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Species-Specific Segregation of Gender-Associated Mitochondrial DNA Types in an Area Where Two Mussel Species (*Mytilus edulis* and *M. trossulus*) Hybridize

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ABSTRACT

In each of the mussel species *Mytilus edulis* and *M. trossulus* there exist two types of mtDNA, the F type transmitted through females and the M type transmitted through males. Because the two species produce fertile hybrids in nature, F and M types of one may introgress into the other. We present the results from a survey of a population in which extensive hybridization occurs between these two species. Among specimens classified as "pure" *M. edulis* or "pure" *M. trossulus* on the basis of allozyme analysis, we observed no animal that carried the F or the M mitotype of the other species. In most animals of mixed nuclear background, an individual's mtDNA came from the species that contributed the majority of the individual's nuclear genes. Most importantly, the two mtDNA types in post-F_1 male hybrids were of the same species origin. We interpret this to mean that there are intrinsic barriers to the exchange of mtDNA between these two species. Because such barriers were not noted in other hybridizing species pairs (many being even less interfertile than *M. edulis* and *M. trossulus*), their presence in Mytilus could be another feature of the unusual mtDNA system in this genus.

CASES of introgression of mitochondrial DNA (mtDNA) between hybridizing taxa in nature are known in most major divisions of the animal kingdom. The list includes Drosophila [members of the *D. pseudoobscura* subgroup (POWELL 1983) and members of the *D. simulans* clade (SOLIGNAC and MONNEROT 1986; SATTA *et al.* 1988)], crickets (HARRISON *et al.* 1987), grasshoppers (MARCHANT 1988), fish (AVISE *et al.* 1984), frogs [genus Rana (SPOLSKY and UZZELL 1984) and genus Hyla (LAMB and AVISE 1986)], deer (CARR *et al.* 1986), field mice (AVISE *et al.* 1983) and house mice (FERRIS *et al.* 1983). Transfer of one species' or strain's mtDNA into the nuclear background of another through backcrossing has also been achieved in many laboratory experiments (LANSMAN *et al.* 1983; GYLLENSTEN *et al.* 1985; CLARK and LYCKEGAARD 1988; MACRAE and ANDERSON 1988; FOS *et al.* 1990; GYLLENSTEN *et al.* 1991; HUTTER and RAND 1995; KILPATRICK and RAND 1995). The general observation from these studies is that individuals with nuclear and mitochondrial genomes from different species or strains "develop with seemingly normal viability and fertility" (GYLLENSTEN *et al.* 1985).

Mussels of the genus Mytilus have a peculiar system of mtDNA transmision that adds a new dimension to the study of mtDNA introgression. Females are normally homoplasmic for one type of mtDNA, the F type, that they transmit to both daughters and sons. Males are heteroplasmic for two types, the F type that they inherit from their mother and the M type that they inherit from their father and transmit to their sons (SKIBINSKI *et al.* 1994a,b; ZOUROS *et al.* 1994a,b). This mode of mtDNA transmission, which has been termed "doubly uniparental inheritance" (DUI) (ZOUROS *et al.* 1994a), explains previous observations of high incidence of heteroplasmy (FISHER and SKIBINSKI 1990; HOEH *et al.* 1991) and high rate of biparental inheritance (ZOUROS *et al.* 1992) in Mytilus.

We have studied this exceptional mtDNA system in two members of the *M. edulis* species-complex, *M. edulis* and *M. trossulus* (ZOUROS *et al.* 1992, 1994b; STEWART *et al.* 1995). In areas of sympatry the two taxa hybridize and there is evidence that at least some of the hybrids are fertile (GOSLING 1992a,b; ZOUROS *et al.* 1992). Allelic frequencies at most examined allozyme loci do generally differ in the two taxa, and in at least two allozyme loci (*Mpi* and *Est-D*) the allele frequency overlap is small enough to allow these loci to be used as diagnostic tools (MCDONALD *et al.* 1991). Most authors have treated *M. edulis* and *M. trossulus* as recognizable taxa, but there is no agreement whether they represent separate species (GOSLING 1992a). For convenience we will use the term "species" to refer to these taxa, while recognizing that they may have not, indeed, achieved the species status.

The coupling of sex and mtDNA inheritance in mussels implies a network of nuclear/cytoplasmic interactions (SKIBINSKI *et al.* 1994b; ZOUROS *et al.* 1994b). Such interactions are also implied by the different patterns of distribution of M and F types in the male's somatic

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tissues (Skibinski et al. 1994b; Stewart et al. 1995). Together, these observations raise the possibility that in Mytilus the constraints for compatibility between nuclear and mtDNA genomes are stronger than in species with a standard uniparental mtDNA inheritance. The possibility of introgression of mtDNA between Mytilus species would depend not only on the compatibility of one species' nuclear DNA with the F or the M mtDNA type of the other species, but also on the compatibility of one species' F type with the other species' M type in hybrid males. In laboratory crosses viable hybrids are produced (Zouros et al. 1992, 1994b), but there have been, as yet, no detailed studies on viability or fertility depression in these hybrids. It is, therefore, difficult to assess the possibility of introgression in nature from laboratory studies. In this paper we have addressed these questions by examining a natural population in which *M. edulis* and *M. trossulus* coexist and hybridize.

MATERIALS AND METHODS

Adult animals were collected from a site near Lunenburg, Nova Scotia, Canada, in spring 1993. In the laboratory the animals were dissected and the sex determined by microscopic examination of the gonad. Gonad and digestive gland were stored at −80° until used for mtDNA and allozyme scoring, respectively.

Five polymorphic enzyme loci with different levels of diagnostic power for the two taxa studied were scored in all animals following the protocols of McDonald and Koehn (1988) with some modifications (Zouros et al. 1994b): *mannose phosphate isomerase* (*Mpi;* EC 5.3.1.8), *esterase-D* (*Est-D;* EC 3.1.1.1), *leucine amino peptidase* (*Lap-1;* EC 3.4.11.1), *octopine dehydrogenase* (*Odh;* EC 1.5.1.11) and *phosphoglucomutase* (*Pgm;* EC 5.4.2.2).

We used two methods for mtDNA scoring. In the "mtDNA restriction fragment length polymorphism (RFLP)" method, total DNA was extracted from the gonad by the salt extraction procedure (Miller et al. 1988) and digested with *Eco*RI or *Hin*dIII. The mtDNA fragments were visualized after hybridization to a probe that consisted of seven cloned fragments. Five of these were the same used by Hoffmann et al. (1992) to obtain the sequence of the *M. edulis* mitochondrial genome. These fragments together comprise the whole F molecule of this species. The other two clones consisted of a 13- and a 7-kb fragment of the *M. trossulus* mitochondrial genome (see Zouros et al. 1992 for details). The mtDNA RFLP method was sufficient to score the mitotypes of females and of most males. For a number of males, however, it failed to detect the presence of F type, which occurs in low amounts in the male gonad (Skibinski et al. 1994b; Stewart et al. 1995). To detect the F mitotype of these apparently homoplasmic males, we used a "PCR/restriction assay". Total DNA from these males was amplified using a set of primers for the *cytochrome oxidase c subunit III* (*COIII*) gene FOR1 5′-TATGTACCAGGTCCA-AGTCCGTG-3′ and REV1 5′-ATGCTCTTCTTGAATATA-AGCGTACC-3′ that correspond to nucleotide positions 460–482 and 1362–1301 of segment #5 of the *M. edulis* FB mitotype in Hofmann et al. (1992). This primer pair amplifies the corresponding mtDNA fragment from the F and M types of both species. PCR conditions were the same as described in Zouros et al. (1994). Different aliquots of the amplification product were digested with *Eco*RI and *Acc*I to identify the various mtDNA genomes in apparently homoplasmic males (Figure 1).

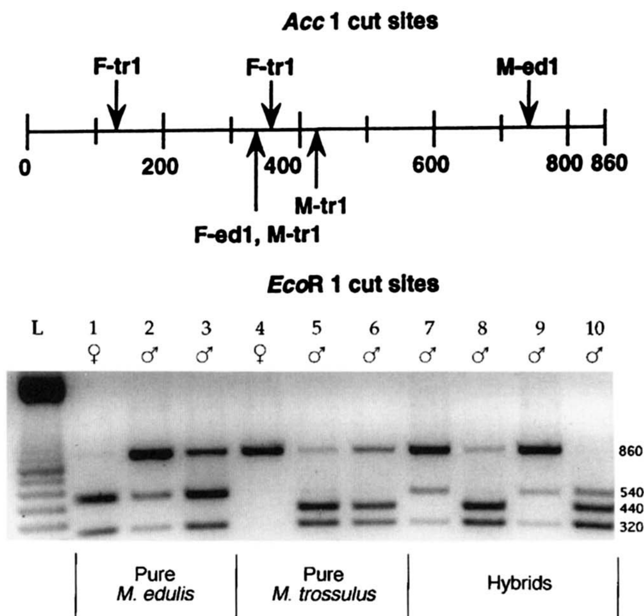

Figure 1.—The *COIII* gene. Top: Restriction sites of an 860-bp segment of the *COIII* gene from the two major mtDNA types of *M. edulis* (F-ed1, M-ed-1) and the two major mtDNA types of *M. trossulus* (F-tr1, M-tr1). Arrows above the line point to *Acc*I sites, arrows below to *Eco*RI sites. Bottom: *Eco*RI profiles of the 860-bp *COIII* segment amplified from 10 individuals: 1, *M. edulis* female scored as F-ed1 both by the mtDNA RFLP and the PCR/restriction assays; 2, *M. edulis* male scored as F-ed1/M-ed1 (heteroplasmic) by both assays; 3, *M. edulis* male scored as M-ed1 (apparently homoplasmic) by the first assay and as F-ed1/M-ed1 by the second; 4, *M. trossulus* female scored as F-tr1 by both assays; 5, *M. trossulus* male scored as F-tr1/M-tr1 by both assays; 6, *M. trossulus* male scored as M-tr1 by the first assay and as F-tr1/M-tr1 by the second assay; 7, hybrid male (backcross to *M. edulis*) scored as M-ed1 by the first assay and as F-ed1/M-ed1 by the second; 8, hybrid male (backcross to *M. trossulus*) scored as M-tr1 by the first assay and as F-tr1/M-tr1 by the second; 9, hybrid male (F_2) scored as M-ed1 by the first assay and as F-ed1/M-ed1 by the second; 10, hybrid male (backcross to *M. edulis*) scored as M-tr1 by the first assay and as F-ed1/M-tr1 by the second. Animal 10 was one of the four males with a heterospecific mtDNA combination.

RESULTS

Classification of animals into "pure species" and "hybrids": In total, 201 animals were scored for sex, mtDNA and allozymes. From the allozyme surveys of Varvio et al. (1988) and McDonald et al. (1990, 1991) (which together cover the whole geographic distribution of *M. edulis* and *M. trossulus*), the *Mpi* locus appears to be completely diagnostic for *M. edulis* (alleles 90 and 100) and *M. trossulus* (alleles 94 and 105). In the same surveys, *Est-D* alleles 100 and 110, comprising together above 96% of the *M. edulis* gene pool, were not found in *M. trossulus* whose populations contained alleles 90 (frequency above 90%) and 95 (frequency less than 5%). Of these, allele 90 was found in *M. edulis* in frequencies between 2 and 4%. We have used these two loci to classify the animals in our sample as "pure

TABLE 1

Allozyme frequencies in the pure *edulis*, pure *trossulus* and hybrid components of a sample of wild mussels

Locus	Allele	Allele frequencies			S_{ej}^{a}
		Pure *edulis*	Pure *trossulus*	Hybrids	
Mpi	90	0.068	0.000	0.022	1.000
	94	0.000	0.993	0.587	0.000
	100	0.932	0.000	0.391	1.000
	105	0.000	0.007	0.000	0.000
	N^{b}	81	74	46	
	I^{c}	0.000	0.555	0.832	
Est-D	80	0.006	0.014	0.011	0.300
	90	0.000	0.946	0.489	0.000
	95	0.000	0.041	0.011	0.000
	100	0.988	0.000	0.489	1.000
	110	0.006	0.000	0.000	1.000
	N	81	74	46	
	I	0.000	0.707	0.707	
Lap-1	80	0.006	0.000	0.011	1.000
	85	0.025	0.178	0.130	0.123
	90	0.025	0.205	0.174	0.109
	95	0.099	0.541	0.261	0.155
	100	0.821	0.068	0.413	0.924
	105	0.012	0.000	0.000	1.000
	110	0.006	0.007	0.011	0.462
	120	0.006	0.000	0.000	1.000
	N	81	73	46	
	I	0.236	0.842	0.700	
Odh	80	0.012	0.000	0.012	1.000
	90	0.019	0.092	0.012	0.171
	95	0.006	0.000	0.023	1.000
	100	0.827	0.134	0.453	0.861
	110	0.031	0.704	0.453	0.042
	120	0.105	0.049	0.023	0.682
	130	0.000	0.021	0.012	0.000
	140	0.000	0.000	0.012	0.500
	N	81	71	43	
	I	0.231	0.731	0.822	
Pgm	93	0.093	0.007	0.043	0.930
	97	0.006	0.000	0.011	1.000
	100	0.790	0.096	0.337	0.892
	106	0.080	0.205	0.207	0.281
	111	0.031	0.610	0.359	0.048
	114	0.000	0.082	0.033	0.000
	120	0.000	0.000	0.011	0.500
	N	81	73	46	
	I	0.213	0.693	0.843	

[a] S_{ej} is the *edulis* specific index as defined in the text.
[b] *N* is the number of animals.
[c] *I* is NEI's genetic identity index. The first *I* value in each locus is the identity value between pure *edulis* and pure *trossulus*, the second between pure *edulis* and hybrids and the third between pure *trossulus* and hybrids.

edulis", "pure *trossulus*" and "hybrids" (Table 1). An exception was made for an animal that was 100/100 for *Mpi* and 80/100 for *Est-D*. This animal was classified as *M. edulis* because allele 80 of *Est-D* occurs in very low frequencies in both species. Hybrids were subsequently classified as "F_1-like" (when an *M. edulis* and an *M. trossulus* allele were observed at both diagnostic loci), "F_2-like" (when both allozymes at one of the two diagnostic loci were of one species, but both allozymes at the other diagnostic locus were of the other species) and "backcross-like" (when both alleles at one locus belonged to one species, but the other locus contained an allele from each species). Backcross-like animals were further classified as "*edulis*-biased" or "*trossulus*-biased" if three of the four allozymes at the diagnostic loci belonged to one or the other species.

This classification provides only tentative information about the real history of any particular hybrid animal.

For example, heterospecificity at both diagnostic loci is compatible with the F_1, F_2 or the backcross state. More importantly, with only two diagnostic loci, the probability that a real F_2 hybrid will be misclassified as a pure species animal is $^1/_8$ and the same probability for a real backcross progeny is $^1/_4$. Thus, several individuals classified as pure species may in fact carry genetic material from both species. This bias strengthens our conclusion that mtDNA introgression from one species into another is a rare event.

To obtain an estimation of each species' contribution to the nuclear genome of a hybrid individual based on all five allozyme loci, we devised an index, the species-specific index (S), that can be measured with reference to either *M. edulis* (S_e) or *M. trossulus* (S_t). For each allozyme (j) we define S_{ej} as $S_{ej} = p_{ej}/(p_{ej} + p_{tj})$, where p_{ej} and p_{tj} are the frequencies of the allozyme among the pure *edulis* and pure *trossulus* groups, respectively. S_{ej} has the desired properties that it varies from one (when the allozyme occurs only in *M. edulis*) to zero (when the allozyme occurs only in *M. trossulus*), takes the value 0.5 when the allozyme is equally frequent in the two species and that the summation of S_{ej} and S_{tj} is equal to one. The value of rare allozymes that are found only in hybrids is defined as 0.5. The S_e index of an individual animal is the mean of the individual's S_{ej} values, *i.e.*, $S_e = (^1/_2n)\Sigma_j S_{ej}$, where n is the number of allozyme loci for which the animal was scored. The S_{ej} value for each allozyme is given in Table 1 together with the allozyme's frequency in the pure *edulis*, pure *trossulus* and hybrid groups. The table also gives NEI's genetic identity (NEI 1972) between pure *edulis* and pure *trossulus* and between each of these groups and the hybrid group. The fact that for all three partially diagnostic loci the identity between each pure species and the hybrid group is much larger than the identity between the two pure species provides independent support for the division of the sample into these three classes.

Sex and nuclear genotype distribution among hybrids: The sex and the S_e value of all 46 hybrids are given in Figure 2. For three individuals the *Odh* locus could not be scored, so the S_e is based on four loci. The two sexes were equally frequent in our sample (21 females *vs.* 25 males). Moreover, there was no substantial difference in the number of males or females whose nuclear background was dominated by one or the other species ($S_e < 0.5$ in 14 females and in 13 males). Thus, there is no evidence in our data for differential hybrid viability between sexes or that backcrosses were dominated by one or the other species. These observations provide further support for the view that the postzygotic isolation between *M. edulis* and *M. trossulus* is weak and accentuate the problem of how the two species maintain their nuclear and mtDNA integrity.

Mitochondrial DNA of pure species animals: An individual was characterized by one or two mitotype symbols, depending on whether it was found to be homoplasmic or heteroplasmic (Table 2). F-ed, M-ed, F-tr and M-tr are generic names introduced by STEWART *et al.* (1995) to refer jointly to the gender (M or F) and species (*M. edulis* or *M. trossulus*) affiliations of a mussel mtDNA molecule.

All but one of the 47 pure *M. edulis* females were found to be homoplasmic (Table 2). The most common *M. edulis* F *Eco*RI mitotype, F-ed1, was found in 44 of these individuals. Each of the other two females contained an *Eco*RI profile (named F-ed2 and F-ed3) that differed from F-ed1 by one or two restriction sites. In a previous study designed to measure levels of molecular divergence among mitotypes of same or different species and gender affiliation (STEWART *et al.* 1995), the sequence divergence between F-ed1 and F-ed3 at a 300-bp segment of the *COIII* gene was found to be 3%, $\sim$$^1/_6$ of the divergence between the two most common *M. edulis* and *M. trossulus* F types (F-ed1 and F-tr1). Thus, F-ed2 and F-ed3 are variants of the main *M. edulis* F type and do not represent introgressions from *M. trossulus*, where they were not found. The same conclusion can be drawn from the pure *M. edulis* males. All 34 of these males were heteroplasmic for an F-ed and an M-ed type. We observed six different M *Eco*RI profiles in *M. edulis*, each of which differed from the most common profile (M-ed1) by one or two restriction sites. The nucleotide sequence divergence of one of these variants, M-ed3, from the most common type, M-ed1, at the *COIII* gene was found to be $\sim$3% (STEWART *et al.* 1995), which is only $\sim$$^1/_9$ of the divergence between the two most common *M. edulis* and *M. trossulus* M types (M-ed1 and M-tr1). We conclude that no pure *M. edulis* animal contained an *M. trossulus* mtDNA, either of the F or the M type.

Eleven females and 12 males, which on the basis of diagnostic allozymes were classified as pure *M. trossulus*, contained an mtDNA *Eco*RI mitotype that clearly distinguished them from the other *M. trossulus* individuals. This mitotype, which we have provisionally called "*trossulus* O" (trO) type, is size-variable. *Eco*RI profiles from animals with a trO mtDNA type share the same set of bands except the largest band, which may vary in size among animals. The trO types carried by females (*i.e.*, F-trO) can be distinguished from those carried by males (M-trO) through the mtDNA RFLP assay, but they cannot be distinguished by the PCR/restriction assay that was designed for standard (*i.e.*, non-O) *M. trossulus* mitotypes. As a result, *M. trossulus* males that on the basis of mtDNA RFLP analysis were classified as apparently homoplasmic for an M-trO type could not subsequently be examined for the presence of an F-trO type. Thus, the heteroplasmic state of these animals remained uncertain and they were listed simply as M-trO (Table 2). Size variation of mtDNA was also observed in *M. trossulus* from the Baltic Sea, where, again, only a subset of males could be unambiguously classified as heteroplas-

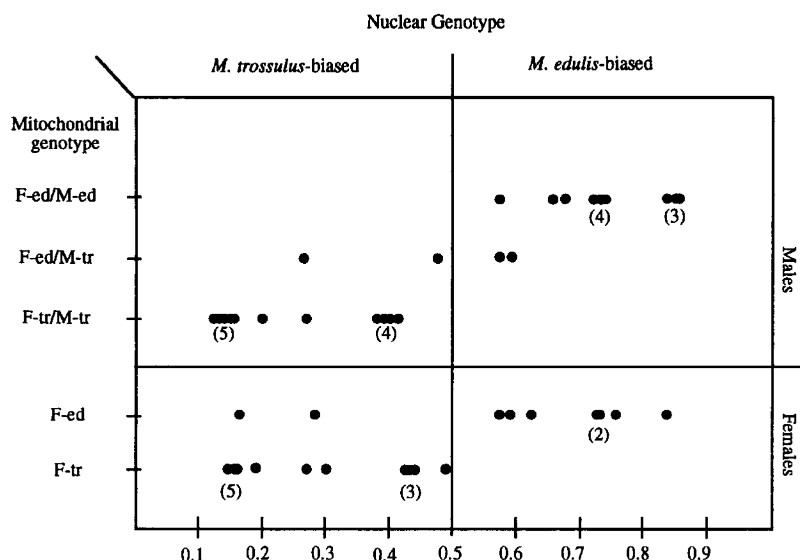

FIGURE 2.—Distribution of mtDNA genotypes of hybrids according to sex and the species-specific index of the nuclear genotype. Abscissa: the *edulis*-specific value. Two males whose heteroplasmy could not be confirmed (type M-trO) were plotted as F-tr/M-tr, as they did not contain an *M. edulis* mtDNA type.

mic (WENNE and SKIBINSKI 1995). This difficulty did not interfere with the objective of our study, which was the detection of one species' mitotypes in the other. No *M. edulis* mitotype was found in any of the 23 *M. trossulus* animals with a trO mtDNA. All 19 *M. trossulus* females with a standard *M. trossulus* mitotype were homoplasmic, 17 for the most common F type (F-tr1) and two for a variant (F-tr2). These two types differ by only 1.5% at the *COIII* gene. All 32 pure *M. trossulus* males with standard *M. trossulus* mitotypes were heteroplasmic for an F-tr and an M-tr type. Three M-tr *Eco*RI profiles were observed among these animals, with an average divergence rate of ~4.8% at the *COIII* gene (STEWART *et al.* 1995), which is ~$^1/_5$ of the divergence between the common M types of *M. edulis* and *M. trossulus* (M-ed1 and M-tr1). We conclude that no *M. edulis* mtDNA type could be found among pure *M. trossulus* animals.

Exceptions to the doubly uniparental mtDNA inheritance in Mytilus, with males carrying only an F type and females carrying an F and an M type, were observed in laboratory crosses (ZOUROS *et al.* 1994b) and in natural populations where *M. edulis* and *M. galloprovincialis* coexist and hybridize (FISHER and SKIBINSKI 1990). Also, animals that were apparently heteroplasmic for more than two mitochondrial genomes were observed by HOEH *et al.* (1991). The pure *edulis* female with both the F and the M *M. edulis* types seen in this study (Table 2) adds to the list of exceptions. In their laboratory study ZOUROS *et al.* (1994b) observed exceptions only in interspecific (*M. edulis* × *M. trossulus*) crosses and suggested that the phenomenon of doubly uniparental inheritance is prone to breakdown in hybrids. If this is true, the exceptional female may be a recent descendant of a hybrid.

TABLE 2

Distribution of mtDNA genotypes among pure species animals

	Pure *edulis*		Pure *trossulus*	
Mitotype	Female	Male	Female	Male
F-ed	46	—	—	—
F-tr	—	—	19	—
F-ed/M-ed	1	34	—	—
F-tr/M-tr	—	—	—	32
F-trO	—	—	11	—
M-trO	—	—	—	12
Total	47	34	30	44

Heteroplasmy is denoted by two mitotypes separated by slash. Other notation as in text. The heteroplasmic status of the 12 M-trO males could not be confirmed (see text for explanation).

Mitochondrial DNA analysis of hybrids: The mitotypes of hybrids were symbolized the same way as those of pure species animals (Table 3). For F_1-like males it was possible to determine if an individual was not a true F_1, because if it was, its two mtDNA types should each come from different species. Of the seven males that were classified as F_1-like, only one fulfilled this condition and could have been a true F_1. This suggests that the overwhelming majority of hybrids in our sample were products of backcrosses to one or the other species. A second observation is that, as with the pure species, females were homoplasmic and males were heteroplasmic (with the exception of two animals with an M-trO mitotype whose heteroplasmic status could not be determined). This is an interesting observation in view of the several deviations from the doubly uniparental mode of mtDNA transmission observed in interspecific crosses (ZOUROS *et al.* 1994b). However, the limited number of true F_1 animals in our sample does not allow us to conclude whether the deviations are less common in nature.

The first question that can be asked from the hybrid class is "do the M and F mtDNA mitotypes in hybrid males tend to be more often homospecific than ex-

TABLE 3

Distribution of mtDNA genotypes among hybrids

Mitotype	F_1-like Female	F_1-like Male	F_2-like Female	F_2-like Male	BC/edulis Female	BC/edulis Male	BC/trossulus Female	BC/trossulus Male	Total
F-ed	2	—	1	—	4	—	2	—	9
F-tr	1	—	1	—	1	—	4	—	7
F-ed/M-ed	—	2	—	4	—	4	—	—	10
F-tr/M-tr	—	4	—	—	—	—	—	5	9
F-ed/M-tr	—	—	—	—	—	1	—	1	2
F-ed/M-trO	—	1	—	—	—	—	—	1	2
F-trO	1	—	—	—	—	—	4	—	5
M-trO	—	—	—	—	—	—	—	2	2
Total	4	7	2	4	5	5	10	9	46

Notation as in Table 2. The heteroplasmic status of some M-trO males could not be determined (see text for details).

pected?" To answer this question we needed to ask, first, what is the probability that an F_1, an F_2 or a backcross male will have a heterospecific combination of F and M mitotypes under DUI. Assuming equal probabilities for survival and fertility for all hybrid types and that all types of crosses are equally possible, this probability is 1 for F_1, $1/2$ for F_2 and $1/2$ for progeny from the first and subsequent backcrosses (Table 4). We have observed four males with heterospecific M and F types out of 25 (Table 3). If we assume that all hybrid males are backcross progeny (or F_2), the probability of this occurring by chance is 0.0007 ($\chi^2 = 11.56$, d.f. = 1). If one of the four males is excluded as a true F_1 (in which case it must carry a heterospecific combination), the probability of observing three males with a heterospecific combination out of 24 is even smaller ($\chi^2 = 13.5$, $P = 0.0002$, d.f. = 1). A second question regarding the hybrid class is, "is there a tendency for a hybrid's mtDNA type (or types, for males) to come from the species that has contributed the majority of nuclear genes?" To answer this question, we note that true F_1 and F_2 animals provide no information because they contain an equal amount of nuclear genes from each species. For backcross progeny the probability of discordance between an animal's mitotype and the majority of its nuclear genes under the mode of doubly uniparental inheritance is 0.25 (Table 4). Among 15 backcross females, there were three discordances (Table 3), which is very close to expectation ($\chi^2 = 0.20$, $P = 0.65$, d.f = 1). To ask the same question in males, we have to consider only those which carry homospecific F and M types. There were nine such backcross males. In all of them both mitochondrial DNA types matched the species that contributed the majority of nuclear genes (Table 3). Again, this observation is not statistically different from expectation ($\chi^2 = 3.00$, $P = 0.083$, d.f. = 1).

The analysis above was based on the subclassification of hybrids in F_1, F_2 and backcross, which, as noted, may not accurately reflect the actual history of each animal and takes no account of the contribution of the three partially diagnostic loci to the hybrid's genotype. The introduction of the species-specific index allows one to ignore this subclassification and ask directly if there is a correlation between a hybrid's nuclear affinity with one or the other parental species and the origin of its mtDNA. This analysis (Figure 2) produced a strong indication of concordance between mtDNA and nuclear DNA origins. Among 21 females, we would expect 5.25 to be on the "wrong" side of the 0.5 line of the species-specific index, but we have observed two ($\chi^2 = 2.68$, $P = 0.10$, d.f. = 1). There were 19 males with

TABLE 4

Expected combinations of mitotypes and nuclear backgrounds of male and female progeny in backcrosses of F_1 hybrids to pure species

Pure species males	F_1 females (F-ed)[a]	F_1 females (F-tr)
M. edulis (F-ed/M-ed)	F-ed [ed][b]	*F-tr [ed]
	F-ed/M-ed [ed]	*F-tr/M-ed [ed]
M. trossulus (F-tr/M-tr)	*F-ed [tr]	F-tr [tr]
	*F-ed/M-tr [tr]	F-tr/M-tr [tr]
Pure species females	**F_1 males (F-ed/M-tr)**	**F_1 males (F-tr/M-ed)**
M. edulis (F-ed)	F-ed [ed]	F-ed [ed]
	F-ed/*M-tr [ed]	F-ed/M-ed [ed]
M. trossulus (F-tr)	F-tr [tr]	F-tr [tr]
	F-tr/M-tr [tr]	F-tr/*M-ed [tr]

Discordances are marked by *. For F mitotypes discordances occur in backcrosses involving F_1 females, and for M mitotypes in backcrosses involving F_1 males. The overall expected rate of discordance is 1/4 for both F and M types. The expectation of heterospecific combinations of F and M mitotypes in males (in bold) is 1/2.

[a] Mitotypes are indicated as F-ed, F-tr, M-ed and M-tr. A single mitotype indicates a female; two mitotypes separated by a slash indicate a male.

[b] The species that contributes the majority (3/4) of the nuclear genes to the progeny of backcrosses is indicated in brackets [ed, *M. edulis*; tr, *M. trossulus*].

homospecific F and M types (these do not include the two apparently homoplasmic males with M-trO mitotypes). None of them was on the "wrong" side of the 0.5 line, while the expected number was 4.75 (χ^2 = 6.33, P = 0.012, d.f. = 1). Thus, there is evidence for concordance between mitochondrial DNA and nuclear DNA in males, but not in females. Because the ratio of discordance to concordance is not different in the two sexes (two to 19 in females, zero to 19 in males; P = 0.24 from Fisher's exact test), the two sexes can be pooled to give an overall rate of discordance of 0.05 that is lower than the expected 0.25 (χ^2 = 22.4, $P = 2 \times 10^{-6}$, d.f. = 1).

Interestingly, three of the four males with heterospecific mtDNA types have species-specific indices close to 0.5 and, indeed, these four males form a group that is different from the other hybrid males with regard to the distance from the 0.5 line of the species-specific index (u = 2.454, P = 0.007 from Wilcoxon's two sample rank test). This suggests that heterospecific combinations of M and F types would rarely be found in nature among males whose nuclear genotype is dominated by one or the other species.

DISCUSSION

Interactions between nuclear and mitochondrial DNA have been proposed as the most likely explanation of results from experimental populations that appear to be incompatible with the hypothesis of mtDNA neutrality (CLARK and LYCKEGAARD 1988; MACRAE and ANDERSON 1988; FOS *et al.* 1990; HUTTER and RAND 1995). But as noted by these authors and others (NIGRO and PROUT 1990; SINGH and HALE 1990), other factors, besides mtDNA, may cause the same pattern of nuclear-cytoplasmic response. The numerous cases of mtDNA introgression among species or races seen in nature or created in the laboratory (cited in the Introduction) suggest that the deleterious effects of nuclear/mtDNA incompatibilities, if they exist, must be minor (with the notable exception of cytoplasmic male sterility in plants; see SAUMITOU-LAPRADE 1994 for review). Lack of intrinsic barriers to mtDNA introgression is an implicit assumption in studies that attempt to delineate the geographical history of hybridizing taxa by examining the joint distribution of nuclear and mtDNA variants (*e.g.*, SCRIBNER and AVISE 1993). Introgression studies in Drosophila have, indeed, failed to produce a convincing case of an interspecific nuclear-cytoplamic incompatibility leading to hybrid breakdown but succeeded in demonstrating such incompatibilities between interspecific nuclear genes (ZENG and SINGH 1993; WU and PALOPOLI 1994; GOULIELMOS and ZOUROS 1995). It would appear that for mtDNA-nuclear incompatibilities to have a measurable effect, the two genomes must come from taxa that have evolved beyond the level of crossability.

This study was motivated from the realization that DUI allows the question of mtDNA incompatibility to be asked at two different levels: compatibility between nuclear and mtDNA genomes and compatibility between M and F mtDNA genomes. To answer these questions we studied individuals from an area in which two mussel taxa are known to exist and hybridize extensively. Consistent with previous observations (PEDERSEN 1991), our results demonstrate that *M. edulis* and *M. trossulus* hybridize in areas of sympatry and that hybrids produce progeny through backcrosses with one or the other species. Yet, the two species appear to be able to maintain their genetic integrity, as evidenced from the existence of nuclear genes that segregate for nonoverlapping sets of alleles and from the fact that they contain different and highly divergent sets of M and F mtDNA molecules (STEWART *et al.* 1995). Maintenance of nuclear integrity in the face of extensive hybridization is not a rare phenomenon (ENDLER 1977). The maintenance of mtDNA integrity is more unique and challenging.

In the surveys of mtDNA variation by RAWSON and HILBISH (1995) and STEWART *et al.* (1995), no *M. edulis* mtDNA type was found in *M. trossulus,* and vice versa. These observations imply that no mtDNA introgression between the two taxa occurs in nature. The present study provides evidence that the introgression is blocked early in the hybridization process. All four males with a heterospecific combination of F and M mitotypes had intermediate species-specific values, which suggests that they were either F_1, F_2 or products of F_1 backcrosses. Also, the two hybrid females whose mtDNA and the majority of nuclear genes did not come from the same species had species-specific values expected from first backcross progeny (Figure 2).

The observation that hybrids with discordant combinations of mitochondrial and nuclear genomes occur in much smaller numbers than expected invites the speculation that a degree of incompatibility exists between mtDNA molecules from one species and nuclear genes from the other. Similarly, the observation that hybrid males with a heterospecific mtDNA combination occur in much smaller numbers than expected invites the speculation that these combinations suffer a disadvantage and are eliminated by natural selection. We note, however, that the incompatibility between M and F types cannot be studied independently of the incompatibility between M or F and the nuclear genome in backcross males. In such individuals the nuclear background will be dominated by one or the other species, so that a male with a heterospecific mtDNA combination will always be exposed to a nuclear-mtDNA incompatibility. The F/M incompatibility can be independently studied in F_2 males whose nuclear background is expected to come from the two species in equal amounts. If F_2 are produced by random mating of F_1, 50% of the F_2 males would carry heterospecific mtDNA

combinations. In our sample four hybrid males could unambiguously be classified as F_2, and all of them carried a homospecific mtDNA combination (Table 3).

Other explanations are, however, possible that may not entail an active role for mtDNA. Assuming that the most probable route to introgression is through a backcross of an F_1 individual to one or the other species, we can identify two symmetrical backcrosses (F_1 females from a *M. edulis* mother crossed to *M. trossulus* and F_1 females from a *M. trossulus* mother crossed to *M. edulis*) that produce sons with heterospecific F/M combinations and daughters with discordant mitochondrial and nuclear genomes. Two other backcrosses (F_1 males from a *M. edulis* mother crossed to *M. edulis* and F_1 males from a *M. trossulus* mother crossed to *M. trossulus*) will also produce sons with a heterospecific combination of mtDNA molecules but will produce daughters with concordant nuclear and mitochondrial DNA (see Table 4). Our results suggest that backcrosses producing heterospecific mtDNA combinations or heterospecific nuclear and mtDNA combinations either do not occur in nature (or occur very rarely) or that their progeny do not survive to adulthood (or survive in low numbers). If these crosses are prevented from happening, the factors that may be responsible for this (ethological, ecological, different times of spawning, gamete recognition, or others) may not be under the influence of mtDNA. Likewise, if progeny from such crosses have low probabilities of survival, this may be the outcome of incompatibilities between interspecific nuclear genes alone.

The actual mechanisms for blocking the passage of mtDNA molecules from one species into the other remain to be resolved. MtDNA introgression is a common occurrence in a wide variety of hybridizing animal species, even among ones whose level of hybridization is lower than that between *M. edulis* and *M. trossulus*. The mussel mtDNA system is, however, profoundly different from that of other species. M and F mtDNA molecules have been evolving along different paths for a long period of time (longer than the origin of the two species; STEWART *et al.* 1995) and their level of divergence of more than 20% (FISHER and SKIBINSKI 1990; HOEH *et al.* 1991; SKIBINSKI *et al.* 1994b; STEWART *et al.* 1995) far exceeds the levels seen among intraspecific molecules or even among mtDNA molecules from different species. They have different patterns of transmission and when they occur in the same individual (males) they have different tissue distributions. These observations imply that the two molecules have acquired special functions that set them apart from each other and from the mtDNA of other animals. The same observations suggest strong nuclear-mtDNA interactions. It is entirely possible that these special function and interactions break down in individuals whose mitochondrial and nuclear genomes come from different, even if closely related, species.

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LITERATURE CITED

AVISE, J. C., J. F. SHAPIRA, S. W. DANIEL, C. F. AQUADRO and R. A. LANSMAN, 1983 Mitochondrial DNA differentiation during the speciation process in *Peromyscus*. Mol. Biol. Evol. **1:** 38–56.

AVISE, J. C., E. BERMINGHAM, L. G. KESSLER and N. C. SAUNDERS, 1984 Characterization of mitochondrial DNA variability in a hybrid swarm between subspecies of sunfish (*Lepomis macrochirus*). Evolution **38:** 931–941.

CARR, S. M., S. W. BALLINGER, J. N. DERR, L. H. BLANKENSHIP and J. W. BICKHAM, 1986 Mitochondrial DNA analysis of hybridization between sympatric white-tailed deer and mule deer in west Texas. Proc. Natl. Acad. Sci. USA **83:** 9576–9580.

CLARK, A. G., and E. M. S. LYCKEGAARD, 1988 Natural selection with nuclear and cytoplasmic transmission. III. Joint analysis of segregation and mtDNA in *Drosophila melanogaster*. Genetics **118:** 471–482.

ENDLER, J. A., 1977 *Geographic Variation, Speciation, and Clines*. Princeton University Press, Princeton, NJ.

FERRIS, S. D., R. D. SAGE, C.-M. HUANG, J. T. NEILSON, U. RITTE *et al.*, 1983 Flow of mitochondrial DNA across a species boundary. Proc. Natl. Acad. Sci. USA **80:** 2290–2294.

FISHER, C., and D. O. F. SKIBINSKI, 1990 Sex-biased mitochondrial DNA heteroplasmy in the marine mussel *Mytilus*. Proc. Roy. Soc. Lond. B **242:** 149–156.

FOS, M., M. A. DOMINGUEZ, A. LATORRE and A. MOYA, 1990 Mitochondrial DNA evolution in experimental populations of *Drosophila subobscura*. Proc. Natl. Acad. Sci. USA **87:** 4198–4201.

GOSLING, E. M., 1992a Systematics and geographic distribution of *Mytilus*, pp. 1–20 in *The Mussel Mytilus: Ecology, Physiology, Genetics and Aquaculture*, edited by E. M. GOSLING. Elsevier, Amsterdam.

GOSLING, E. M., 1992b Genetics of *Mytilus*, pp. 309–382 in *The Mussel Mytilus: Ecology, Physiology, Genetics and Aquaculture*, edited by E. M. GOSLING. Elsevier, Amsterdam.

GOULIELMOS, G., and E. ZOUROS, 1995 Incompatibility analysis of male hybrid sterility in two *Drosophila* species: lack of evidence for maternal, cytoplasmic, or transposable element effects. Am. Nat. **145:** 1006–1014.

GYLLENSTEN, U. B., D. WHARTON and A. C. WILSON, 1985 Maternal inheritance of mitochondrial DNA during backcrossing of two species of mice. J. Heredity **76:** 321–324.

GYLLENSTEN, U. B., D. WHARTON, A. JOSEFSSON and A. C. WILSON, 1991 Paternal inheritance of mitochondrial DNA in mice. Nature **325:** 255–257.

HARRISON, R. G., D. M. RAND and W. C. WHEELER, 1987 Mitochondrial DNA variation in field crickets across a narrow hybrid zone. Mol. Biol. Evol. **4:** 144–158.

HOEH, W. R., K. H. BLAKLEY and W. M. BROWN, 1991 Heteroplasmy suggests limited biparental inheritance of *Mytilus* mitochondrial DNA. Science **251:** 1488–1490.

HOFFMANN, R. J., J. L. BOORE and W. M. BROWN, 1992 A novel mitochondrial genome organization for the Blue Mussel *Mytilus edulis*. Genetics **131:** 397–412.

HUTTER, C. M., and D. M. RAND, 1995 Competition between mitochondrial haplotypes in distinct nuclear genetic environments: *Drosophila pseudoobscura vs. D. persimilis*. Genetics **140:** 537–548.

KILPATRICK, S. T., and D. M. RAND, 1995 Conditional hitchhiking of mitochondrial DNA: frequency shifts of *Drosophila melanogaster* mtDNA variants depend on nuclear genetic background. Genetics **141:** 1113–1124.

LAMB, T., and J. C. AVISE, 1986 Directional introgression of mito-

chondrial DNA in a hybrid population of tree frogs: the influence of mating behaviour. Proc. Natl. Acad. Sci. USA **83:** 2526–2530.

LANSMAN, R. A., J. C. AVISE and M. D. HUETTEL, 1983 Critical experimental test of the possibility of "paternal leakage" of mitochondrial DNA. Proc. Natl. Acad. Sci. USA **80:** 1969–1971.

MACRAE, A. F., and W. W. ANDERSON, 1988 Evidence for non-neutrality of mitochondrial DNA haplotypes in *Drosophila pseudoobscura*. Genetics **120:** 485–494.

MARCHANT, A. D., 1988 Apparent introgression of mitochondrial DNA across a narow hybrid zone in the *Caledia captiva* species complex. Heredity **60:** 39–46.

MCDONALD, J. H., and R. K. KOEHN, 1988 The mussels *Mytilus galloprovincialis* and *M. trossulus* on the Pacific coast of North America. Mar. Biol. **99:** 111–118.

MCDONALD, J. H., R. K. KOEHN, E. S. BALAKIREV, G. P. MANCHENKO, A. I. PUDOVKIN *et al.*, 1990 Species identity of the "common mussel" inhabiting the Asiatic coasts of the Pacific Ocean. Biol. Morya **1990(1):** 13–22.

MCDONALD, J. H., R. SEED and R. K. KOEHN, 1991 Allozyme and morphometric characters of three species of *Mytilus* in the Northern and Southern hemispheres. Mar. Biol. **111:** 323–335.

MILLER, S. A., D. D. DYKES and H. F. POLESKY, 1988 A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. **16:** 1215.

NEI, M., 1972 Genetic distance between populations. Am. Nat. **106:** 283–292.

NIGRO, L., and T. PROUT, 1990 Is there selection on RFLP differences in mitochondrial DNA? Genetics **125:** 551–555.

PEDERSEN, E. M., 1991 Population genetics of mussels from a developing community in Nova Scotia, Canada. M.Sc. Thesis. Dalhousie University, Halifax, Nova Scotia, Canada.

POWELL, J. R., 1983 Interspecific cytoplasmic gene flow in the absence of nuclear gene flow: evidence from *Drosophila*. Proc. Natl. Acad. Sci. USA **80:** 492–495.

SATTA, Y., N. TOYOHARA, C. OHTAKA, Y. TATSUNO, T. K. WATANABE *et al.*, 1988 Dubious maternal inheritance of mitochondrial DNA in *Drosophila simulins* and evolution of *D. mauritiana*. Genet. Res. **52:** 1–6.

SAUMITOU-LAPRADE, P., J. CUGUEN and P. VERNET, 1994 Cytoplasmic male-sterility in plants—molecular evidence and the nucleocytoplasmic conflict. TREE **9:** 431–435.

SCRIBNER, K. T., and J. C. AVISE, 1993 Cytonuclear genetic architecture in mosquitofish populations and the possible roles of introgressive hybridization. Mol. Ecol. **2:** 139–149.

SINGH, R. S., and L. R. HALE, 1990 Are mitochondrial DNA variants selectively nonneutral? Genetics **124:** 995–997.

SKIBINSKI, D. O. F., C. GALLAGHER and C. M. BEYNON, 1994a Mitochondrial DNA inheritance. Nature **368:** 817–818.

SKIBINSKI, D. O. F., C. GALLAGHER and C. M. BEYNON, 1994b Sex-limited mitochondrial DNA transmission in the marine mussel *Mytilus edulis*. Genetics **138:** 801–809.

SOLIGNAC, M., and M. MONNEROT, 1986 Race formation, speciation, and introgression within *Drosophila simulans, D. mauritiana,* and *D. sechellia* inferred from mitochondrial DNA analysis. Evolution **40:** 531–539.

SPOLSKY, C. M., and T. UZZELL, 1984 Natural interspecies transfer of mitochondrial DNA in amphibians. Proc. Natl. Acad. Sci. USA **81:** 5802–5805.

STEWART, D. T., C. SAAVEDRA, R. R. STANWOOD, A. O. BALL and E. ZOUROS, 1995 Male and female mitochondrial DNA lineages in the Blue Mussel (*Mytilus edulis*) species group. Mol. Biol. Evol. **12:** 735–747.

VARVIO, S.-L., R. K. KOEHN and R. VÄINÖLÄ, 1988 Evolutionary genetics of the *Mytilus edulis* complex in the North Atlantic region. Mar. Biol. **98:** 51–60.

WENNE, R., and D. O. F. SKIBINSKI, 1995 Mitochondrial DNA heteroplasmy in European populations of the mussel *Mytilus trossulus*. Mar. Biol. **122:** 619–624.

WU, C.-I., and M. F. PALOPOLI, 1994 Genetics of postmating reproductive isolation in animals. Annu. Rev. Genet. **27:** 283–308.

ZENG, L.-W., and R. S. SINGH, 1993 The genetic basis of the Haldane's rule and the nature of asymmetric male sterility among *Drosophila simulans, Drosophila mauritiana* and *Drosophila sechellia*. Genetics **134:** 251–260.

ZOUROS, E., K. R. FREEMAN, A. O. BALL and G. H. POGSON, 1992 Direct evidence for extensive paternal mitochondrial DNA inheritance in the marine mussel *Mytilus*. Nature **359:** 412–414.

ZOUROS, E., A. O. BALL, C. SAAVEDRA and K. R. FREEMAN, 1994a Mitochondrial DNA inheritance. Nature **368:** 817–818.

ZOUROS, E., A. O. BALL, C. SAAVEDRA and K. R. FREEMAN, 1994b An unusual type of mitochondrial DNA inheritance in the blue mussel *Mytilus*. Proc. Natl. Acad. Sci. USA **91:** 7463–7467.

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