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A molecular approach to the ecology of a mussel (*Mytilus edulis – M. trossulus*) hybrid zone on the east coast of Newfoundland, Canada

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Abstract *Mytilus trossulus* Gould and *M. edulis* L. coexist in mixed populations in Atlantic Canadian waters. Although there is evidence that the two species hybridize in natural populations and that hybrids produce progeny through backcrosses, no study of the microgeographic distribution of the two forms and their hybrids has been made. Here we examine subtidal samples of mussels taken in July 1997 from two locations in eastern Newfoundland (Canada) and from wave-exposed and protected environments within each location. Shell lengths ranged from 15 to about 90 mm. Mussels were classified as pure forms or hybrids (F_1, F_2 and from backcrosses) based on four diagnostic markers, two allozyme loci (*Mpi* and *Est-D*) and two nuclear PCR-based DNA markers (*ITS* and *Glu-5*). In addition, a PCR-based mtDNA marker (*COIII*) was used to characterize the distribution of mtDNA mitotypes among pure and hybrid individuals. There were differences in the proportions of pure *M. edulis* and *M. trossulus* between sites and between environments at one location. *M. trossulus* was the predominant species at one of the two exposed sites. In all four samples, *M. trossulus* was also the predominant form among small individuals. The frequency of hybrids was 26% overall and did not differ among samples. Hybrids consisted mostly of backcrosses that were *M. trossulus*-biased among small mussels and *M. edulis*-biased among large ones. We conclude that both intrinsic genetic factors and extrinsic environmental factors influence the relative frequency of *M. edulis*, *M. trossulus* and their hybrids.

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Introduction

The micro- and macrogeographic distribution of the genus *Mytilus* on the Atlantic coast of North America has been studied by examining genetic variation at several polymorphic enzyme loci (Gartner-Kepkay et al. 1980; Koehn et al. 1984; Varvio et al. 1988; McDonald et al. 1991; Bates and Innes 1995). Early genetic studies of *Mytilus* populations on the east coast of North America suggested that *Mytilus edulis* was the only species present (Koehn et al. 1976; Gartner-Kepkay et al. 1980) but a more detailed investigation (Koehn et al. 1984) showed that populations of *Mytilus* spp. in Atlantic Canada consisted of two genetically distinct forms that were found sympatrically at some locations, but without evidence of interbreeding. These two genetically distinct forms of *Mytilus* (groups II and III of Koehn et al. 1984) were later confirmed to belong to the species *Mytilus edulis* and *M. trossulus* (Varvio et al. 1988; McDonald et al. 1991; Bates and Innes 1995; Mallet and Carver 1995). The situation in Atlantic Canada appears to be similar to that found in southwest England and the Atlantic coast of France, where two genetically distinct species of *Mytilus* (*M. edulis* and *M. galloprovincialis*) have overlapping ranges and at some locations occur sympatrically (Skibinski et al. 1978; Coustau et al. 1991; Gardner 1994). Natural hybridization occurs wherever any two species of this *Mytilus* species complex are found sympatrically (Gosling 1992; Gardner 1994). In southwest England and the Atlantic coast of France, the relative proportions of the two species and the amount of natural hybridization and introgression depend on the location sampled and environmental variables, such as

wave exposure and position of attachment in the intertidal and subtidal zones (Gardner and Skibinski 1990, 1991; Skibinski and Roderick 1991; Willis and Skibinski 1992; Gardner et al. 1993; Comesaña and Sanjuan 1997). In Atlantic Canada, there is limited evidence for interbreeding between *M. edulis* and *M. trossulus* in nature (Koehn et al. 1984; Varvio et al. 1988), despite the sympatric occurrence of both species and the successful production of viable hybrids in laboratory crosses (Zouros et al. 1992, 1994b; Saavedra et al. 1996). Undetected hybrids may in part be due to the failure to use highly diagnostic allozyme markers and/or an insufficient sample size. A recent study on the genetic variation of these two species of mussels in Newfoundland showed that the distribution of a hybrid index based on three partially diagnostic loci (*Est-D*, *Pgm*, *Lap*) provided no evidence for hybridization (Bates and Innes 1995). Two studies in Lunenburg Bay, Nova Scotia estimated the degree of natural hybridization to be $< 5\%$ (Mallet and Carver 1995) using the *Mpi* locus and 22.8% (Saavedra et al. 1996) using *Mpi* and *Est-D* loci. In the latter study *Mpi* and *Est-D* were used to classify the mussels as "pure" *M. edulis*, "pure" *M. trossulus*, or hybrids, and mtDNA variation was used to characterize the gender-associated mtDNA present in each individual. No "pure" *M. edulis* mtDNA type was found in "pure" *M. trossulus* individuals and vice versa, suggesting that no mtDNA introgression between the two species occurs in nature. Furthermore, the study provides evidence that introgression is blocked early in the hybridization process, although the mechanism remains unknown. Saavedra et al. (1996) did not provide any information about the environmental characteristics of the sites sampled or the size of the mussels sampled. It is possible that these two factors play an important role in determining the proportions of the pure forms and their hybrids present in the sample.

The objective of the present study was to determine the possibility of microgeographic genetic differentiation of *Mytilus edulis* and *M. trossulus* and to detect the presence of naturally occurring hybrids. We report the degree of natural hybridization at two different locations and, at each location, for two different environments (exposed and protected from wave action). Mussels with a range of shell lengths were sampled to determine if both *Mytilus* species and their hybrids are distributed differentially according to size. A final objective was to examine further the possible intrinsic incompatibilities between *edulis* and *trossulus* nuclear and mitochondrial genomes in mussels of different size from different environments.

Materials and methods

Study sites and sampling

Mussel populations (*Mytilus edulis* Linnaeus, 1758, *M. trossulus* Gould, 1850) were sampled subtidally (approximately 1.5 m below mean low tide) by SCUBA at two locations (Bellevue and Chance Cove) on the east coast of Newfoundland, Canada (Fig. 1) during July 1997. At each location, mussels were sampled from a wave-exposed environment and from a protected, sheltered one. Mussels with shell lengths from 15 to 92 mm were used in the genetic analysis. The mussels were brought alive to the laboratory, dissected, and the sex of each individual determined by microscopic examination of the gonad. Gonad and digestive gland were stored at −80 °C for PCR (polymerase chain reaction) and allozyme analysis. A small piece of the mantle border tissue was stored in ethanol (95%) at −20 °C for PCR analysis.

Allozyme analysis

Horizontal starch-gel electrophoresis was carried out on 11% gels (Sigma starch) at 4 °C. The digestive gland was homogenized in an equal volume of 0.01 *M* dithiothreitol solution and centrifuged at $8000 \times g$ for 7 min. The supernatant was used as the source for five enzyme loci that show different levels of diagnostic power for the two *Mytilus* taxa: esterase-D (*Est-D*; EC 3.1.1.-), mannose-6-phosphate isomerase (*Mpi*; EC 5.3.1.8), leucine aminopeptidase (*Lap*; EC 3.4.11.1), octopine dehydrogenase (*Odh*; EC 1.5.1.11) and phosphoglucomutase (*Pgm*; EC 5.4.2.2). Electrophoretic procedures were conducted following Bates and Innes (1995) for *Est-D*, *Lap* and *Pgm*, and Väinölä and Hvilsom (1991) for *Odh* and *Mpi*. Allele terminology was that used by McDonald and Koehn (1988) and McDonald et al. (1991). The *Mpi* locus appears to be

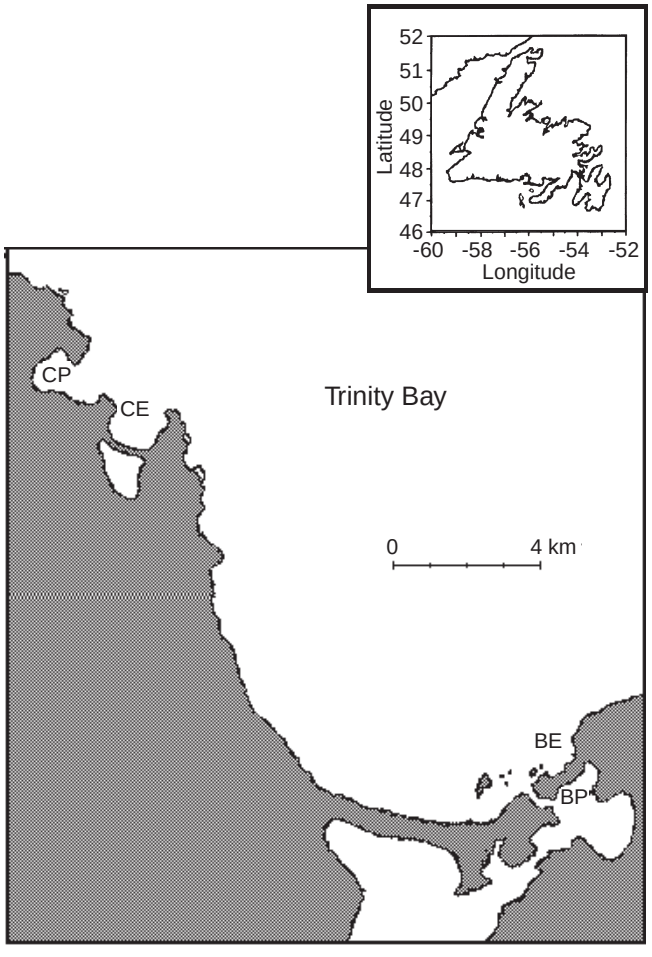

Fig. 1 Map of sample sites in eastern Newfoundland: Bellevue Protected (*BP*), Bellevue Exposed (*BE*), Chance Cove Protected (*CP*), Chance Cove Exposed (*CE*)

completely diagnostic between *Mytilus edulis* and *M. trossulus*, and *Est-D* is also a highly diagnostic locus for these two taxa (see McDonald and Koehn 1988; Varvio et al. 1988; McDonald et al. 1991; Väinölä and Hvilson 1991). Nevertheless, *Odh* does not appear to be diagnostic as reported in Saavedra et al. (1996) because of the different buffer system used in the present study.

DNA analysis

Mantle border tissue from each mussel was used for total DNA extraction following Heath et al. (1995). Two nuclear markers (*Glu-5* and *ITS*) and one mitochondrial DNA marker (*COIII*) were analyzed after PCR amplification. *Glu-5* and *ITS* are co-dominant DNA markers producing two specific *Mytilus edulis* and *M. trossulus* patterns and distinct patterns for hybrids. We applied similar DNA amplification conditions for both *Glu-5* (Rawson et al. 1996) and *ITS* (Heath et al. 1995) markers. Approximately 0.2 μg of total DNA was incubated for 35 cycles at 94 °C for 20 s, 50 °C for 20 s, and 72 °C for 2 min. The initial denaturation period was 3 min (modified from Heath et al. 1995; Rawson et al. 1996). The PCR products were run in a 3% agarose gel (2% Sigma and 1% NuSieve GTG agarose). The amplified product of *Glu-5* was directly resolved in an agarose gel, while the *ITS* PCR product was first digested with *HhaI* restriction endonuclease (Heath et al. 1995; Rawson et al. 1996) before being visualized on agarose gels. The mtDNA marker (*COIII*) is based on an 860-bp fragment of the cytochrome *c* oxidase subunit III gene, and the amplification protocol used was modified from Zouros et al. (1994b). Approximately 0.2 μg total DNA was incubated for 30 cycles at 94 °C for 1 min, 54 °C for 10 s, and 72 °C for 1 min. The initial denaturation period was 2 min and a final extension period was not used. The primer set used (FOR1 and REV1) amplifies the corresponding mtDNA fragment from the F and M types of both species. Different aliquots of the amplification product were digested with *EcoRI* restriction enzyme to identify the various mtDNA genomes in males and females (Zouros et al. 1994b; Saavedra et al. 1996). For those males in which it was not possible to identify the M genome in mantle tissue, the previously frozen gonad was used for total DNA extraction.

Classification of mussels

The two allozyme loci (*Mpi* and *Est-D*) and the two nuclear DNA markers (*Glu-5* and *ITS*) were used to classify the mussels as "pure" *edulis*, "pure" *trossulus* or hybrids (F_1-like, F_2-like, backcross-like). The allozyme alleles *90* and *100* at the *Mpi* locus and allele *100* at the *Est-D* locus (typical alleles for *M. edulis*) and *84*, *94* and *104* at the *Mpi* locus and allele *90* at the *Est-D* locus (typical alleles of *M. trossulus*) were used to classify individuals following Saavedra et al. (1996). The restriction analysis of the *ITS* PCR product with *HhaI* produced species-specific banding patterns. In *M. edulis*, the 1250-bp amplified fragment was cut into two 450-bp fragments and two 180-bp fragments, while in *M. trossulus* the PCR product was cut into two 280-bp fragments, two 180-bp fragments and several < 100-bp fragments. Hybrids showed three bands in the 3% agarose gels (Heath et al. 1995). The *Glu-5* PCR-assay in *M. edulis* produced a single 350-bp band in most individuals, although a few mussels showed a single 380-bp band and others exhibited both bands, while in *M. trossulus* a single 240-bp band was observed. The hybrids produced a banding pattern with a 240-bp band, and another 350-bp band (rarely a 380-bp band instead of 350-bp band or both) (Rawson et al. 1996). Individuals with alleles at four loci belonging to the same taxon were classified as pure species. Hybrids were classified as follows: when an individual was found to be heterozygous for one *edulis* and one *trossulus* allele at all four diagnostic markers, it was considered to be an F_1. When alleles for two of the four loci were from one species, but alleles for the remaining two loci were from the other, the individual was considered an F_2. When alleles at one, two, or three loci belonged to one species, but the fourth locus contained an allele from each species, the individual was classified as a backcross biased towards one or the other species. This classification, based on the four markers, provides reliable information about the identity of any particular pure or hybrid mussel (see Saavedra et al. 1996; Boecklen and Howard 1997).

A species-specific hybrid index was used to study the relationship between genotype and size. The index was calculated according to Sanjuan et al. (1994) using the four markers for the *Mytilus* species. For all markers, each typical *trossulus* allele was given the value −1, each *edulis* allele the value +1 and all other alleles the value 0. The hybrid index value for each individual consists of the sum of the eight values (two alleles for each of four diagnostic markers). The index ranges between −8 (pure *trossulus*) and +8 (pure *edulis*). Individuals with value 0 (but only tetraheterozygotes) are considered as F_1. Those individuals with values between −8 and 0 were classified as *trossulus*-biased backcrosses, and those with values between 0 and +8 were classified as *edulis*-biased backcrosses. All genetic analyses outlined above were performed using BIOSYS-1 (Swofford and Selander 1981) and Zaykin and Pudovkin (1993) computer programs.

Size–frequency analysis

Shell lengths of all sampled mussels were measured to the nearest 0.01 mm using electronic digital calipers. Size classes consisted of 5 mm shell length intervals.

Results

The allele frequencies of the most diagnostic loci (*Est-D* and *Mpi*) varied among the four populations. In contrast to the findings of Saavedra et al. (1996), the *Odh* locus was not diagnostic. The typical *Mytilus trossulus* alleles (*Mpi 94* and *Est-D 90*) reached the highest frequency in the Chance Cove exposed sample (CE). For example, the frequency of the *Mpi 94* allele was 0.390 for Bellevue protected and 0.765 for Chance Cove exposed. For *Est-D*, *Mpi* and *Pgm* loci, all samples showed a significant deficit of heterozygotes (significantly positive *F*-values), which suggests a Wahlund-like effect because it occurs in the enzyme loci where the differences in allele frequencies between pure *M. edulis* and pure *M. trossulus* are larger.

The allele frequencies for all enzyme loci in each genotypic group for each sample are shown in Table 1. In all populations, the hybrid class had intermediate allele frequencies for *Est-D, Mpi,* and *Pgm* loci for those alleles with larger differences in frequency between pure *edulis* and pure *trossulus*, as expected. For example, in Bellevue protected, the allele *Mpi 94* had a frequency of 0.960 and 0.000 in pure *trossulus* and pure *edulis*, respectively, while its frequency in the hybrid class was 0.636. At Bellevue there was no significant difference ($G = 2.38$, $df = 2$, $P > 0.05$) in the occurrence of the two species and hybrids between the protected and exposed sites. At Chance Cove, there was a significant difference ($G = 7.22$, $df = 2$, $P < 0.05$) in the frequency of the two species and hybrids, with *Mytilus trossulus* most frequent (61%) at the exposed site and *M. edulis* most frequent (44%) at the protected site. There was also a significant difference ($G = 30.53$, $df = 2$, $P < 0.001$) in the frequency of both species and hybrids between Chance Cove and Bellevue, pooling samples

Table 1 *Mytilus* spp. Allele frequencies at *Est-D*, *Lap*, *Mpi*, *Odh* and *Pgm* loci for mussels from four locations classified as "pure" *M. edulis*, "pure" *M. trossulus*, or hybrids using four diagnostic markers (*Est-D*, *Mpi*, *ITS*, *Glu-5*) (*N* sample size)

Locus, allele	Bellevue protected			Bellevue exposed			Chance Cove protected			Chance Cove exposed		
	edul	tros	hybr	edul	tros	hybr	edul	tros	hybr	edul	tros	hybr
Est-D												
80	0.010	0.000	0.000	0.000	0.016	0.000	0.000	0.023	0.000	0.000	0.008	0.019
85	0.000	0.000	0.000	0.012	0.016	0.000	0.000	0.000	0.000	0.000	0.000	0.000
90	0.000	0.960	0.586	0.000	0.938	0.759	0.000	0.965	0.607	0.000	0.958	0.635
95	0.019	0.040	0.068	0.000	0.031	0.019	0.018	0.012	0.018	0.000	0.025	0.058
100	0.951	0.000	0.364	0.976	0.000	0.222	0.946	0.000	0.375	0.964	0.000	0.288
105	0.010	0.000	0.000	0.000	0.000	0.000	0.018	0.000	0.000	0.036	0.008	0.000
110	0.010	0.000	0.000	0.012	0.000	0.000	0.018	0.000	0.000	0.000	0.000	0.000
(*N*)	(52)	(25)	(22)	(41)	(32)	(27)	(28)	(43)	(28)	(14)	(59)	(26)
Lap												
90	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.017	0.019
92	0.000	0.060	0.068	0.000	0.016	0.019	0.000	0.119	0.054	0.000	0.076	0.077
94	0.048	0.380	0.432	0.012	0.469	0.352	0.056	0.381	0.286	0.107	0.381	0.250
96	0.250	0.520	0.318	0.268	0.469	0.500	0.278	0.429	0.518	0.250	0.432	0.538
98	0.692	0.020	0.182	0.707	0.047	0.130	0.648	0.060	0.143	0.607	0.093	0.115
100	0.009	0.020	0.000	0.012	0.000	0.000	0.019	0.012	0.000	0.036	0.000	0.000
(*N*)	(52)	(25)	(22)	(41)	(32)	(27)	(27)	(42)	(28)	(14)	(59)	(26)
Mpi												
84	0.000	0.020	0.023	0.000	0.016	0.019	0.000	0.047	0.000	0.000	0.034	0.019
90	0.144	0.000	0.068	0.183	0.000	0.019	0.125	0.000	0.036	0.179	0.000	0.019
94	0.000	0.960	0.636	0.000	0.953	0.704	0.000	0.953	0.768	0.000	0.958	0.750
100	0.856	0.000	0.273	0.817	0.000	0.259	0.875	0.000	0.196	0.821	0.000	0.192
104	0.000	0.020	0.000	0.000	0.031	0.000	0.000	0.000	0.000	0.000	0.008	0.019
(*N*)	(52)	(25)	(22)	(41)	(32)	(27)	(28)	(43)	(28)	(14)	(59)	(26)
Odh												
90	0.000	0.060	0.000	0.037	0.063	0.000	0.089	0.047	0.018	0.000	0.068	0.000
95	0.000	0.000	0.000	0.012	0.000	0.000	0.018	0.000	0.000	0.036	0.000	0.019
100	0.962	0.920	0.886	0.878	0.891	0.889	0.750	0.930	0.893	0.857	0.881	0.942
105	0.010	0.000	0.000	0.000	0.031	0.056	0.000	0.000	0.018	0.000	0.008	0.019
110	0.029	0.020	0.114	0.073	0.016	0.056	0.143	0.023	0.071	0.071	0.042	0.019
120	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.036	0.000	0.000
(*N*)	(52)	(25)	(22)	(41)	(32)	(27)	(28)	(42)	(28)	(14)	(59)	(26)
Pgm												
90	0.000	0.000	0.000	0.012	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
93	0.077	0.020	0.000	0.098	0.031	0.000	0.036	0.000	0.018	0.000	0.000	0.019
100	0.808	0.020	0.273	0.780	0.094	0.296	0.929	0.058	0.214	0.857	0.042	0.173
106	0.115	0.300	0.227	0.098	0.234	0.203	0.036	0.128	0.232	0.143	0.263	0.212
108	0.000	0.060	0.000	0.000	0.000	0.019	0.000	0.012	0.018	0.000	0.008	0.000
111	0.000	0.580	0.455	0.012	0.625	0.426	0.000	0.686	0.464	0.000	0.576	0.538
114	0.000	0.020	0.045	0.000	0.016	0.056	0.000	0.105	0.054	0.000	0.093	0.038
118	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.012	0.000	0.000	0.017	0.019
(*N*)	(52)	(25)	(22)	(41)	(32)	(27)	(28)	(43)	(28)	(14)	(59)	(26)

from exposed and protected sites. *M. trossulus* was most frequent (53%) at Chance Cove, *M. edulis* was most frequent (46%) at Bellevue and the frequency of hybrids was similar (26%, 24%) at both locations.

The distribution of mtDNA mitotypes among pure *Mytilus edulis* and *M. trossulus* individuals from all sites combined are shown in Table 2. Mussels were first classified as pure forms or hybrids based on *Mpi* and *Est-D* to be comparable to the results reported by Saavedra et al. (1996), and then, using both allozyme loci and the *ITS* and *Glu-5* DNA markers (numbers in parentheses in tables). The mussels were then characterized by one or two mitotype symbols (F-ed, F-tr, M-ed, M-tr) (Stewart et al. 1995) if they were found to be homoplasmic or heteroplasmic, respectively. The sex ratios (males:females) were 1:1.23 (*M. edulis*) and 1:1.26 (*M. trossulus*) when classification was based on the four markers (*ITS*, *Glu-5*, *Mpi* and *Est-D*). All *M. edulis* and *M. trossulus* females were found to be homoplasmic (F-ed or F-tr, respectively) (Table 2). All *M. edulis* males were heteroplasmic for the F and M *edulis* genome combination (F-ed/M-ed), however, two individuals (classified using the two allozyme loci) showed a heterospecific combination of F *edulis* genome and M *trossulus* genome (F-ed/M-tr). One of these heterospecific males was hybrid for both *ITS* and *Glu-5* nuclear-DNA markers, while the other was hybrid only for the *ITS* marker (Table 2). Both individuals were then reclassified as hybrids instead of pure forms when classification was based on four diagnostic markers. All 84

Table 2 *Mytilus* spp. Distribution of mtDNA genotypes by sex among "pure" *M. edulis* and *M. trossulus* as established by two allozyme loci (*Mpi*, *Est*) or as established by the two allozyme loci and the two nuclear-DNA markers (in parentheses), pooling four populations (discussion of mitotype symbols, see "Results")

Mitotype	Pure *M. edulis*		Pure *M. trossulus*	
	female	male	female	male
F-ed	58 (56)	–	–	–
F-tr	–	–	84 (69)	–
F-ed/M-ed	–	71 (69)	–	1[b] (0)
F-ed/M-tr	–	2[a] (0)	–	1[c] (0)
F-tr/M-tr	–	–	–	64 (52)
M-trO/F-tr	–	–	–	42 (35)
Total	58 (56)	73 (69)	84 (69)	108 (87)

[a] From the two "pure" *M. edulis* males that showed the heterospecific combination, one was hybrid for *ITS* and the other was hybrid for both nuclear-DNA markers
[b] This individual was *edulis* for both nuclear-DNA markers
[c] This individual was hybrid for the *ITS* nuclear-DNA marker

pure *M. trossulus* females classified according to two allozyme loci were homoplasmic for the F-tr mitotype. However, using the *ITS* and *Glu-5* nuclear-DNA markers, three individuals were hybrids for *ITS*, ten were hybrid for *Glu-5*, one was hybrid for both *ITS* and *Glu-5*, and two were *edulis* for the *Glu-5* marker. Therefore, using the four markers, 15 individuals were reclassified as hybrids. From the 108 pure *trossulus* males (based on two allozyme loci), 64 were heteroplasmic for the F-tr/M-tr combination; another 42 *M. trossulus* males showed a mitotype of only one 860-bp band for *EcoRI* and were classified as M-trO according to Saavedra et al. (1996). One individual showed the heteroplasmic combination of F and M *edulis* genomes (F-ed/M-ed), and was also found to be *edulis* for both *ITS* and *Glu-5* nuclear-DNA markers; this individual was reclassified as an F_2 hybrid using the four diagnostic markers (*M. trossulus* for *Mpi* and *Est-D* and *M. edulis* for *ITS* and *Glu-5*). Another individual showed the heterospecific combination F-ed/M-tr and was found to be one of the three individuals classified as hybrids by the *ITS* marker. With the use of four diagnostic markers, no pure *M. edulis* or *M. trossulus* contained F or M mtDNA from the other species. Nevertheless, if the classification were based only on two allozyme loci, four male individuals would be identified as "pure" species having a heterospecific nuclear- and mtDNA combination.

The distribution of mtDNA mitotypes among the hybrid individuals from all the samples is shown in Table 3. Of the three F_1-like females (based on two diagnostic allozyme loci), one had a pattern that could be an M-ed/F-ed homospecific combination or a heterospecific combination (type F-ed/M-trO) (see Saavedra et al. 1996). This female had the *edulis* pattern for both *ITS* and *Glu-5* markers. Another two (one with F-ed and one with F-tr mitochondrial mitotypes) were *edulis* and *trossulus* for *ITS* and *Glu-5* nuclear markers, respectively. Thus, according to the four markers, these females were considered as backcrosses. All five males classified as F_1 hybrids using *Mpi* and *Est-D* were also hybrids for *ITS* and *Glu-5* markers. Two of them had a clear heterospecific combination of F and M genomes (F-ed/M-tr, see Saavedra et al. 1996) whereas the other had only one 860-bp band for *EcoRI*. This pattern could be considered as heterospecific, combining the *trossulus* F genome and the *edulis* M genome, a pattern with only one 860-bp band (Saavedra et al. 1996). Thus among all mussels sampled (400 individuals) only five could be considered as true F_1 hybrids based on four diagnostic loci and one mtDNA marker. Among males considered F_2-like and backcrosses to *edulis* and *trossulus,* only five had a heterospecific M and F mtDNA genome combination. There was one backcross to *trossulus* that had the homospecific F and M *edulis* mtDNA genome combination. The remaining F_2 and backcross individuals had the same species combination of mitochondrial and nuclear DNA. Nevertheless, it is worth noting the significant increase in the number of *trossulus*-biased

Table 3 *Mytilus* spp. hybrids. Distribution of mtDNA genotypes by sex, based on *Mpi* and *Est-D* or using four diagnostic markers (in parentheses) for four populations pooled (*Bc-tr*, *trossulus*-biased backcross; *Bc-ed*, *edulis*-biased backcross)

mtDNA genotype	F_1-like		F_2-like		Bc-tr		Bc-ed	
	female	male	female	male	female	male	female	male
F-ed	1 (0)	–	–	–	–	–	12 (14)	–
F-tr	1 (0)	–	2 (0)	–	16 (36)	–	–	–
F-ed/M-ed	–	–	–	0 (1)	–	2[b] (1)	–	7 (8)
F-ed/M-tr	–	2 (2)	–	–	–	1 (2)	–	0 (2)
F-ed/M-trO	1[c] (0)	–	–	–	–	–	0 (1)	1[a] (1)
F-tr/M-ed	–	3 (3)	–	–	–	–	–	–
F-tr/M-tr	–	–	–	–	–	3 (15)	–	–
M-trO	–	–	–	1 (0)	–	6 (14)	–	–
Total	3 (0)	5 (5)	2 (0)	1 (1)	16 (36)	12 (32)	12 (15)	8 (11)

[a] This individual could be M-trO and not M-ed because it is hybrid for the *ITS* and *Glu-5* nuclear-DNA markers
[b] One individual is a male, hybrid for *ITS*, *trossulus* for *Glu-5* and M-ed/F-ed, backcross *trossulus*-biased; the other is a male, *edulis* for both nuclear-DNA markers, M-ed/F-ed and backcross *trossulus*-biased
[c] This individual is *edulis* for both nuclear-DNA markers

backcrosses when classification of individuals was based on four diagnostic markers rather than two (Table 3). Most of these came from individuals classified as pure *trossulus* according to the two allozyme loci.

Among males identified as backcrosses or F_2, an equal number of heterospecific and homospecific M and F mtDNA types is expected (Saavedra et al. 1996). Of the 44 backcross and F_2 males identified, only five were heterospecific for F and M mtDNA ($\chi^2 = 26.3$, $df = 1$, $p < 0.001$). Backcross females have a 0.25 chance of having a mitotype different from the majority of their nuclear genes (Saavedra et al. 1996). Of the 51 backcross females identified, 12.5 were expected to have a discordance between mtDNA and the majority of their nuclear genes but none were observed ($\chi^2 = 8.33$, $df = 1$, $p = < 0.005$). A similar analysis for 24 backcross males carrying homospecific F and M mitotypes found one discordance where six were expected ($\chi^2 = 2.78$, $df = 1$, $p > 0.05$).

The relationship between frequency of the *Mytilus edulis*, *M. trossulus*, and hybrid individuals (based on four markers) and shell length from the four populations is shown in Fig. 2. *M. trossulus* was the predominant species in the smallest size classes for all samples. At the Bellevue site *M. trossulus* was most frequent only in the smallest size categories (between 15 and 26 mm), whereas it was the predominant species in almost all size classes at Chance Cove. An increase in frequency of *M. edulis* individuals and a decrease in frequency of *M. trossulus* individuals with shell length was observed in both environments at both Bellevue and Chance Cove. The change in frequency of both species was gradual with increasing size, except at the Bellevue exposed site in which there was an abrupt change in the frequencies between the 26 to 30 mm and 31 to 35 mm size classes (Fig. 2). The frequency of hybrids tended to decrease in the larger size classes, although no clear pattern was observed.

The relationship between the hybrid index values and shell length for all individuals in four pooled samples is shown in Fig. 3. Pure *M. trossulus* individuals (hybrid index value −8) were represented in small size classes, as were all the *trossulus*-biased backcrosses (values between −7 and 0). Pure *M. edulis* individuals (value of +8) and *edulis*-biased backcrosses (values between 0 and 7) were distributed in the whole size range, and pure *M. edulis* dominated the largest size classes. When this size range was divided in two parts (from 15 to 35 mm and from 36 to 80 mm), there was a significantly different ($G = 41.51$, $df = 1$, $P < 0.001$) distribution of the number of *edulis*-biased backcrosses (values between 0 and 8) and *trossulus*-biased backcrosses (values between −8 and 0). Most of the backcross individuals (80%) were grouped in the smallest size classes (< 35 mm), and of these 88% were *trossulus*-biased backcrosses. In the largest size classes (> 35 mm), 89% of the backcrosses were *edulis*-biased. The six dots with a value of zero in the hybrid index corresponded to five F_1 individuals and one F_2.

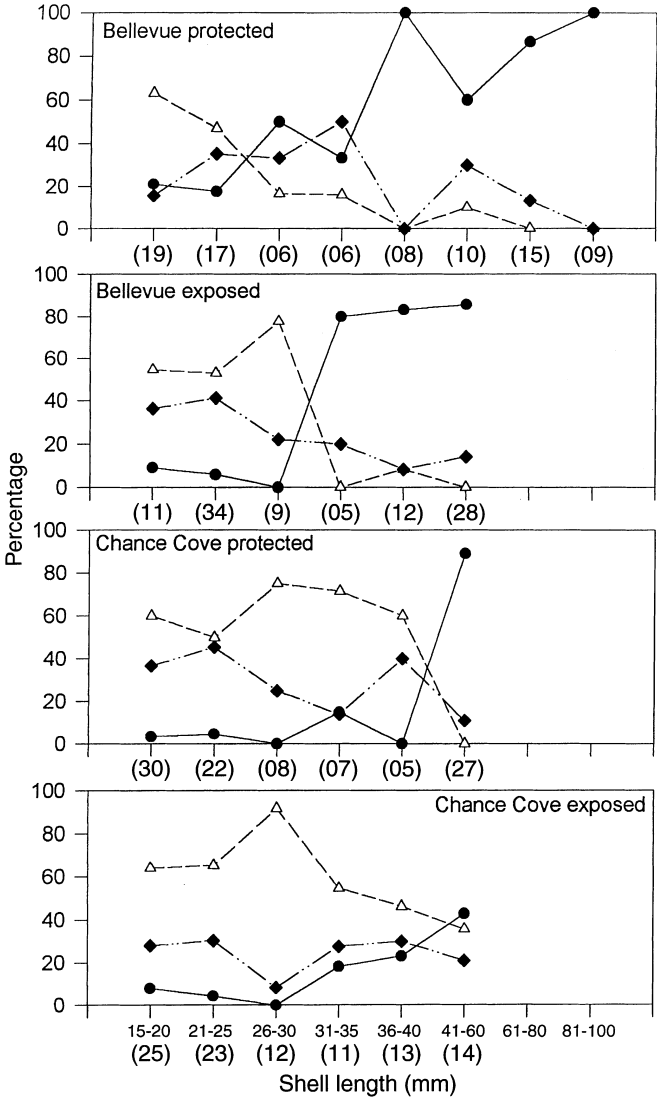

Fig. 2 *Mytilus* spp. Frequencies of putative *M. edulis* (●), *M. trossulus* (△) and hybrids (◆) plotted against shell length classes for four populations in eastern Newfoundland. Number of individuals in each length class in parentheses

Discussion

Hybrid zones provide unique opportunities to study evolutionary processes that maintain reproductive isolation between species (Barton and Hewitt 1985; Harrison 1990). Hybrid zones between pairs of species within the *Mytilus edulis* species complex (*M. edulis*, *M. trossulus,* and *M. galloprovincialis*) have received increasing attention, particularly after the recent discovery of double uniparental inheritance (DUI) of the mitochondrial genome and the occurrence of separate maternal and paternal mtDNA lineages (Zouros et al. 1992, 1994a, b; Skibinski et al. 1994; Stewart et al. 1995; Saavedra et al. 1996). DUI adds an additional layer of complexity to assessing the fitness of hybrid individuals in which fitness may be reduced due to incompatible mixtures of genetic

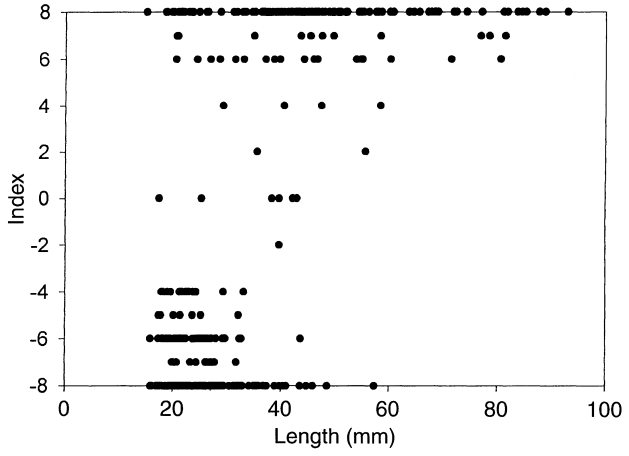

Fig. 3 *Mytilus* spp. Species-specific hybrid index values for all individuals (four populations pooled) plotted against shell length. Individuals with a value −8 were regarded as *M. trossulus*, those with a value +8 *M. edulis* and those with a value 0 (and tetraheterozygotes) as F_1. Individuals with values from −8 to 0 and 0 to 8 were regarded as *trossulus*-biased backcrosses and *edulis*-biased backcrosses, respectively

material from two species in the nuclear genome, between F and M mitotypes from different species in males, and incompatibility between mitochondrial and nuclear genomes derived from different species in both sexes. A critical requirement for such studies is the ability to identify individual species and their hybrids. Boecklen and Howard (1997) examined the relationship between the number of genetic markers and the error in discriminating among pure species, F_1, and backcross individuals within a hybrid zone. They concluded that as few as four markers were useful for most applications, with about 5% of backcross individuals being misclassified as either F_1 or pure species. In the present study, the addition of the two DNA markers to the two enzyme markers resulted in about 13% of those individuals initially assigned to pure species being reclassified as hybrids. The largest change was a reclassification of pure *M. trossulus* individuals to *trossulus*-biased backcrossed individuals. Based on the four diagnostic markers, the Bellevue/Chance Cove area had a greater frequency of *M. trossulus* (41%) than of *M. edulis* (33%), with about 26% of individuals of hybrid origin. This compares with a value of 23% hybrids between *M. edulis* and *M. trossulus* detected by Saavedra et al. (1996) for a site in Nova Scotia, which may, however, be an underestimate since only two diagnostic markers were used. In these areas of Atlantic Canada, hybridization and introgression appear to be much lower than between *M. edulis* and *M. galloprovincialis* at various locations in Europe. For instance, Sanjuan et al. (1994) reported 25 to 50% hybrids, Hilbish et al. (1994) about 80%, and Comesaña and Sanjuan (1997) 27 to 49% hybrids (see also Coustau et al. 1991; Gardner 1994). Low levels of hybridization have also been reported for *M. trossulus* and *M. galloprovincialis* on the Pacific coast of North America (about 5.7% according to Sarver and Foltz 1993; 4 to 29% according to Suchanek et al. 1997). However, it is difficult to make general comparisons between studies using different numbers of markers which vary in their power to discriminate between pure species and hybrids, and the different degree of hybridization between locations even in the same areas.

Gardner (1996) proposed that species of *Mytilus* may maintain their integrity, despite hybridization, through adaptation to different environments. He noted that the areas of hybridization among pairs of *Mytilus* species occur at ecotones between major marine biogeographical provinces where environmental variation may allow the parental species and their hybrids to coexist in different microhabitats. Some information is available on the differential distribution of mussel species in relation to environmental conditions. For example, *M. galloprovincialis* and *M. edulis* in Europe appear to have different distributions related to the degree of wave exposure and the level of attachment in the intertidal zone (Gosling 1992; Gardner 1994; Comesaña and Sanjuan 1997). *M. galloprovincialis* is more frequent on exposed environments and higher up the shore compared with *M. edulis*, probably because of its increased strength of attachment to the substrate (Gardner and Skibinski 1991; Willis and Skibinski 1992; Gardner et al. 1993). In addition, Hilbish et al. (1994) showed that the two species have different physiological responses to higher temperatures. No significant difference in the effect of wave exposure or height in the intertidal zone was observed between *M. galloprovincialis* and *M. trossulus* in California, but the distribution of the two species did differ as a function of the temperature and salinity regime (Sarver and Foltz 1993). Bates and Innes (1995) sampled mussels from the intertidal zone and found the highest frequency of *M. trossulus* at two wave-exposed sites and a higher frequency of *M. edulis* at several sheltered sites. In the present study, there was no consistent pattern in the distribution of *M. trossulus* or *M. edulis* according to wave exposure. Although *M. trossulus* was more common at the exposed Chance Cove site, no difference in the relative frequency of the two species was found between exposed and sheltered sites at Bellevue. All samples were collected subtidally, which may decrease the likelihood of detecting any differences due to wave exposure. Furthermore, the observed differences between the two environments at Chance Cove may simply reflect the high degree of microgeographic variation in the occurrence of these species observed in a previous study (Bates and Innes 1995). Further sampling across a larger number of sheltered and exposed and intertidal sites would be required in order to establish the importance of wave exposure for determining the distribution of each species.

None of the 281 individuals classified as pure species, based on four markers, contained mtDNA from the other species. This observation is consistent with the data of Saavedra et al. (1996) for a hybrid population of *Mytilus edulis* and *M. trossulus* in Nova Scotia. The early block to the introgression of mtDNA between these species was also detected when only two markers

were used, since only four males that were misclassified as members of a pure species contained mixtures of nuclear and mitochondrial genomes from each species. Furthermore, no F_1 hybrid females were detected and only five males were identified as F_1 hybrids, having heterozygous genotypes for the four nuclear markers and heterospecific mitotypes. Two of the F_1 hybrids had an *M. edulis* mother and an *M. trossulus* father, and three came from reciprocal matings. Only one F_2 hybrid was detected, probably due to the low frequency of matings expected among the very rare F_1 hybrids. Most of the hybrid individuals (94/100) were classified as backcrosses. This would be expected if only a few F_1 hybrids survive to reproduce and suggests that backcrossed individuals have a greater survival than F_1 individuals. *M. trossulus*-biased backcrosses were more than twice as common as *M. edulis*-biased backcrosses. This probably reflects the greater frequency of pure *M. trossulus* in this area. Among the backcross individuals, males with heterospecific mitotypes and species-specific discordances between mitotype and the nuclear genome of both sexes occurred less frequently than would be expected if there were equal fertility and survival among all classes. These observations are consistent with those of Saavedra et al. (1996) who found approximately equal frequencies of both *M. edulis*-biased and *M. trossulus*-biased backcrosses in a sample, again probably a function of the approximately equal frequencies of the two species in their sample.

Samples from coexisting populations of *Mytilus galloprovincialis* and *M. edulis* showed a consistent pattern, where *M. galloprovincialis* was more frequent among larger individuals and *M. edulis* more frequent among smaller individuals (Gardner and Skibinski 1988; Gardner et al. 1993). Data collected on size at given age showed that *M. galloprovincialis* had a slightly higher growth rate and a greater survivorship than *M. edulis*. Higher growth and survival of *M. edulis* compared with *M. trossulus* may explain the increase in the frequency of *M. edulis* with increasing shell length observed in the Newfoundland populations. Mallet and Carver (1995) recorded only small differences in growth rate between *M. trossulus* and *M. edulis*, suggesting that the observed changes in species frequency with shell length may reflect differential survival. The various size classes include different cohorts of larvae settling in the area over several years. The observed increase in frequency of *M. edulis* with increasing shell length may simply reflect a much higher frequency of *M. edulis* in the oldest cohort at the time of settlement. Although the frequency of each species in the newly settled spat may vary from year to year and from site to site, the consistency of the pattern in each of the four samples, the distribution of *M. edulis* and *edulis*-biased backcrosses in the whole size range (Fig. 3), and the regular occurrence of both species in this area (Koehn et al. 1984; Bates and Innes 1995) do not support this explanation. Bates (1992) also found a higher frequency of *M. edulis* in the larger size classes and a higher frequency of *M. trossulus* in the smaller size classes at a different site in eastern Newfoundland. The environmental factor or factors responsible for viability differences between the two mussel species remain to be determined. Interestingly, backcross individuals with a nuclear gene composition biased towards one species or the other supported the trends observed in the pure species.

Factors responsible for blocking the introgression of mtDNA between *Mytilus edulis* and *M. trossulus* in Atlantic Canada do not appear to be operating as strongly in the hybrid zones of European *Mytilus* species. Populations of both *M. trossulus* and *M. galloprovincialis* contain individuals with *M. edulis* mtDNA (Rawson and Hilbish 1998). The asymmetric introgression of mtDNA between *M. edulis* and *M. galloprovincialis* was explained by previous observations of directional selection in the hybrid zone favoring alleles of *M. galloprovincialis* (Gardner and Skibinski 1988; Skibinski and Roderick 1991; Gardner et al. 1993). Thus hybrid individuals with *M. edulis* mtDNA and a predominance of *M. galloprovincialis* nuclear alleles would be favored over hybrid individuals with *M. galloprovincialis* mtDNA and a predominance of *M. edulis* nuclear alleles. This mechanism might predict that any introgression of mtDNA in Atlantic Canada mussel populations would be in the direction of *M. trossulus* into *M. edulis*. Moreover, in small individuals we would expect a greater number of backcrosses with a *trossulus* nuclear genome and an *edulis* mtDNA genome (introgression in the direction of *M. edulis* into *M. trossulus*). Thus, it seems that the incompatibility between mitochondrial and nuclear genomes is greater among *M. trossulus* and *M. edulis* than *M. edulis* and *M. galloprovincialis*. The greater degree of mtDNA introgression observed in Europe than in North America may be due to a longer period of contact between these *Mytilus* species compared with the Atlantic Canada *M. edulis* and *M. trossulus*, or simply to a different degree to which natural selection is acting against each species on each continent. In addition, the different degree of divergence between the *Mytilus* species results in a different relationship between mitochondrial DNA and nuclear genomes for each *Mytilus* taxon. Further studies are required of the different life history stages of both *M. trossulus* and *M. edulis* in relation to the environment to identify the factors responsible for maintaining the integrity of each species.

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References

Barton NH, Hewitt GM (1985) Analysis of hybrid zones. A Rev Ecol Syst 16: 113–148

Bates JA (1992) Genetic and morphological variation in the mussel *Mytilus* in Newfoundland. M.Sc. thesis, Memorial University of Newfoundland, St. John's

Bates JA, Innes DJ (1995) Genetic variation among populations of *Mytilus* spp. in eastern Newfoundland. Mar Biol 124: 417–424

Boecklen WJ, Howard DJ (1997) Genetic analysis of hybrid zones: numbers of markers and power of resolution. Ecology 78: 2611–2616

Comesaña AS, Sanjuan A (1997) Microgeographic allozyme differentiation in the hybrid zone of *Mytilus galloprovincialis* Lmk. and *M. edulis* L. on the continental European coast. Helgoländer Meeresunters 51: 107–124

Coustau C, Renaud F, Delay B (1991) Genetic characterization of the hybridization between *Mytilus edulis* and *Mytilus galloprovincialis* on the Atlantic coast of France. Mar Biol 111: 87–93

Gardner JPA (1994) The structure and dynamics of naturally occurring hybrid *Mytilus edulis* Linnaeus, 1758 and *Mytilus galloprovincialis* Lamarck, 1819 (Bivalvia: Mollusca) populations: review and interpretation. Arch Hydrobiol 99: 37–71

Gardner JPA (1996) The *Mytilus edulis* species complex in southwest England: effects of hybridization and introgression upon interlocus associations and morphometric variation. Mar Biol 125: 385–399

Gardner JPA, Skibinski DOF (1988) Historical and size-dependent genetic variation in hybrid mussel populations. Heredity 61: 93–105

Gardner JPA, Skibinski DOF (1990) Genotype-dependent fecundity and temporal variation of spawning in hybrid mussel (*Mytilus*) populations. Mar Biol 105: 153–162

Gardner JPA, Skibinski DOF (1991) Biological and physical factors influencing genotype-dependent mortality in hybrid mussel populations. Mar Ecol Prog Ser 71: 235–243

Gardner JPA, Skibinski DOF, Bajdik CD (1993) Shell growth and viability differences between the marine mussels *Mytilus edulis* (L.), *Mytilus galloprovincialis* (Lmk.) and their hybrids from two sympatric populations in S.W. England. Biol Bull mar biol Lab, Woods Hole 185: 405–416

Gartner-Kepkay KE, Dickie LM, Freeman KR (1980) Genetic differences and environments of mussel populations in Maritime Provinces. Can J Fish aquat Sciences 37: 775–782

Gosling EM (1992) Genetics of *Mytilus*. In: Gosling E (ed) The mussel *Mytilus*: ecology, physiology, genetics and culture. Elsevier, Amsterdam, pp 309–382

Harrison RG (1990) Hybrid zones: windows on evolutionary process. Oxf Surv evolut Biol 7: 69–128

Heath DD, Rawson PD, Hilbish TJ (1995) PCR-based nuclear markers identify alien blue mussel (*Mytilus* spp.) genotypes on the west coast of Canada. Can J Fish aquat Sciences 52: 2621–2627

Hilbish TJ, Bayne BL, Day A (1994) Genetics of physiological differentiation within the marine mussel genus *Mytilus*. Evolution 48: 267–286

Koehn RK, Hall JG, Innes DJ, Zera AJ (1984) Genetic differentiation of *Mytilus edulis* in eastern North America. Mar Biol 79: 117–126

Koehn RK, Milkman R, Mitton JB (1976) Population genetics of marine pelecypods. IV. Selection, migration and genetic differentiation in the blue mussel *Mytilus edulis*. Evolution 30: 2–32

Mallet AL, Carver CE (1995) Comparative growth and survival patterns of *Mytilus trossulus* and *Mytilus edulis* in Atlantic Canada. Can J Fish aquat Sciences 52: 1873–1880

McDonald JH, Koehn RK (1988) The mussels *Mytilus galloprovincialis* and *M. trossulus* on the Pacific Coast of North America. Mar Biol 99: 111–118

McDonald JH, Seed R, Koehn RK (1991) Allozymes and morphometric characters of three species of *Mytilus* in the northern and southern hemispheres. Mar Biol 111: 323–333

Rawson PD, Hilbish TJ (1998) Asymmetric introgression of mitochondrial DNA among European populations of blue mussels (*Mytilus* spp.). Evolution 52: 100–108

Rawson PD, Joyner KL, Meetze K, Hilbish TJ (1996) Evidence for intragenic recombination within a novel genetic marker that distinguishes mussels in the *Mytilus edulis* species complex. Heredity 77: 599–607

Saavedra C, Stewart DT, Stanwood RR, Zouros E (1996) Species-specific segregation of gender-associated mitochondrial DNA types in an area where two mussel species (*Mytilus edulis* and *M. trossulus*) hybridize. Genetics 143: 1359–1367

Sanjuan A, Zapata C, Alvarez G (1994) *Mytilus galloprovincialis* and *M. edulis* on the coast of the Iberian peninsula. Mar Ecol Prog Ser 113: 131–146

Sarver SK, Foltz DW (1993) Genetic population structure of a species' complex of blue mussels (*Mytilus* spp.). Mar Biol 117: 105–112

Skibinski DOF, Ahmad M, Beardmore JA (1978) Genetic evidence for naturally occurring hybrids between *Mytilus edulis* and *Mytilus galloprovincialis*. Evolution 32: 354–364

Skibinski DOF, Gallagher C, Beynon CM (1994) Mitochondrial DNA inheritance. Nature 368: 817–818

Skibinski DOF, Roderick EE (1991) Evidence of selective mortality in favour of the *Mytilus galloprovincialis* Lmk phenotype in British mussel populations. Biol J Linn Soc 42: 351–366

Steward DT, Saavedra C, Stanwood RR, Ball AO, Zouros E (1995) Male and female mitochondrial DNA lineages in the blue mussel (*Mytilus edulis*) species group. Molec Biol Evolut 12: 735–747

Suchanek TH, Geller JB, Kreiser BR, Mitton JB (1997) Zoogeographic distributions of the sibling species *Mytilus galloprovincialis* and *M. trosssulus* (Bivalvia: Mytilidae) and their hybrids in the North Pacific. Biol Bull mar biol Lab, Woods Hole 193: 187–194

Swofford DL, Selander RB (1981) BIOSYS-1, FORTRAN program for the comprehensive analysis of electrophoretic data in population genetics and systematics. J Hered 72: 281–283

Varvio SL, Koehn RK, Väinölä R (1988) Evolutionary genetics of the *Mytilus edulis* complex in the North Atlantic region. Mar Biol 98: 51–60

Väinölä R, Hvilson MM (1991) Genetic divergence and a hybrid zone between Baltic and North Sea *Mytilus* populations (Mytilidae: Mollusca) Biol J Linn Soc 43: 127–148

Willis GL, Skibinski DOF (1992) Variation in strength of attachment to the substrate explains differential mortality in hybrid mussel (*Mytilus galloprovincialis* and *Mytilus edulis*) populations. Mar Biol 112: 403–408

Zaykin DV, Pudovkin AI (1993) Two programs to estimate chi-square values using pseudo-probability test. J Hered 84: p 152

Zouros E, Ball AO, Saavedra C, Freeman KR (1994a) An unusual type of mitochondrial DNA inheritance in the blue mussel *Mytilus*. Proc natn Acad Sci 91: 7463–7467

Zouros E, Ball AO, Saavedra C, Freeman KR (1994b) Mitochondrial DNA inheritance. Nature 368: p 818

Zouros E, Freeman KR, Ball A, Pogson GH (1992) Direct evidence for extensive paternal mitochondrial DNA inheritance in the marine mussel *Mytilus*. Nature 359: 412–414