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Sex-Limited Mitochondrial DNA Transmission in the Marine Mussel *Mytilus edulis*

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ABSTRACT

Mitochondrial DNA (mtDNA) was thought to be inherited maternally in animals, although paternal leakage has been reported in mice and Drosophila. Recently, direct evidence of extensive paternal inheritance of mtDNA has been found in the marine mussel Mytilus. We give evidence that whereas female mussels are homoplasmic for a genome that is transmitted to eggs, male mussels are heteroplasmic for this genome and for a second genome that is transmitted preferentially to sperm. The results provide support for the existence of separate male and female routes of mtDNA inheritance in mussels. The two genomes show a base sequence divergence exceeding 20% at three protein coding genes, consistent with long term maintenance of the heteroplasmic state. We propose that the two genomes differ in fitness in males and females, possibly as a result of interaction with nuclear genes.

ALTHOUGH reports of heteroplasmy of mitochondrial DNA (mtDNA) were rare at first, the phenomenon is now thought to be widespread, with heteroplasmic individuals often occurring at high frequencies within populations (BERMINGHAM *et al.* 1986; DENSMORE *et al.* 1985; RAND and HARRISON 1986; SNYDER *et al.* 1987). Most reports of heteroplasmy involve mtDNA molecules that differ in length (HARRISON *et al.* 1985; SOLIGNAC *et al.* 1983; WALLIS 1987).

In theory, many factors might influence the level of mtDNA heteroplasmy. These include mutation rate, paternal leakage, selection at the level of the individual or organelle, the effective number of mtDNA molecules per cell, and the number of cell divisions per organism generation (CLARK 1984; 1988). Several factors have been identified as the cause of high observed levels of length heteroplasmy in animals. These include mutation rates high enough to counteract sorting out (BIJU-DUVAL *et al.* 1991; ARNASON and RAND 1992; BROWN *et al.* 1992), a balance of organelle and individual selection (WALLACE 1992), and a balance between the rate of organelle replication and degradation (VOLZ-LINGENHOHL *et al.* 1992). Because the number of mtDNA molecules within the germ line is low, heteroplasmy will tend to be converted to homoplasmy by sorting out, despite recurrent mutation (TAKAHATA and MARUYAMA 1981; BIRKY *et al.* 1983; SOLIGNAC *et al.* 1984). Thus, there will be little time for genomes within a heteroplasmic line to diverge at the nucleotide level. Consequently, hybridization and paternal transmission between different lineages is thought to be the cause of heteroplasmy for highly diverged mitochondrial genomes (GYLLENSTEN *et al.* 1991; HOEH *et al.* 1991; MAGOULAS and ZOUROS 1993).

The results of backcross experiments (LANSMAN *et al.* 1983; GYLLENSTEN *et al.* 1985) and studies of hybridizing natural populations (LAMB and AVISE 1986) suggested that the inheritance of mtDNA is predominantly maternal. However, paternal transmission does occur in some animals (KONDO *et al.* 1990; GYLLENSTEN *et al.* 1991; KONDO *et al.* 1992) and plants (ERICKSON *et al.* 1989; NEALE *et al.* 1989). In experiments with Drosophila, paternal leakage occurred at a low level, but frequently enough to explain observed levels of heteroplasmy in natural populations (KONDO *et al.* 1990, 1992).

Recent evidence from laboratory crosses suggests extensive paternal inheritance of mtDNA in the marine mussel Mytilus (ZOUROS *et al.* 1992). It appears that this may be the cause of the high incidence of heteroplasmy in mussels collected from diverse geographic locations (FISHER and SKIBINSKI 1990; HOEH *et al.* 1991).

In Mytilus, most heteroplasmic individuals have two genomes called *F* and *M* (FISHER and SKIBINSKI 1990; ZOUROS *et al.* 1992). In this report we give evidence to support the hypothesis (FISHER and SKIBINSKI 1990) that inheritance of the *F* genome is maternal and inheritance of the *M* genome is paternal.

MATERIALS AND METHODS

Construction of clones: For cloning, mtDNA fragments were separated on a 1% agarose gel, excised, and purified using Prepagene (Bio-Rad). The Bluescript vector was used with reagents and protocols supplied by Stratagene. The entire *F* genome was cloned in two *Pst*I fragments obtained from two different females (clones 43 and 48). Fifty percent of the *M* genome was cloned in two *Bam*HI fragments obtained from four different males (clones 25, 27, 32 and 35). Clones 25 and 27 are replicates of the same fragment (but derived from different individuals) as are clones 32 and 35 (see Figure 1B).

DNA mapping and sequencing: The clones were mapped for the enzymes *Ava*I, *Bam*HI, *Eco*RI, *Nco*I and *Xba*I using single and double digests of cloned DNA and separation of fragments on 2.0% agarose gels stained with ethidium bro-

mide. Double-stranded sequencing was carried out on the ends of the cloned fragments using the T7 gene *6* exonuclease protocol and Sequenase 2.0 kit from U.S. Biochemical Corp. and using both Bluescript and mtDNA primers. *Hin*dIII deletion subclones were used to obtain internal sequence from the clones.

Production of gametes: *Mytilus edulis* individuals were collected from Swansea Bay, South Wales (SS624888) in March 1993. Mussels were sexed by microscopic examination of mantle tissue (containing gonads) for the presence of eggs or sperm. Stripped gametes were obtained by placing mantle tissue, scored with a razor blade, in filter-sterilized seawater. After 2 hr, 2–3 ml of seawater were removed and microcentrifuged for a few seconds to remove debris. The supernatant was then checked microscopically for eggs or viable sperm. Gametes were pelleted and resuspended in STE100 (0.1 M NaCl, 0.1 M EDTA, 0.05 M Tris-HCl, pH 8.0). Sperm were also obtained from three male mussels induced to spawn by injection of 3 ml of 0.5 M KCl into the mantle cavity.

DNA extraction: DNA preparations enriched in mtDNA used for *Eco*RI digestions were prepared from whole body tissue or mantle tissue using a CTAB procedure as described previously (Fisher and Skibinski 1990). For eggs and sperm, 20 μl of 10% sodium dodecyl sulfate (SDS) were mixed with 600 μl of gamete suspension, and nucleic acid was purified by phenol/chloroform extraction. Nucleic acid was precipitated with isopropanol at room temperature, washed with 70% then absolute alcohol, dried and dissolved in TE buffer (1 mM EDTA, 0.01 M Tris-HCl, pH 8.0). Genomic DNA was prepared from gill or mantle by chopping finely about 0.25 cm^2 tissue in 50 μl STE100 with a scalpel on a microscope slide. Following transfer to an Eppendorf tube, the volume was made to up to 600 μl with STE100, and nucleic acid was prepared by SDS lysis and phenol/chloroform extraction as above.

Filter hybridization: *Eco*RI-digested mtDNA fragments were separated on 1.0% agarose gels stained with ethidium bromide, then Southern blotted onto nylon membrane (Bio-Rad Zetaprobe). Digoxigenin labeling of cloned mtDNA fragments and hybridization were carried out using chemiluminescence protocols and kits supplied by Boehringer Mannheim. Membranes were washed 2 × 5 min in 2 × SSC, 0.1% SDS at room temperature, then 2 × 15 min in 0.1 × SSC, 0.1% SDS at 68°. The membrane was probed first with the *F* clone, stripped, then reprobed with the *M* clone. For slot blots, 1 μg of gill or sperm DNA and 1 ng of egg DNA were applied per slot. Hybridization was carried out as described above, except that replicate blots for *F* and *M* probes and colorimetric detection methods were used.

Polymerase chain reaction (PCR) amplification: Primers specific for the *F* genome were constructed using sequence obtained by Hoffman *et al.* (1992). Primers specific for the *M* genome were constructed from sequence obtained in this study for clones 25 and 27. The *F* genome primers are 5′-TCTTGGTACAACTGCGGGAA-3′ (COIII gene, nucleotide positions 959–978 of segment 5) and 5′-ACCAAGAAACGGAGGCATC-3′ (ND2 gene, nucleotide positions 21–39 of segment 6). The *M* genome primers are 5′-AAACCCTTCGTCCACAAGG-3′ (homologous with COIII gene, nucleotide positions 806–824 of segment 5) and 5′-AGCCTTTTTGTCATCATTCTGT-3′ (homologous with ND2 gene, nucleotide positions 143–164 of segment 6). About 40 ng of genomic DNA and less than 1 ng of egg DNA was used for PCR amplifications which were carried out in a volume of 50 μl with primers at 0.4 μM, nucleotides at 200 μM, magnesium chloride at 2.5 mM, and *Taq* polymerase (1 unit) and reaction buffer from Promega. Thirty-three cycles were used with denaturation at 94° (for 1 min, but 3 min for first cycle), annealing at 53° (for 30 sec), and extension at 72° (for 1 min 30 sec, but 5 min for final cycle). PCR product (15 μl of each amplification) was visualized on 1.6% agarose gels stained with ethidium bromide.

Restriction analysis of PCR product: Aliquots of PCR amplifications (1–4 μl) were digested with the restriction enzymes *Dde*I and *Rsa*I and the products separated on 6% nondenaturing polyacrylamide gels. For silver staining, gels were washed in 10% ethanol/0.5% acetic acid for 2 × 3 min, in 0.1% silver nitrate for 10 min, rinsed twice in deionized water, then washed in 1.5% sodium hydroxide/0.1% sodium borohydride/0.15% formaldehyde until bands appeared.

RESULTS

DNA maps and sequence: The restriction maps of the *F* and *M* clones (Figure 1C) and the sequence derived from them (Figure 1B, segments a to i) are colinear with the *F* genome segments sequenced by Hoffman *et al.* (1992) (Figure 1A), and with the *a* and *c* genomes mapped by Hoeh *et al.* (1991) (Figure 1C). The *F* and *a* genomes have similar maps and probably have a close ancestor. The *M* and *c* genomes do not have sites of homology distinct from the other maps, thus their relationship is uncertain. The *c* genome is longer than the *M* genome; the likely region of increased length, flanked by *Nhe*I (Nh) and *Nco*I (N) sites, corresponds in position to an unidentified (?) region in segment 1.

Comparisons between the *F* and *M* genome for three protein coding genes (ATPase6, ND2 and COIII) (Table 1), reveal nucleotide substitutions exceeding 20%, which should be contrasted with lower values of 1–2% for comparisons within the *F* and *M* genomes. Lower values for small rRNA are in line with expectations for this more highly conserved gene (Moritz *et al.* 1987; Harrison 1989; Meyer 1992). For the protein coding genes, nucleotide substitutions are more frequent at the third than at the first position, and more frequent at the first than at the second position. Of amino acid replacements, there are 11 non-synonymous and 4 synonymous for ATPase6 (for both segments h and i, Table 1), 8 non-synonymous and 4 synonymous for ND2 (segment g), and 3 non-synonymous and either 5 (segment e) or 4 (segment f) synonymous for COIII.

The data provide no evidence for domains of high and low divergence between the *F* and *M* genomes, as postulated for the *a* and *c* genomes by Hoeh *et al.* (1991). Nor is there evidence that tissue specific methylation (Hoeh *et al.* 1991) might be the cause of the very different restriction maps of the two genomes. The hypothesis that the *M* genome might not be mtDNA but an episome (Fisher and Skibinski 1990) is not supported.

Hybridization experiments: The *F* and *M* genomes both occur in sufficiently large quantity in some individuals to be detectable on agarose gels stained with ethidium bromide (Figure 2, lane 6). Polymorphism of the *F* genome is revealed by using cloned *F* fragments as hybridization probes (Figure 2, lanes 1–4). *M* genome genotypes are detected in a similar fashion with an *M*

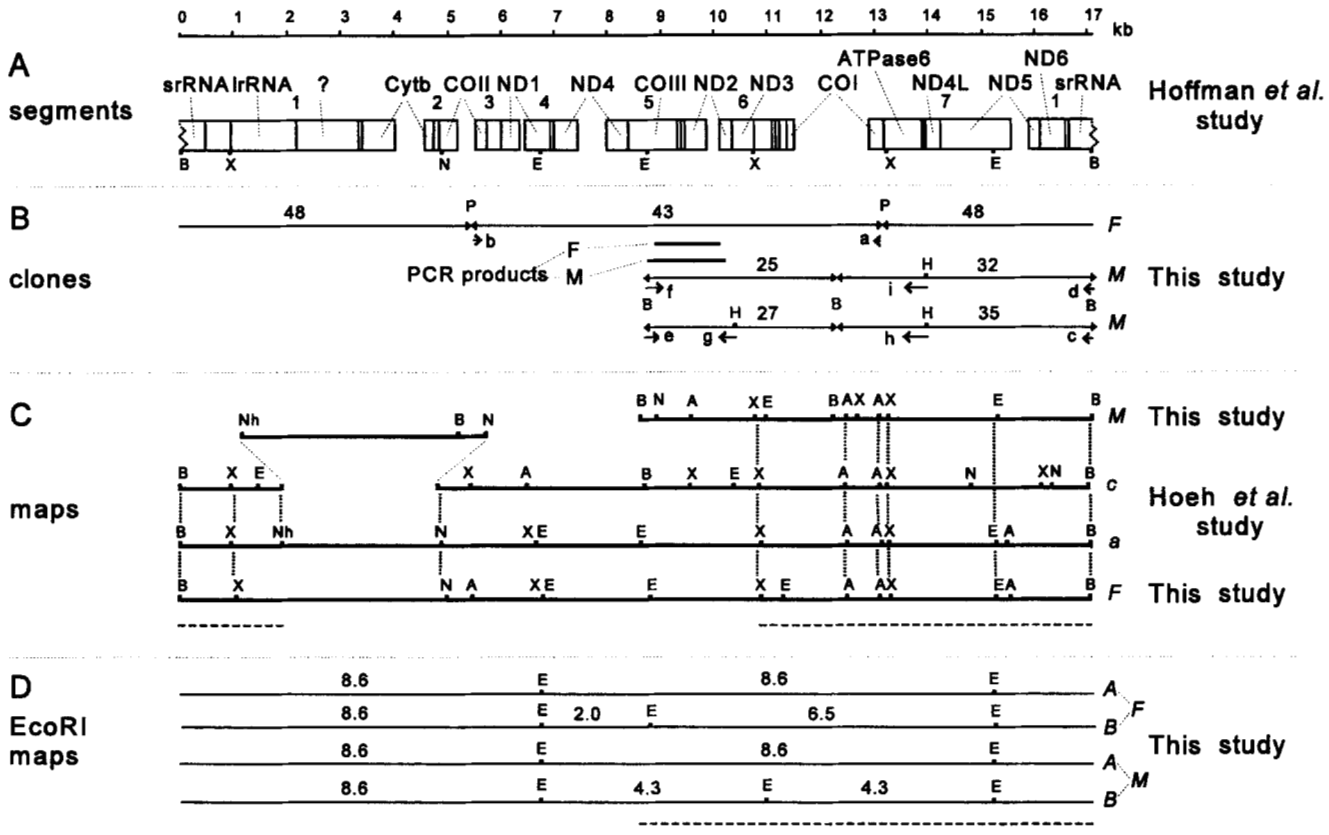

FIGURE 1.—Comparison of Mytilus mtDNA genomes. (A) Segments 1 to 7 (GenBank accession nos. M83756 to M83762) of an entire *F* genome cloned into the phage λEMBL3 vector (SKIBINSKI and EDWARDS 1987) then subcloned and partially sequenced (HOFFMAN *et al.* 1992). The segments are positioned in relation to the maps beneath (in C) using the restriction sites marked underneath the segments. Unlabeled regions are for tRNA or unassigned sequence. (B) The *F* clones (43 and 48) and *M* clones (25, 27, 32 and 35) constructed in this study. The position and extent of sequence (indicated by arrows a to i) and PCR products are shown. (C) Restriction maps for the cloned *F* and *M* genome fragments (this study) and for the *a* and *c* genomes (HOEH *et al.* 1991). Vertical lines link potentially homologous sites. The broken horizontal line indicates a domain with postulated higher homology of the *a* and *c* genomes (HOEH *et al.* 1991). (D) *Eco*RI restriction maps of genotypes *A* and *B* of the *F* and *M* genomes shown in Figure 2. The broken horizontal line marks the cloned region of the *M* genome (see B). [Restriction enzyme sites shown in A–D are *Ava*I (A), *Bam*HI (B) *Eco*RI (E), *Hin*dIII (H), *Nco*I (N), *Nhe*I (Nh), *Pst*I (P), *Xba*I (X).]

probe (Figure 2, lanes 9–12). The interpretation of the banding patterns of Figure 2 is shown in Figure 1D.

In slot blots of genomic DNA, both sexes gave strong *F* signals, but males alone gave a strong *M* signal (Figure 3A). Although both probes gave signals with male somatic DNA, only the *M* probe gave a signal with sperm DNA, suggesting preferential transmission of the *M* genome to sperm. In a similar experiment using genomic DNA from sperm and mantle tissue from three males, all tissues gave an *M* signal, but mantle tissue alone gave an *F* signal (Figure 3B). This suggests that the mechanism underlying the differential transmission operates within mantle tissue during gametogenesis. Females do give a weak *M* signal. This is presumably the result of cross hybridization because the *M* genome is undetectable in females using PCR (see below).

PCR analysis: The *F* and *M* primers are highly specific (Figure 4A) and give a PCR product of the same size with cloned DNA and with DNA extracted from mussels (Figure 4B). Digestion of PCR product with restriction enzymes reveals polymorphisms and band sharing between PCR product from cloned DNA and mussel DNA (see Figure 5A).

PCR analysis using *F* and *M* specific primers gave sex and tissue specific signals (Figure 4B) in line with those obtained in the hybridization experiments (Figure 3A). Both sexes gave a strong *F* signal with DNA from somatic tissue. Males gave an *M* signal for DNA from somatic tissue and sperm, but females gave no *M* signal. No exceptions were observed in a sample of 40 females and 48 males (Table 2). Sperm DNA gave a strong *M* signal, but a much weaker *F* signal (Figure 4B); in some males there was no detectable *F* signal. Consistent results of this kind were observed for 17 stripped males tested (Table 2). The second and third of three spawned males also gave a stronger *F* signal with DNA from mantle tissue than from sperm and a strong *M* signal with both tissues (Figure 4C and Table 2). These results are in line with those obtained in the slot blots (Figure 3B). The weak *F* signal for sperm DNA was often associated with smearing and minor PCR products. Thus the failure to develop a strong *F* signal might be because the *F* genome occurs

TABLE 1

Sequence comparisons within and between *F* and *M* genomes

Sequence comparison			Nucleotide sites compared	Nucleotide differences at codon positions			Estimated nucleotide substitutions[b] (%)
Genome	DNA segment[a]	Gene		First	Second	Third	
Within *F* genome	7 and a	COI	76	0	0	1	1.3
	3 and b	COII	185	0	0	0	0
Within *M* genome	c and d	srRNA	200	—	—	—	0
	h and i	ATPase6	423	0	0	5	1.2
	e and f	COIII	312	2	0	5	2.3
Between *F* and *M* genomes	1 and c	srRNA	217	—	—	—	6.7
	1 and d	srRNA	200	—	—	—	6.8
	7 and h	ATPase6	423	17	6	60	22.7
	7 and i	ATPase6	423	17	6	59	22.4
	6 and g	ND2	186	11	3	29	27.6
	5 and e	COIII	312	12	3	53	25.8
	5 and f	COIII	312	11	3	52	24.9

[a] Segments 1, 3, 5, 6 and 7 (Figure 1A) are from the study of HOFFMAN *et al.* (1992); segments a to i refer to the sequence obtained in this study (Figure 1B). Sequence alignments were carried out using the Gap program from the University of Wisconsin Genetics Computer Group package (DEVEREUX *et al.* 1984). Over all the alignments, only one gap (of one nucleotide) in the comparison of c and d with segment 1 was observed.

[b] Estimated nucleotide substitutions (d) is defined as: $d = -100b \ln(1 - D/b)$, where D is the observed proportion of nucleotide sites in which two sequences differ and $b = 1 - \sum_{i=1}^{4} f_i^2$, *where* f_i is the frequency of the ith of the four bases A, C, G and T in the sequences being compared (TAJIMA and NEI 1984).

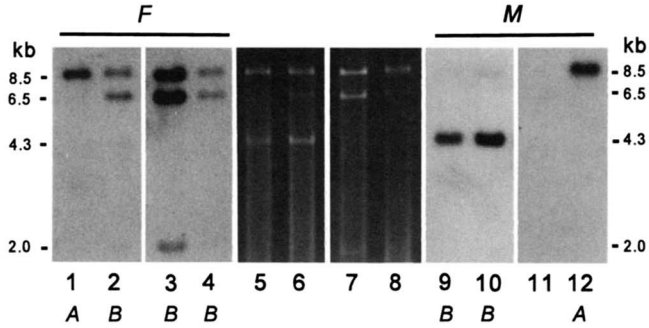

FIGURE 2.—*Eco*RI digests of mtDNA extracted from one homoplasmic female (lanes 3, 7 and 11) and three heteroplasmic males (other lanes). Lanes 5–8: ethidium bromide stained agarose gel; lanes 1–4: Southern blot of the gel probed with *F* genome (clones 43 and 48 pooled); lanes 9–12: same blot probed with *M* genome (clones 25, 27, 32, 35 pooled). Lane 1: *F* genotype *A* (two 8.5-kb fragments); lanes 2–4: *F* genotype *B* (8.5-, 6.5- and 2.0-kb fragments); lanes 9–10: *M* genotype *B* (8.5-kb and two 4.3-kb fragments); lane 12: *M* genotype *A* (two 8.5-kb fragments). The genotypes (*A* or *B*) are indicated beneath the lanes.

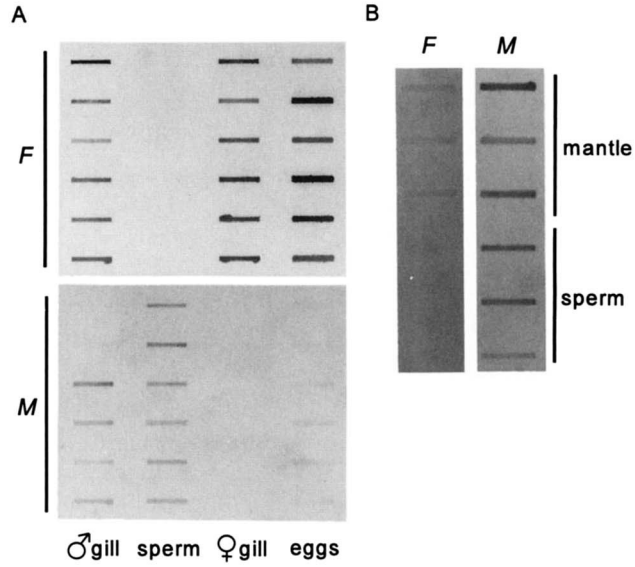

FIGURE 3.—Slot blots. (A) Genomic DNA from gill tissue and either eggs or sperm from each of six female and six male mussels probed with *F* (upper panel) and *M* (lower panel) clones. (B) Genomic DNA from mantle tissue and sperm from three mussels probed with *F* (left panel) and *M* (right panel) clones.

at such a low level in sperm that heterologous priming dominates during PCR amplification.

For four males, the weak *F* band from sperm DNA was excised from the gel and the DNA reamplified using *F* primers. Restriction enzyme analysis with the enzymes *Dde*I and *Rsa*I gave patterns for the reamplified product that were identical to *F* patterns obtained for gill DNA from the same individual mussels (results not shown). This suggests that the weak band is not an artefact and that the *F* genome does occur at a low level in sperm. However, it is possible that the weak *F* signal arises from cellular debris released during the stripping process. This cannot easily explain the stronger *F* than *M* signal with DNA from both mantle tissue and sperm for the first spawned male (Figure 4C). Although the results for the spawned males are few, they suggest that there might be differences between males in the relative proportions of the two mtDNA genomes allocated to sperm.

Within genome heteroplasmy: Heteroplasmy for different *F* genotypes or for different *M* genotypes was not observed in the Swansea mussels when the PCR products from both genomes were digested with the restriction

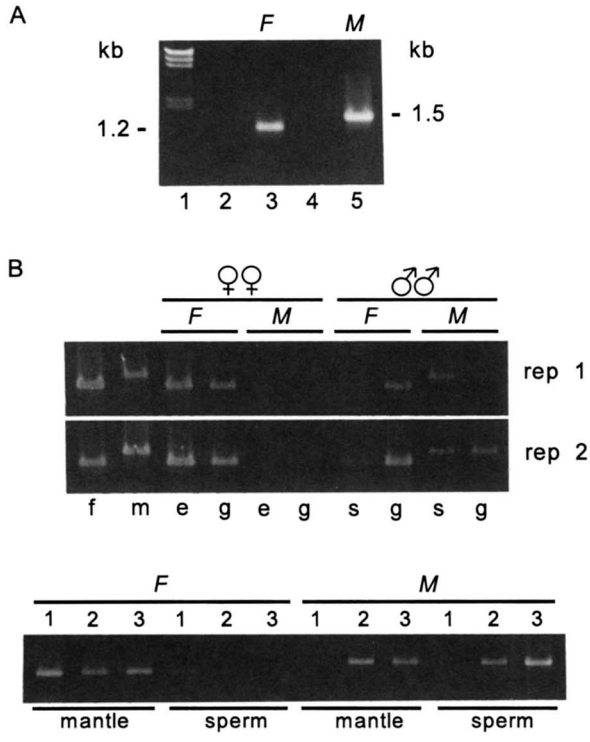

FIGURE 4.—PCR analysis using *F* and *M* specific primers. (A) Lane 1: λ*Hin*dIII marker fragments; lane 2: negative control, *F* primers with *M* DNA (clone 25); lane 3: positive control, *F* primers with *F* DNA (clone 43); lane 4: negative control, *M* primers with *F* DNA (clone 43); lane 5: positive control, *M* primers with *M* DNA (clone 25). (B) Amplifications of DNA from gill (g), eggs (e), and sperm (s) from male and female mussels using *F* and *M* primers. Each lane contains pooled aliquots of equal volume for four different mussels. The experiment is repeated twice with different mussels (reps 1 and 2). Lane f: positive control with *F* primers and cloned *F* DNA. Lane m: positive control with *M* primers and cloned *M* DNA. (C) Amplifications of genomic DNA from mantle tissue and sperm from three spawned mussels (labeled 1, 2 and 3) using *F* and *M* specific primers.

enzymes *Dde*I and *Rsa*I. The possibility that heteroplasmy for the *F* and *M* genomes is more easily detectable because these genomes are highly diverged, that is that there is an ascertainment artefact (CLARK 1988), can be rejected. This is because genotype diversity was high for both genomes (Table 3) and the restriction patterns for most combinations of *F* or *M* genotypes are sufficiently different to make heteroplasmy easily detectable. This is so for the genotypes shown in Figure 5A. The failure to detect within genome heteroplasmy is also unlikely to be a PCR artefact as it proved possible to detect artificially generated heteroplasmy in amplifications using mixed DNA samples (results not shown). In a recent study of geographic variation in other British mussel populations using *F* primers (C. GALLAGHER and D. O. F. SKIBINSKI, unpublished results), four individuals out of 403 were heteroplasmic for different *F* genotypes. The restriction pattern for one of these individuals is shown in Figure 5B. Overall, the data suggest that within genome heteroplasmy does occur, but at a much lower frequency than heteroplasmy involving the *F* and *M* genomes. However the possibility that a high level of heteroplasmy does exist at specific sites within either the *F* or *M* genome, but is undetected by the restriction enzymes used in this study, cannot be ruled out.

Dilution experiments and quantification: Equal amounts of gill DNA was mixed from eight female mussels to make a female genomic DNA pool; gill DNA from eight male mussels was used to make a male pool. In dilution experiments, the *M* genome could be detected by PCR in 100pg of pooled male DNA, but not in 1 μg of pooled female DNA. Thus, if the *M* genome is present in females it occurs at a level less than 10^{-4} of that in males. Agarose gel electrophoresis and ethidium bromide staining was used to quantify nuclear DNA and mtDNA in egg genomic DNA preparations. The staining intensity of the nuclear DNA exceeded that of the mtDNA by about an order of magnitude. Assuming that the Mytilus diploid genome weighs 3 pg (HINEGARDNER 1974), eggs should therefore contain 0.3 pg mtDNA corresponding to about 3×10^8 base pairs and 2×10^4 mtDNA molecules. Given that five mitochondria occur in the sperm midpiece (LONGO and DORNFELD 1967), *F* should outnumber *M* molecules by a factor of about 10^4 in the fertilized egg. Thus the predominance of the *M* over the *F* genome in somatic tissue from some males (for example in Figure 2, lane 6, the 4.3-kb band from the *M* genome is much stronger than the 6.5-kb band from the *F* genome) points to a fitness advantage of the *M* genome in males.

DISCUSSION

Sex-limited transmission and inheritance of mtDNA: This study gives evidence of a qualitative difference in the incidence of heteroplasmy between male and female mussels taken from a natural population. Females are homoplasmic for the *F* genome, males are heteroplasmic for the *F* and *M* genomes. In addition, in males, the *M* genome occurs at a much higher level, relative to the *F* genome, in sperm than in somatic tissue. The results suggest that the *F* genome is transmitted preferentially to eggs and that the *M* genome is transmitted exclusively to sperm.

These results support the hypothesis (FISHER and SKIBINSKI 1990) that the *F* genome is inherited maternally and the *M* genome is inherited paternally. If females lack the *M* genome, males must inherit it from the father. If this happens, and because sperm mitochondria are in a minority in the fertilized egg, there should also be a way of ensuring that the *M* genome is included in sperm in preference to the *F* genome during spermatogenesis. Two mechanisms appear to have been selected in Mytilus. The *M* genome has a fitness advantage during development, and it is transmitted preferentially to sperm.

Crosses provide support for separate routes of inheritance: Paternal inheritance of the *M* genome is sup-

TABLE 2

PCR analysis of DNA from gametes and somatic tissue using genome specific primers (*F* and *M*)

	Gametes (eggs or sperm)		Gill tissue		Mantle tissue		Gill+mantle tissue (mixed)		
	F	*M*	*F*	*M*	*F*	*M*	*F*	*M*	No. of mussels
Stripped females	+	–	+	–					16
Stripped males	–	+	+	+					14
	–	+	+	–					3
Spawned males	–	+			+	+			2
	+	–			+	–			1[a]
Females							+	–	24
Males							+	+	28

The final column gives the number of mussels showing the patterns of tissue and genome specific PCR signals represented by the + and – signs in the rows of the table. +, a strong PCR signal; –, no detectable PCR signal (for females), or weak signal compared with that obtained with other primers for same tissue (for males).

[a] For this male, sperm DNA did give an *M* signal, but this was weaker than the *F* signal.

TABLE 3

Diversity and number of genotypes for *F* and *M* genomes

		Restriction enzyme				
		*Dde*I		*Rsa*I		
	Genome	Genotype diversity	No. of genotypes	Genotype diversity	No. of genotypes	No. of mussels
Females	*F*	0.57	3	0.78	6	20
	M	—	—	—	—	
Males	*F*	0.22	3	0.66	6	17
	M	0.96	13	0.83	9	17

Genotype diversity is defined as: $(n/(n-1))(1-\sum_{i=1}^{k} p_i^2)$, where n is sample size, and p_i is the frequency of the ith of k genotypes (NEI and TAJIMA 1981).

ported by the results obtained from crossing an *M. edulis* female with *M. edulis* and *Mytilus trossulus* males (ZOUROS *et al.* 1992; Figure 1). The *M. edulis* father and some of his progeny possessed the *M* genome 4.1-kb *Eco*RI fragment (shown here in Figure 2 as a 4.3-kb fragment, lanes 5, 6, 9 and 10). The half-sib progeny from the *M. trossulus* father lacked this fragment, but some inherited a different fragment possessed by their father. This result casts some doubt on the alternative hypothesis that the *M* genome occurs at a low level in females, undetectable by PCR, but is nevertheless inherited maternally. It is possible that paternal transmission of the *M* genome occurs more readily in interspecific crosses. Thus in intraspecific crosses, the alternative hypothesis can be tested directly only if population data on genotype frequencies is available. Then, if the *M* genotype of the male progeny matches that of the father, and if this genotype is rare in the population, inheritance from the mother can be confidently excluded.

If it can be demonstrated by DNA methods that the *M* and *F* genomes are absent from eggs and sperm respectively, then the need for crosses is bypassed. Moreover, from the viewpoint of assessing the generality of the phenomenon, analysis of gametes from a single male and female will provide information equivalent to that from investigation of the parents and progeny of a single cross, but demand considerably fewer resources.

Leakage of male and female routes of inheritance: It cannot be assumed at present that the *F* and *M* genomes show exclusive uniparental inheritance. In this study, PCR analysis reveals that the *F* genome does occur in sperm DNA preparations. This suggests some paternal leakage of the *F* genome. If it is assumed that females do inherit the *M* genome from sperm, maternal leakage of this genome also cannot be discounted. In future studies, the estimation of low levels of leakage might best be approached by applying PCR or *in situ* hybridization techniques to individual gametes. The possibility of maternal leakage of the *M* genome receives support from an earlier study (FISHER and SKIBINSKI 1990) in which five out of 99 females, from four populations from southwest England and Scotland, were observed to be heteroplasmic for the *F* and *M* genomes. Despite these results, the observation of high levels of heteroplasmy for diverged genomes in Mytilus populations over a wide geographic area (FISHER and SKIBINSKI 1990; HOEH *et al.* 1991) lends

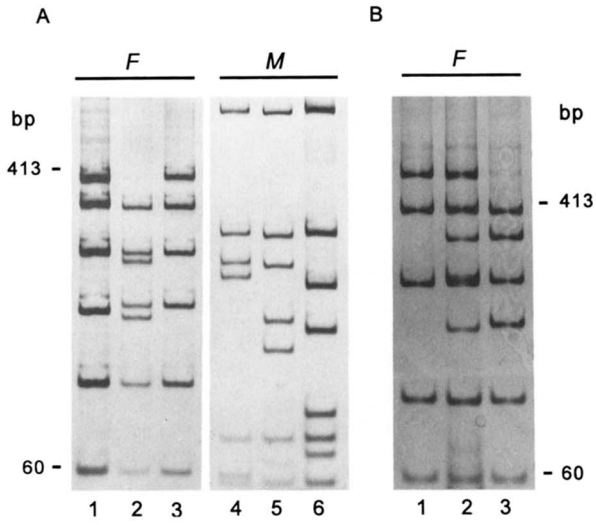

FIGURE 5.—PCR product restriction digest patterns. (A) Silver stained polyacrylamide gels showing *Dde*I *F* genome genotypes (lanes 1–3) and *Dde*I *M* genome genotypes (lanes 4–6) for two males (lanes 1, 4 and lanes 2, 5), and for *F* clone 43 (lane 3) and *M* clone 25 (lane 6). (B) *Dde*I *F* genome genotypes (lanes 1 and 3), and pattern for an individual heteroplasmic for these two genotypes (lane 2).

support to the hypothesis that separate male and female transmission routes are a basic feature of mtDNA inheritance in Mytilus.

High divergence of *F* and *M* genomes: The estimated level of nucleotide substitutions between the *F* and *M* genomes exceeds 20% at three protein coding genes. This is far greater than the 2–3% reported in studies of Drosophila (SATTA *et al.* 1988) and anchovies (MAGOULAS and ZOUROS 1993) where high divergence of mtDNA molecules within heteroplasmic individuals was associated with paternal inheritance. In fact, if mtDNA evolves at a rate of several percent per Myr (BROWN *et al.* 1979; AVISE 1986), the two genomes would have a divergence time that is much more ancient than the Pleistocene ancestor postulated (BARSOTTI and MELUZZI 1968) for *M. edulis* and its closest relative *Mytilus galloprovincialis*. If there are independent maternal and paternal transmission routes, the *F* and *M* genomes could be maintained in a population indefinitely and protected from loss by sorting out. This could result in the evolution of the unusually high level of nucleotide substitutions between the two genomes. The high divergence would be consistent with more recent origin of male heteroplasmy if one of the genomes is derived by interspecific transfer, for which there are precedents in other organisms (POWELL 1983; TEGELSTROM 1987). The study of phylogenetic affinities of mtDNA in related taxa is likely to throw light on the causes of the high divergence between the genomes and the origin of heteroplasmy.

Another consideration when assessing the time of divergence of the two genomes is that the *M* genome could be evolving very rapidly. Sperm mtDNA might suffer a high level of free radical damage (LAMBERT and BATTAGLIA 1993) that could result in a high mutation rate and hence high evolutionary rate for the *M* genome. The high observed genotype diversity for the *M* genome (Table 3) is certainly consistent with a high mutation rate, given that effective population size is not greater for the *M* than *F* genome.

Selfish mtDNA genomes and uniparental inheritance: It has been suggested that Mytilus might possess selfish mitochondria (HASTINGS 1992). Uniparental inheritance of cytoplasm associated with anisogamy might have been selected in evolution because it slows down the spread of selfish and deleterious mtDNA genotypes throughout a population (GRUN 1976; HASTINGS 1992; LAW and HUTSON 1992). In fact, the male sex has recently been defined as ". . . . that which resigns attempts to contribute cytoplasmic genes to the next generation" (HURST and HAMILTON 1992). In this context the discovery of high levels of biparental transmission of mtDNA is surprising. However, the example of *M. edulis* shows that biparental inheritance of mtDNA is consistent with uniparental inheritance of individual mtDNA genomes. If nuclear modifiers causing unconnected male and female mtDNA transmission routes are selected, individual mtDNA genotypes can be confined to either male or female clones. Populations could thus be protected against the spread of selfish and deleterious mtDNA genotypes. It is possible that heteroplasmy in Mytilus is largely absent from females because the potential for leakage is much greater as a result of the large number of mitochondria in eggs. Theoretical models of the relationship between the level of protection afforded and the amount of leakage between routes might in future be tested using empirical estimates of leakage.

In many animals, uniparentally inherited cytoplasmic genes increase their own fitness by causing a sex ratio bias (HURST 1993). In a sample of 160 mussels collected for the present study, 142 could be sexed, and of these 72 were male. The sex ratio in this population is therefore close to 1:1. Thus there is no evidence that selfish behavior of the *M* genome is manifest in a biassing of the population sex ratio.

Sex-specific fitness differences of *F* and *M* genomes: The fitness advantage of the *M* over the *F* genome in males probably involves both faster replication and preferential transmission to sperm. Similar phenomena might occur in Sequoia where pollen mtDNA dominates in the progeny of crosses (NEALE *et al.* 1989). In some human diseases that are caused by mtDNA deletions, the deleted mtDNA molecules have a replicative advantage assumed to be a result of their small size (WALLACE 1992). This same advantage for small mtDNA molecules has been implicated in other animals (SOLIGNAC *et al.* 1984; BOURSOT *et al.* 1987). The *M* genome appears to be of equal length to the *F* genome and to have no large deletions, thus this factor can be excluded.

Any advantage that the *M* has over the *F* genome in males must be suppressed in females. Unless *M* bearing

mitochondria are destroyed in females following fertilization, or their average fitness reduced below that of *F* bearing mitochondria, there would be a danger of the *F* genome being eliminated from populations. The available DNA sequence for both genomes should permit future empirical analysis of the fate of *F* and *M* genomes during male and female development and provide data that can be compared with the results of theoretical models.

Interactions with sex determining factors: The difference in behavior of the *M* and *F* genomes in males and females implies interactions with sex determining factors. The mechanisms of sex determination in Mytilus is unknown but the observation of a 1:1 sex ratio is consistent with a monofactorial switch. Evidence does exist for the evolution of nuclear cytoplasmic interactions involving mtDNA in animals (CLARK and LYCKEGAARD 1988; MACRAE and ANDERSON 1988; MATSUURA 1991; WALLACE 1992). Thus a plausible hypothesis is that the sex differences are caused by interactions between mtDNA and factors associated with a nuclear switch.

Differential replication of mtDNA genotypes within heteroplasmic lines of *Drosophila melanogaster* has been shown to be temperature dependent (MATSUURA *et al.* 1991). Thus it is also possible that the interactions involve environmental stimuli that have a role in sex determination.

Is the *M* genome functional mtDNA?: The *M* genome appears to be functional mtDNA. It is co-linear with the *F* genome and is of normal length. There are no deletions or rearrangments that might be symptomatic of loss of function. The pattern of nucleotide substitution between the *F* and *M* genomes in relation to codon position in regions from three protein coding genes is consistent with the operation of selective constraint and purifying selection, although the possibility that some of the amino acid replacements are caused by positive selection cannot be excluded. What is unknown is whether the *M* genome has a function that is selfishly directed toward increasing the fitness of the *M* genome in males, or whether the function provides a benefit to male mussels beyond that provided by the *F* genome. The high divergence between the two genomes should facilitate future studies of tissue specific and temporal expression. Moreover, it might be possible to relate components of individual fitness to the relative abundance or differential expression of the two genomes. Given the external mode of reproduction in Mytilus there might also be a strong selection pressure in favor of mutants that increase the fitness of sperm. To test this, differences in viability, fertility, motility or other fitness components or fitness related characters of *F* and *M* carrying sperm could easily be measured in the laboratory.

Phylogenetic analysis and population genetics: The analysis of mtDNA has been particularly useful for establishing matriarchal phylogenies of individuals and for estimating divergence times in evolution (WILSON *et al.* 1985; HARRISON 1989; AVISE 1986). The example of Mytilus throws doubt on the assumption that biparental inheritance of mtDNA necessarily weakens these methods. With isolated transmission routes, separate maternal and paternal phylogenies could be constructed. In phylogeographic analysis (AVISE *et al.* 1987; AVISE 1992) broadly similar conclusions should be obtained from *F* and *M* phylogenies unless differences in male and female gametic dispersal or mtDNA effective population size are influential. In population genetic analysis, study of variation at the two genomes might throw light on the the relative importance of male and female gametic dispersal. Furthermore, if panmixia generates random combinations of *F* and *M* genotypes (FISHER and SKIBINSKI 1990), the high levels of diversity for mtDNA should facilitate the identification of recruits.

Is Mytilus a special case?: When, in previous studies of mtDNA variation in Mytilus, ripe female gonads were used as the source of mtDNA (EDWARDS and SKIBINSKI 1987), the *M* genome was not detected. Later, the separate analysis of males and females and the fortuitous high divergence between the genomes led quickly to the detection of sex biased heteroplasmy (FISHER and SKIBINSKI 1990). At present there must remain some uncertainty about whether the mode of mtDNA inheritance reported in this study is rare or whether it occurs widely but has been missed in other species. The search for other examples might well be directed toward species that are closely related to, or that have similar modes of reproduction to Mytilus.

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