; Wonham 2004); if the two European species compete here, as reported in native areas (Hilbish et al. 2002), the more invasive *M. galloprovincialis* (Braby and Somero 2006) would out-compete *M. edulis*. In any case, our results suggest that the level of introgression is increasing in VI, or at least in aquaculture spots. Heath et al. (1995) reported 6.2 % NIS, whereas Shields et al.'s (2010) figures were 4.2 and 6.6 % in 2005 and 2006 respectively. The value of 8.7 % found in our work for the 2012 samples obtained from farming areas (0.6 % in areas with little farming), although not strictly comparable with previous studies due to a different sampling strategy, suggests that farms are acting as vectors of exotic mussels in Canada and also confirm the invasive condition of *M. galloprovincialis* (Geller 1999; Braby and Somero 2006).

Differences in habitat preferences among species have been reported for the *Mytilus* complex (Bierne et al. 2003; Gilg and Hilbish 2003; Shields et al. 2010), and also between the two native species of this region. *M. californianus* has an advantage in exposed habitats (Bell and Gosline 1997), and that was reflected in our

samples. Within the *M. edulis* complex, NIS-native heterozygotes seem to also prefer exposed zones (they are negatively associated with sheltering). On the other hand, and contrary to expectations (e.g. Shields et al. 2008; Pysek et al. 2010), the degree of substrate artificiality (anthropogenic substrate) did not contribute to the NIS distribution found in our survey in any analysis, nor did algae coverage or wave exposure. This result strongly suggests that they could be introduced even in well-preserved habitats with little anthropogenic disturbance, making their potential control more difficult.

Although we did not find farming to be a main factor for the spreading of exotic mussels on VI there are other possible vectors. Marine currents have been previously shown to be important dispersal vectors (Gilg and Hilbish 2003; Shields et al. 2010). In our study there are some hints of their importance. For example, Sooke has no farms nearby but contained some NIS. This area is affected by marine currents that may carry mussel larvae from more intensely farmed areas like Crofton and mainland British Columbia farms (Fig. 1). However, another well recognized vector for transferring exotic mollusc species such as fouling or boat transport (Carlton 1992; Geller et al. 1994; Molnar et al. 2008) was not positively associated with NIS, rather the opposite, which is logical if farms are the primary source of NIS because aquaculture facilities are not located within or near ports. These results support Heath et al. (1995) who suggested that fouling is not a major factor of mussel dispersal in the region.

The dynamics of NIS introgression on VI cannot be explained from known environmental gradients alone (Shields et al. 2008). Dias et al. (2009) reported higher levels of hybridization between *M. trossulus* and *M. galloprovincialis* in Scottish aquaculture sites than in natural intertidal populations. Heath et al. (1995) reported a decrease in the incidence of alien mussel alleles with the distance to farms in southeastern VI, similar to our results found near the intensely farmed areas of Cortes Island and Fanny Bay. Although there are no available data concerning the proportion of each species in each farm, previous studies report much higher incidence of *M. galloprovincialis* farming (Heath et al. 1995; Hilbish 1999; Anderson et al. 2002). Our results consistently indicate that farms are contributing to NIS introduction and may help to understand the hybridization patterns, likely in combination with a mixture of selection and barriers to dispersal (Veliz et al. 2006). Our results suggest that such selection may be driven by exposure to open habitats and perhaps by salinity, two factors that have been previously reported to to explain mussel distribution patterns in the intertidal (e.g. Riginos and Cunningham 2005; Robinson et al. 2005; Elliott et al. 2008; Shields et al. 2010).

In conclusion, our results suggest that the hybrid zones of invasive and native mussels formed on the Pacific coast of Canada in areas of intense farming may be considered gates for introgression of NIS alleles, seemingly not related to maritime traffic in this case. In combination with marine currents that may facilitate larvae dispersal, NIS mussels seem to be expanding in some open coastal areas of VI although the invasion is not intense yet. The present study adds to the understanding of the effects of aquaculture escapes and their implications in invasion processes.

Management implications and recommendations

The studied area is near the northern limit of the distribution of *M. galloprovincialis* on this coastline. However, the natural limits are not clear for this species which has acclimated to cold areas as far the Kerguelen Islands in the Subantarctic Ocean (e.g. Lewis et al. 2004), and to the Massachusetts and Scotland coasts in the Atlantic Ocean (e.g. Hilbish et al. 2000). Moreover, under the current conditions of climate change the expansion of this highly adaptable species is expected to continue northwards (e.g. Wonham 2004). Although it is probably too late because the species is already present in the coast, this expansion should be recognized as problematic by managers and farmers; following a precautionary approach, the Canadian blue mussel aquaculture industry should not be developed further north. The results of this study suggest preference of individuals with *M. galloprovincialis* introgression for open areas, as reported for the pure species. Locating aquaculture facilities in sheltered non-exposed zones could perhaps delay (although probably not prevent) the settlement and population expansion of blue mussels around farms. On the other hand the apparent preference of introgressed individuals for locations nearby freshwater sources would suggest considering salinity when planning containment measures.

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