The Effects of Natural Hybridization on the Regulation of Doubly Uniparental mtDNA Inheritance in Blue Mussels (*Mytilus* spp.)

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Manuscript received January 28, 1996
Accepted for publication May 30, 1996

ABSTRACT

Blue mussels in the *Mytilus edulis* species complex have a doubly uniparental mode of mtDNA inheritance with separate maternal and paternal mtDNA lineages. Female mussels inherit their mtDNA solely from their mother, while males inherit mtDNA from both parents. In the male gonad the paternal mtDNA is preferentially replicated so that only paternal mtDNA is transmitted from fathers to sons. Hybridization is common among differentiated blue mussel taxa; whenever it involves *M. trossulus*, doubly uniparental mtDNA inheritance is disrupted. We have found high frequencies of males without and females with paternal mtDNA among hybrid mussels produced by interspecific matings between *M. galloprovincialis* and *M. trossulus*. In contrast, hybridization between *M. galloprovincialis* and *M. edulis* does not affect doubly uniparental inheritance, indicating a difference in the divergence of the mechanisms regulating mtDNA inheritance among the three blue mussel taxa. Our data indicate a high frequency of disrupted mtDNA transmission in F_1 hybrids and suggest that two separate mechanisms, one regulating the transmission of paternal mtDNA to males and another inhibiting the establishment of paternal mtDNA in females, act to regulate doubly uniparental inheritance. We propose a model for the regulation of doubly uniparental inheritance that is consistent with these observations.

MARINE mussels in the genus Mytilus have an unusual form of mitochondrial DNA (mtDNA) inheritance in which there are independently transmitted female and male mitochondrial genomes. This mode of inheritance has been called both sex-limited mitochondrial inheritance (SKIBINSKI *et al.* 1994) and doubly uniparental mitochondrial inheritance (ZOUROS *et al.* 1994). As with most animals, female mussels inherit mtDNA from their mother, which they transmit to both their daughters and sons. Male mussels, however, inherit mtDNA from both parents and, because the paternally inherited mtDNA is preferentially amplified during the development of the gonad, they transmit only paternal mtDNA to their sons. The origin of doubly uniparental inheritance predates speciation in the genus Mytilus (RAWSON and HILBISH 1995a; STEWART *et al.* 1995), and the sequence divergence between the male and female mtDNA genomes is quite high (FISHER and SKIBINSKI 1990; HOEH *et al.* 1991; RAWSON and HILBISH 1995a; STEWART *et al.* 1995). Doubly uniparental inheritance has also been observed in other bivalve taxa, including *Guekensia demissa* and freshwater unionid mussels (LUI and MITTON 1995; W. R. HOEH and D. T. STEWART, personal communication). At present, however, it is not clear whether doubly uniparental inheritance arose independently within these different bivalve taxa or is an ancestral character that has been lost in other bivalve taxa.

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The doubly uniparental inheritance of mtDNA occurs in allopatric populations of all three species of blue mussels belonging to the *M. edulis* species group, *M. edulis*, *M. galloprovincialis* and *M. trossulus* (SKIBINSKI *et al.* 1994; ZOUROS *et al.* 1994; RAWSON and HILBISH 1995a). Recently, ZOUROS *et al.* (1994) have demonstrated that within interspecific crosses of *M. edulis* and *M. trossulus* the doubly uniparental mode of inheritance is disrupted. This breakdown of doubly uniparental inheritance suggests an incompatibility between introgressing nuclear and mitochondrial genomes within hybrid mussels. In the Northern Hemisphere there are several regions where natural hybridization between blue mussel species has been reported (MCDONALD *et al.* 1991; GOSLING 1992a). If doubly uniparental mtDNA inheritance is disrupted by natural hybridization, then this failure may affect the level of nuclear and mitochondrial DNA introgression among blue mussel species.

To determine whether disrupted mitochondrial inheritance is important in maintaining genetic differentiation among blue mussels, we first set out to assess whether doubly uniparental mitochondrial inheritance is disrupted within two hybrid mussel populations, one in San Francisco Bay, CA and the other in Whitsand Bay, UK. In the former population, hybrids are formed from interspecific matings between *M. trossulus* and *M. galloprovincialis*, while in the latter they are produced by matings bewteen *M. edulis* and *M. galloprovincialis*. Data from both the male and female mtDNA lineages (RAWSON and HILBISH 1995a) and from allozyme comparisons indicate that *M. trossulus* is more distantly related to *M. edulis* and *M. galloprovincialis* than the latter

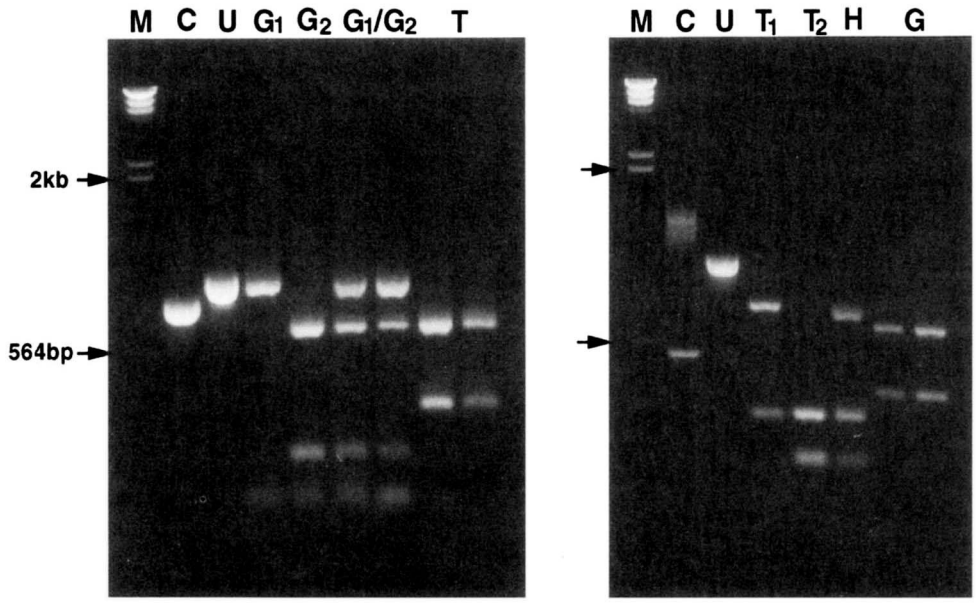

FIGURE 1.—Three PCR-based nuclear markers used to differentiate *M. galloprovincialis* from *M. trossulus*. (A) PCR products for the ITS marker digested with the restriction enzyme *Sau96*I: M, size marker, lambda DNA cut with *Hin*dIII; C, uncut *M. californianus;* U, uncut *M. trossulus;* G_1, G_2 and T, *M. galloprovincialis* and *M. trossulus* restriction fragment patterns, repectively. (B) PCR products for the MAL-I marker digested with the restriction enzyme *Spe*I: M, size marker, lambda DNA cut with *Hin*dIII; C, uncut *M. californianus;* U, uncut *M. trossulus;* T_1, T_2 and G, *M. trossulus* and *M. galloprovincialis* restriction fragment patterns, repectively; H, an individual heterozygous for T_1 and T_2.

two are from each other (SKIBINSKI *et al.* 1980; VÄINÖLÄ and HVILSOM 1991). Thus, we are also particularly interested in examining the role of phylogenetic distance on the disruption of doubly uniparental inheritance in hybrid mussel populations.

MATERIALS AND METHODS

San Francisco Bay: The mussels from San Francisco Bay were genotyped using three PCR-based nuclear markers that we have developed. The first marker, GLU-5′, targets the gene encoding the polyphenolic adhesive protein used by mussels to attach to the substrate (WAITE 1992). The development of this marker has been described by RAWSON *et al.* (1996). Briefly, we have designed oligonucleotide primers that anneal in the 5′ coding region of the "giant exon" that encodes the last 875 amino acid residues of the protein. These primers amplify species-specific primary bands of 300 and 500 bp in *M. galloprovincialis* and 240 bp in *M. trossulus* (RAWSON *et al.* 1996).

The second marker targets the internal transcribed spacer (ITS) regions of the nuclear ribosomal RNA genes. The development and application of this marker is described by HEATH *et al.* (1995). We used the same PCR protocol as HEATH *et al.* (1995) but replaced the restriction enzyme *Hha*I with *Sau96*I. This assay produces species-specific restriction fragment patterns that readily differentiate *M. trossulus* from *M. galloprovincialis* on low density (1.5%) agarose gels (Figure 1A). The ITS PCR products from *M. galloprovincialis* mussels are cut by *Sau96*I to produce two distinct restriction fragment profiles (G_1 and G_2). Most *M. galloprovincialis* individuals carry both types of ITS sequence and produce restriction fragments like those labeled G_1/G_2. In contrast, *M. trossulus* mussels only produce a single ITS restriction profile (T).

The third marker targets an anonymous coding locus that we have designated as Mytilus Anonymous Locus-I (MAL-I). A single random clone 350 bp in length was selected from an *M. edulis* cDNA library and sequenced. A search of GenBank (rel. 91.0; DEVEREUX *et al.* 1984) did not reveal any known related sequences for this stretch of coding sequence. Oligonucleotide primers (JH-2; GCGCAGTGCTTATTGTAGACG and PR-9; CTTCATGGCTTTGACTTTTGCTG) were designed to span 165 bp of the cDNA sequence. These primers were used for PCR amplifications with 50 ng of total DNA, 2.5 nmol of dNTPs, 50 pmol of each primer, 1.5 mM $MgCl_2$ and 0.5 U *Taq* polymerase in a total volume of 12.5 μl using the enzyme buffer supplied by the manufacturer (U.S. Biochemical). The samples were denatured initially for 3 min at 94° and then incubated for 30 cycles of 94° for 20 sec, 52° for 20 sec, and 72° for 45 sec. These reactions produce PCR products that are ~960 bp in length for all three blue mussel species and two products of 1100 and 500 bp in length with the congener *M. californianus* (Figure 1B). Sequence analysis for all three blue mussel species indicates that MAL-I PCR products contain ~790 bp of noncoding sequence from an intron flanked by coding sequence adjacent to each primer. Southern analysis indicates that MAL-I is single copy (data not shown).

Following amplification, MAL-I PCR products were digested with the restriction enzyme *Spe*I to produce species-specific restriction fragment patterns (Figure 1B). *M. trossulus* is polymorphic for two restriction fragment patterns (T_1 and T_2). Heterozygotes express both restriction fragment length polymorphism (RFLP) patterns, and the Newport, OR population of *M. trossulus* does not differ from Hardy Weinberg equilibrium for the three RFLP patterns (Table 1). Allopatric populations of *M. galloprovincialis* contain a single RFLP (G) pattern that is distinct from those observed in *M. trossulus*.

All three of the novel Mytilus-specific markers described above diagnostically identify *M. trossulus* and *M. galloprovincialis* sampled from allopatric populations at Newport, OR and San Diego, CA, respectively (Table 1). In addition, RAWSON (1996) has shown that mussel populations from Cape Mendocino, CA, north to central Oregon are characterized by high frequencies (>95%) of *M. trossulus*-specific alleles,

TABLE 1

Allele frequencies in allopatric populations of *M. galloprovincialis* and *M. trossulus*

	ITS					MAL-I							GLU-5′		
Location	*n*	G_1	G_2	G_1/G_2	T	*n*	G	T_1	T_2	T_1/T_1	T_2/T_2	T_1/T_2	*n*	G	T
San Diego	64	16.0	19.0	65.0	0.0	64	100	0.0	0.0	–	–	–	64	100	0.0
Newport	52	0.0	0.0	0.0	100	49	0.0	71.0	29.0	53.0	10.0	37.0	52	0.0	100
H.W.										G = 0.501; NS					

The allele designations are identical to those used in Figure 1; *n* = number of mussels genotyped at each locus. The genotype frequencies for the two *M. trossulus* MAL-I alleles at Newport were tested for Hardy-Weinberg equilibrium (H.W.).

those from of Monterey Bay to San Diego, CA are fixed for *M. galloprovincialis*-specific alleles, while populations from Monterey Bay to Cape Mendocino contain intermediate frequencies of both species-specific alleles.

These markers were applied to the samples taken from San Francisco Bay. A total of 423 mussels (10–60 mm shell length) were collected from six sites within San Francisco Bay (Figure 2). At the San Rafael, Berkeley Marina breakwater, Sierra Point breakwater and San Mateo Bridge (west end breakwater) sites mussels were collected from mid-intertidal rocks, while at the East Fort Baker and San Leandro sites they were collected from floating docks. Each mussel was dissected and the mantle tissue removed and divided into two equal parts. The first portion was used for DNA extraction. Total cellular DNA was extracted by digesting a small (≈50 mg) subsample of mantle tissue for 3 hr at 55° in 300 μl lysis buffer (50 mM TRIS-HCl pH 8.0, 1% SDS, 25 mM EDTA) with 100 μg of proteinase K. The nucleic acids were isolated by extracting the completely digested tissue once with phenol/chloroform (1:1) followed by precipitation of the DNA with 95% ethanol. The precipitate was washed in 70% ethanol, air dried and resuspended in 100 μl of TE (10 mM Tris-HCl, 1 mM EDTA). The remainder of this portion of the mantle tissue was preserved in 95% ethanol while the second portion of the mantle tissue was frozen at −80°.

The DNA from these mussels was used in PCR reactions for each of the three loci as described above. Individual mussels were assigned a genotypic score based on the number of *M. galloprovincialis* alleles identified at all three loci. While several hundred tandemly arranged copies of the ribosomal gene array are often found in higher eukaryotes (HILLIS and DIXON 1991), the ITS marker behaves as a single gene locus in Mytilus (HEATH *et al.* 1995) and was scored accordingly in this study. For both ITS and MAL-I, multiple alleles within each species were combined to produce a single composite species-specific allele. Thus, mussels that were homozygous for *M. trossulus* or *M. galloprovincialis* alleles at all three loci received a genotypic score of zero or six, respectively, while those that were heterozygous at all three loci received a score of three.

The gender of individual mussels was determined by digesting ~20 mg of ethanol preserved or −80° frozen mantle tissue with thiobarbuturic acid using the method of JABBAR and DAVIES (1987). Because this protocol is intended to be used with fresh tissue, we first checked whether this method was accurate when using ethanol preserved or frozen tissue. We took the ethanol preserved and frozen mantle tissue from mussels for which RAWSON and HILBISH (1995a) determined gender by mantle squashes. In all cases, the thiobarbuturic method gave the same unambiguous result as mantle squashes ($n = 85$).

Whitsand Bay: Adult blue mussels (20–30 mm shell length) were collected from an intertidal population in Whitsand Bay, UK that had previously been shown to contain a high frequency of hybrid individuals (SKIBINSKI *et al.* 1983; WILHELM 1993). Each mussel was dissected, tissue samples taken from the gill, mantle and digestive gland and frozen at −80° and a separate portion of mantle tissue was set aside for histological analysis. Frozen tissue samples were electrophoresed and stained according to the procedures of SKIBINSKI *et al.* (1980) for *Esterase-D* (*Est*, E.C. 3.1.1.1) and *Leucine aminopeptidase* (*Lap*, E.C. 3.4.11) and MCDONALD *et al.* (1991) for *Mannosephosphate isomerase* (*Mpi*, E.C. 5.3.1.8). These allozyme loci have been used extensively to differentiate *M. edulis* from *M. galloprovincialis* (MCDONALD *et al.* 1991). Previous studies have shown that populations within the Mediterranean Sea and along the Iberian peninsula have high frequencies of *M. galloprovincialis*-specific alleles, while mussel populations in the North Atlantic are characterized by high frequencies of *M. edulis*-specific alleles (SKIBINSKI *et al.* 1983; MCDONALD *et al.* 1991). Mixed populations, including mussels with highly introgressed hybrid genotypes have been identified at numerous sites in England and on the Atlantic coasts of Ireland and France (GOSLING 1992b).

Each of these allozyme loci is multi-allelic, therefore, we combined alleles to create compound alleles characteristic of *M. edulis* and *M. galloprovincialis* as described SKIBINSKI *et al.* (1980). The mussels were then classified by the number of *M. galloprovincialis* alleles they carried in a manner analogous to the San Francisco Bay mussels. Because *Est-D*, *Lap* and *Mpi* are each ~95% differentiated between *M. edulis* and *M. galloprovincialis*, it is possible that individuals with genotypic scores of 1 or 5 actually belong to the parental genotypic class. To insure that the mussels selected for this study were of hybrid ancestry we selected 60 individuals who were trilocus heterozygotes or had a genotypic score of between 2 and 4 and were homozygous for alternate species-specific alleles at two loci. The latter genotypes can only be produced by matings in which both parents are hybrids and represent introgressed genotypes.

Gender was determined for the Whitsand Bay mussels using standard histological techniques described by LOWE *et al.* (1982). A portion of the gonad-bearing mantle tissue was embedded in paraffin, cut into 6-μm sections, stained with hematoxylin and counterstained with eosin. These sections were then scored for the presence of egg or sperm follicles.

mtDNA haplotypes: The sex-specific mtDNA lineages have been labeled as the F (female) and M (male) genomes by SKIBINSKI *et al.* (1994), and the remainder of this paper will follow this format. We determined species-specific F genome mt16S rRNA haplotypes for the San Francisco and Whitsand Bay samples using the methods of RAWSON and HILBISH (1995b) with the exception that the forward universal primer mt16S-AR was replaced by an F genome specific primer (PR-19; CTGCCCAGTGCAACTAGAGTAAT). Amplification with this primer and the universal primer mt16S-BR produces a 440-bp product for all three species of blue mussel. Following amplification, F mt16S PCR products were simultaneously di-

TABLE 2

Allele and haplotype frequencies in San Francisco and Whitsand Bay samples

	Nuclear loci							mt16S haplotypes						
	ITS			MAL-I		GLU-5′		Female			Male			
Location	n_1	G	T	G	T	G	T	A	D	B	n_2	C1	C2	C3
San Rafael	54	46.2	53.8	47.2	52.8	50.9	49.1	25.9	33.3	40.7	15	53.2	39.9	6.8
East Ft. Baker	74	69.5	30.5	68.9	31.1	69.6	30.4	27.0	44.6	28.4	38	29.0	65.8	5.2
Berkeley	67	48.6	51.4	50.0	50.0	48.5	51.5	28.4	23.9	47.8	28	53.6	42.9	3.5
Sierra Pt.	43	70.9	29.1	70.9	29.1	70.9	29.1	20.9	46.5	32.6	15	20.0	73.3	6.7
San Mateo Br.	84	73.2	26.8	73.8	26.2	74.4	25.6	34.5	41.7	23.8	36	19.3	72.3	8.4
San Leandro	58	92.2	7.8	94.0	6.0	95.7	4.3	43.1	50.0	6.9	39	0.0	94.9	5.1
SF Bay (total)	380	67.0	33.0	67.6	32.4	68.4	31.6	30.5	39.8	29.7	171	25.7	68.4	5.9
		LAP		EST		MPI								
Whitsand Bay	61	41.0	59.0	47.5	52.5	58.2	41.8	100	0.0	–	33	–	3.0	97.0

The two *M. galloprovincialis* ITS and two *M. trossulus* MAL-I RFLP patterns have been combined into composite species-specific alleles; n_1 = number of mussels genotyped for all three nuclear loci and F mtDNA haplotypes, n_2 = number of mussels from which M mtDNA haplotypes could be amplified. Mitochondrial haplotypes were scored according to RAWSON and HILBISH (1995b); female haplotypes A and D and male haplotypes C2 and C3 are specific to *M. galloprovincialis,* while haplotypes B and C3 are specific to *M. trossulus.*

gested with 0.5 U of *Spe*I and 5 U *Eco*RV restriction enzymes in the digest buffer supplied by the manufacturer (New England Biolabs); the resulting restriction fragments were resolved on 2.5% agarose gels and scored according to RAWSON and HILBISH (1995b). Species-specific male genome haplotypes were also determined for all individuals following the methods of RAWSON and HILBISH (1995b). For mussels in which gender could be determined unambiguously, the association between gender and the presence of M genome haplotypes was tested using a G-test for independence (SOKAL and ROHLF 1981; p. 692).

RESULTS

San Francisco Bay: A total of 380 mussels from the San Francisco Bay population were successfully genotyped at all three PCR-based nuclear loci and the allele frequencies at these loci are given in Table 2. The frequency of alleles specific to *M. galloprovincialis* and *M. trossulus* varied from site to site. Four sites, San Leandro, San Mateo, Sierra Point and East Fort Baker, were characterized by fairly high frequencies of *M. galloprovincialis* alleles, while there were nearly equal frequencies of *M. trossulus* and *M. galloprovincialis* alleles at the remaining two sites, Berkeley and San Rafael. A total of 79 mussels from San Francisco Bay had hybrid genotypes; of these 66% were putative F_1 genotypes. Appreciable frequencies of hybrid genotypes were found at all six sites, with the highest frequencies occurring at the San Rafael and Berkeley sites (Figure 2). Male mtDNA haplotypes were detected in 171 of the 380 mussels from San Francisco Bay. The frequency of *M. trossulus* and *M. galloprovincialis* M and F haplotypes at each site was similar to the frequency of the species-specific alleles at each of the nuclear loci sampled (Table 2).

Gender was determined for 64% of the mussels from San Francisco Bay; 160 with pure *M. galloprovincialis,* 29 with *M. trossulus* and 55 with hybrid nuclear genotypes. The frequencies of the *M. galloprovincialis* and *M. trossulus* M and F haplotypes and nuclear alleles within this subset of mussels did not differ from the frequencies for the whole sample from San Francisco Bay (G = 0.05; NS). Because the number of hybrid mussels for which gender could be determined at individual sites was small (n = 5–15), we investigated the association of paternal mtDNA haplotypes and gender for all San Francisco sites combined. There was a highly significant association between gender and the presence of M haplotypes among mussels homozygous for *M. galloprovincialis* or *M. trossulus* alleles at all three nuclear loci (Table 3). The degree of association for *M. trossulus*-like (genotype 0) and *M. galloprovincialis*-like (genotype 6) mussels was similar to that observed by RAWSON and HILBISH (1995a) for allopatric populations of *M. trossulus* from Newport, OR (G = 1.94; NS) and *M. galloprovincialis* from San Diego, CA (G = 3.45; NS), respectively. These results indicate that mussels with parental genotypes follow the typical pattern of doubly uniparental transmission of mtDNA observed in allopatric populations of each species.

In contrast, there was no statistically significant association between gender and the presence of M haplotypes for mussels with hybrid genotypes (genotypes 1–5; Table 3). The lack of a detectable association was due to the presence of both male mussels for which we could not detect M genome haplotypes and female mussels that were heteroplasmic for M and F genome haplotypes. This observation indicates that doubly uniparental inheritance of mtDNA has been disrupted in both male and female *M. galloprovincialis-M. trossulus* hybrids.

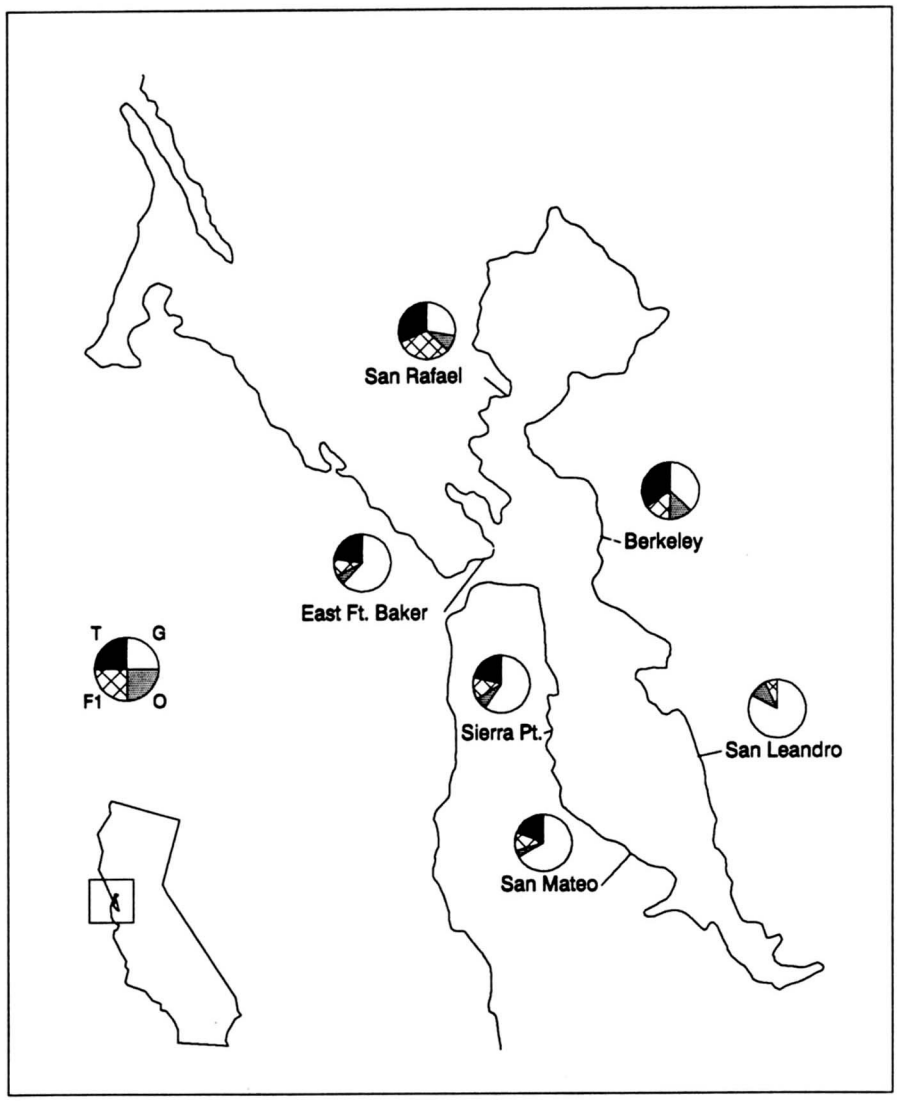

FIGURE 2.—Frequency of *M. trossulus* parental (T), *M. galloprovincialis* parental (G), F_1 and other hybrid (O) genotypes at the six sites sampled within San Francisco Bay, CA.

The majority of mussels with gender-haplotype combinations indicative of the breakdown of doubly uniparental inheritance occurred in genotype class 3. This class contains mostly (85%) mussels with putative F_1 genotypes. For mussels with putative F_1 genotypes, there is no statistical association between gender and the presence of haplotype (G = 0.22; NS). When F_1 hybrids are removed from consideration, there is still no statistical gender-haplotype association for hybrid mussels with introgressed genotypes (G = 1.61; NS). Although the frequency of non-F_1 hybrid genotypes in our dataset is small (n = 21), these observations suggest that the failure of doubly uniparental inheritance occurs in the first generation of hybridization and persists through later stages of introgression. However, we observed no homoplasmic male or heteroplasmic female mussels in genotypic class 1 and 5, suggesting that the failure rate drops in mussels with highly introgressed genotypes.

Although there is a strong correlation between gender and the presence of paternal haplotypes in genotypic classes 0 and 6, there were several male mussels that failed to and females mussels that did inherit M mtDNA in these classes. All of these mussels carried female mtDNA haplotypes that were of the same species origin as their nuclear background. Among females in genotypic classes 0 (two individuals) and 6 (21 individuals) that inherited M haplotypes, all but one carried M mtDNA haplotypes that were of the same species origin as their nuclear background. This observation suggests that M mtDNA introgression is not a prerequisite for the transmission of M mtDNA to female mussels.

We also found that the sex ratio was skewed among *M. galloprovincialis*-*M. trossulus* hybrid mussels. Although the sex ratio for all mussels sampled from San Francisco Bay was not statistically different from 1:1 (G = 2.36; NS), among the hybrid genotypes there was nearly four times as many females as males (G = 13.7; $P < 0.001$). Thus, hybridization between *M. galloprovincialis* and *M. trossulus* is associated with not only the breakdown of doubly uniparental inheritance but with a bias toward the production or survival of females as well.

Whitsand Bay: The allele frequencies at the three

TABLE 3

Occurrence of M and F mtDNA haplotypes in each genotypic class at San Francisco and for Whitsand Bay hybrids

	Nuclear genotypes								
	0	1	2	3	(F_1)	4	5	6	WBH
Gender									
Female	11	1	1	32	28	3	4	82	25
Male	18	1	2	8	6	0	3	78	36
Gender/haplotype									
Female −M	9	1	1	18	17	1	3	61	24
Male +M	16	1	0	4	3	0	4	67	32
Female +M	2	0	1	14	11	2	0	21	1
Male −M	2	0	1	4	3	0	0	11	4
G	14.62				2.11			62.84	50.33
P	<0.001				NS			<0.001	<0.001

Mussels were grouped as females or males having F mtDNA haplotypes but lacking M mtDNA haplotypes (females −M, males −M) and males or females heteroplasmic for both F and M mtDNA haplotypes (males +M, females +M). G values are for test of association between gender and the presence of M mtDNA haplotypes.

nuclear loci assayed for mussels from Whitsand Bay are given in Table 2. This sample contained 54% mussels with putative F_1 genotypes, while the rest were roughly equally distributed among the remaining introgressed genotypes. There was a strong and statistically significant association of gender with the presence of male mtDNA haplotypes in this population (Table 3). This association is similar to that found by RAWSON and HILBISH (1995a) for an allopatric population of *M. edulis* mussels from Lewes, DE (G = 1.59; NS). Compared to the hybrid mussels from San Francisco Bay, sex ratio among *M. galloprovincialis-M. edulis* hybrids does not deviate significantly from 1:1. Thus, hybridization between *M. edulis* and *M. galloprovincialis* does not appear to affect either sex ratio or the transmission of mtDNA typical for blue mussels.

DISCUSSION

Doubly uniparental inheritance of mitochondrial DNA in *Mytilus* spp. creates a strong association between the gender of individual mussels and the presence or absence of paternally inherited mtDNA and is the norm in allopatric populations of all three species of blue mussels, *M. edulis, M. trossulus* and *M. galloprovincialis* (RAWSON and HILBISH 1995a). In contrast, this study has shown that among hybrid mussels produced by interspecific matings between *M. trossulus* and *M. galloprovincialis* there is no association between gender and the presence of M haplotypes, indicating a breakdown in the doubly uniparental transmission of mtDNA. ZOUROS *et al.* (1994) also observed a breakdown of doubly uniparental inheritance in laboratory-reared progeny of heterospecific crosses between *M. edulis* and *M. trossulus,* wherein nearly 40% of male hybrid mussels did not inherit M genomes. Our results differ from those of ZOUROS *et al.* (1994) in that we found not only an appreciable frequency (35%) of male mussels missing M genomes but also a high frequency (43%) of female hybrid mussels who had inherited M genomes. These observations indicate that hybridization between *M. galloprovincialis* and *M. trossulus* can disrupt the mechanisms that inhibit the establishment of sperm mitochondria in females as well as the transmission of M genomes to males. In addition, our results and those of ZOUROS *et al.* (1994) indicate that the failure of M genome transmission to males is not obligatorily associated with the disruption of M genome blockage in females and supports the suggestion of ZOUROS *et al.* (1994) that two separate mechanisms, one in males and one in females, are responsible for regulating doubly uniparental mtDNA inheritance.

Hybridization between blue mussel taxa does not necessarily result in the failure of doubly uniparental mtDNA inheritance. In the *M. edulis-M. galloprovincialis* hybrid population from Whitsand Bay, England, doubly uniparental mtDNA transmission is intact despite hybridization. The association between gender and the presence of M genome haplotypes is as strong among mussels with genotypes indicative of multiple generations of hybridization as it is in allopatric populations of both *M. galloprovincialis* and *M. edulis.* This indicates that relatively little differentiation has occurred between these two taxa at the loci regulating doubly uniparental inheritance. In contrast, whenever interspecific hybridization involves *M. trossulus,* the normal pattern of M genome transmission is at least partly disrupted. Thus, our observations suggest that *M. trossulus* is the most divergent taxa within the *M. edulis* species complex and that *M. trossulus* has diverged farther from *M. galloprovincialis* than it has from *M. edulis* at the loci regulating M mtDNA inheritance. This conclusion is consistent with the blue mussel taxonomy established from allozyme studies (see GOSLING 1992b) and with the phylogenetic relationships for both the M and F mitochon-

drial genomes among the three blue mussel species (RAWSON and HILBISH 1995a).

The observation that doubly uniparental inheritance is frequently disrupted among hybrid mussels, particularly among F_1 hybrids that carry a full complement of nuclear alleles from each parent species, may provide insight into the mechanisms that regulate this novel form of mtDNA inheritance. In male mussels, successful transmission of the M genome must include the following: (1) the penetration of the egg by sperm mitochondria during fertilization, (2) the inclusion of sperm mitochondria into cell lineages that are precursors to the germ line, and (3) preferential replication of the male mitochondria during development of the germ line. Although the M genome is most abundant in the male gonad, both FISHER and SKIBINSKI (1990) and GELLER (1994) have found appreciable levels of the M genome in hepatopancreas and muscle tissue. We have observed that the M genome is as prevalent in the mantle of female *M. galloprovincialis*/*M. trossulus* hybrids as it is in the mantle of nonhybrid male mussels (data not shown), suggesting that the M genome also has a replicative advantage in female hybrids. Thus, the M genome appears to have replicative advantage in most, if not all, tissues in which it occurs. However, replicative advantage is not likely to be the sole mechanism regulating doubly uniparental inheritance. Sperm contain five mitochondria (LONGO and DORNFIELD 1967), and these remain associated with the sperm pronucleus during initial embryonic development (LONGO and ANDERSON 1969). To guarantee the presence of M genomes in sperm, there must be a mechanism ensuring that sperm mitochondria are retained and included in the cell lineage that will eventually form the gonad during the initial cell divisions of male development. Without such a mechanism males would frequently fail to properly inherit and transmit their father's mtDNA.

A second mechanism must act to block M genome transmission to females. Sperm mitochondria may be inhibited from entering the egg, as has been observed for paternal organelles in barley (MOGENSON 1988). This would suggest that the observed penetration of the egg by sperm mitochondria observed by LONGO and ANDERSON (1969) was for male mussels. Alternatively, the paternal mitochondria may be lost by dilution due to the more numerous maternal mitochondria. Dilution, however, would also require that the replicative advantage of the M genome be suppressed in females. A third possibility is that after sperm mitochondria enter the egg they are selectively degraded or expelled from the embryo as has been documented in a variety of organisms, including fungi, slime molds, green algae and sea urchins (ANDERSON 1968; MIRFAKHRAI *et al.* 1990; BECKERS *et al.* 1991; MELAND *et al.* 1991).

For these postulated mechanisms to regulate doubly uniparental inheritance of M mitochondria, it is essential that the sex of an egg be determined before fertilization. Recently, ZOUROS and coworkers (ZOUROS *et al.* 1994; E. ZOUROS and C. SAAVEDRA, personal communication) have reported that the sex ratio of mussel clutches is under maternal control. This observation suggests that in mussels the female determines the gender of an egg before expression of the embryonic genome.

As a working hypothesis, we propose the following model for the regulation of doubly uniparental inheritance. First, sperm mitochondria penetrate and are initially retained in eggs regardless of whether they will develop into males or females. Once sperm mitochondria have entered the egg, interactions between the sperm mitochondrial membrane and the egg cytoskeleton determine whether sperm mitochondria enter the germ line in males or are targeted for elimination in females. Interactions between the mitochondrial membrane and cytoskeleton interactions have been implicated in the proper transmission of mtDNA in yeast (MCCONNELL and YAFFE 1993; SOGO and YAFFE 1994). Presumably, the morphological differences between sperm and egg mitochondria in mussels (LONGO and ANDERSON 1969) are accompanied by biochemical differences that help to mediate their sex-specific fates. Once the M mtDNA becomes established in males it is preferentially replicated.

This model emphasizes that the proper transmission of M genomes is dependent upon communication between the sperm mitochondrial membrane and the cytoskeleton and other components of the egg. Thus, the disruption of doubly uniparental inheritance is not due to the introgression of differentiated M genomes. Rather, the sperm mitochondrial membrane and egg cytoskeleton are determined by the paternal and maternal nuclear genomes, respectively. Because the M genome is packaged within a membrane not recognized by the egg cytoskeleton upon interspecific hybridization, the disruption of doubly uniparental inheritance is due to the interaction of two highly divergent parental genotypes. The high incidence of doubly uniparental inheritance failure observed in the F_1 generation by this study and that of ZOUROS *et al.* (1994) is consistent with our model. The model also predicts that among more introgressive genotypes there should be a lower incidence of doubly uniparental inheritance failure for both males and females than has been observed in the F_1 generation. We are currently developing additional highly differentiated nuclear markers for *M. galloprovincialis* and *M. trossulus* that will allow us to better separate F_1 and other more introgressive genotypes to test this prediction.

Finally, hybridization between *M. galloprovincialis* and *M. trossulus* is also associated with a significantly skewed sex ratio. We found that the sex ratio within the parental genotype classes from San Francisco Bay was not significantly different from 1:1, as is commonly seen in blue mussel populations (HILBISH and ZIMMERMAN 1988; FISHER and SKIBINSKI 1990; SEED and SUCHANEK 1992).

Among hybrids, however, there was a deficit of males so that the sex ratio was nearly 4 to 1 in favor of females. This skewed sex ratio could be due to a lowered production of male eggs within the San Francisco Bay population. However, if this were true, then there should also be a deficit of males among the parental genotype classes. Alternatively, the deficit of males could be caused by increased mortality of male eggs when they are fertilized by heterospecific sperm. The deficit of males, however, does not appear to be associated with the failure of doubly uniparental inheritance per se, since we found equal frequencies of males with and without M genomes. Thus, hybridization would appear not only to disrupt mitochondrial DNA transmission typical in blue mussels but also perhaps to bring about increased male mortality through a separate mechanism.

We thank A. Day, R. Burton and C. Langdon for providing some of the mussels used in this study, L. Bridgman for her help in developing MAL-I and R. Showman for numerous helpful conversations regarding mussel development and for reviewing an earlier version of this manuscript. We greatly appreciate the space and assistance provided by D. Hedgecock and the University of California, Davis' Bodega Marine Laboratory for collecting the San Francisco Bay mussels. This research was supported by Bodega Field Conference and Slocum Lunz fellowships to P.D.R. and by grants OCE-9203320 and DEB-9509742 to T.J. Hilbish from the National Science Foundation.

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Communicating editor: A. G. Clark