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# Heteroplasmy Suggests Paternal Co-transmission of Multiple Genomes and Pervasive Reversion of Maternally into Paternally Transmitted Genomes of Mussel (*Mytilus*) Mitochondrial DNA

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**Abstract.** Marine mussels of the genus *Mytilus* have two types of mitochondrial DNA with separate paternal and maternal inheritance. Females are homoplasmic for an F genome that is transmitted to all offspring, whereas males are heteroplasmic for this F genome and for a highly diverged ( > 20%) M genome that is transmitted only to sons. Here we provide phylogenetic evidence based on lrRNA sequence data that most of the paternal genomes in European *M. trossulus* have an introgressive female *M. edulis* origin and are nearly indistinguishable in sequence from F types of *M. trossulus*. This observation is best explained by the hypothesis that introgressed F type molecules have recently invaded the paternal route and have assumed the role of M molecules, then resetting to zero the time of sequence divergence between M and F lineages. European *M. trossulus* shows a high prevalence of males heteroplasmic for three different mitochondrial DNA types all having the same two paternal types and the same maternal type, consistent with.paternal co-transmission of multiple genomes. Co-transmission of the same genomes must apparently operate uninterruptedly for several generations in spite of the very different evolutionary origin of the specific molecules that are transmitted paternally and maternally in European *M. trossulus*.

**Key words:** *Mytilus* — Mitochondrial DNA — Doubly uniparental inheritance — Heteroplasmy — Paternal leakage

## Introduction

Although strictly maternal inheritance of mitochondrial DNA (mtDNA) has been demonstrated for many species of animals (Avise 1991), an apparent exception has been noted for marine mussels of the genus *Mytilus*. They present a special mode of inheritance in which two highly diverged genomes (>20%) are transmitted separately by males and females (Skibinski et al. 1994a, b; Zouros et al. 1994a, b; Beagley et al. 1997). Females are homoplasmic for an F genome that is transmitted to all offspring, whereas males are heteroplasmic for this F genome and for an M genome that is transmitted only to sons. The occurrence of highly diverged gender-associated mtDNA lineages in freshwater mussels of the family Unionidae and in the venerid clam *Tapes philippinarum* indicates that this doubly uniparental inheritance (DUI) may not be restricted to Mytilidae (Hoeh et al. 1996; Passamonti and Scali 2001). Most of these reports on DUI also show a complex pattern of variation due to the presence of rare animals that display exceptional features. These include females heteroplasmic for an F and an M genome (Fisher and

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Skibinski 1990; Zouros et al. 1994b; Stewart et al. 1995; Quesada et al. 1996; Garrido-Ramos et al. 1998), males apparently homoplasmic for an F genome (Fisher and Skibinski 1990; Zouros et al. 1994b; Rawson and Hilbish 1995b; Wenne and Skibinski 1995; Rawson et al. 1996; Saavedra et al. 1997), and individuals heteroplasmic for three or more genomes (Hoeh et al. 1991). Intraspecific laboratory crosses also indicate the occurrence of rare males showing the standard maternal mtDNA inheritance, as well as the incidental failure of male mtDNA transmission in individuals displaying DUI (Saavedra et al. 1997). The evolutionary dynamics of this mtDNA system is further enigmatic due to the observation that a maternally inherited genome may eventually invade the paternal route becoming what has been termed a masculinized genome (Hoeh et al. 1997; Saavedra et al. 1997). If such masculinized genomes could rise in frequency and quickly replace the formerly paternally transmitted genomes, then the time of divergence between conspecific F and M lineages would be reset to zero. This could result in the failure to find the paternally transmitted mtDNA owing to the difficulty of detecting its extremely small divergence from the female type.

The present study assesses these possibilities in European populations of *Mytilus trossulus*, where many of the above DUI anomalies occur together. In Europe, the maternally inherited F-type of *M. trossulus* has been replaced by the introgressed F-type of *M. edulis* (Quesada et al. 1995b, 1999), whereas mtDNA introgression is blocked in American populations (Rawson et al. 1996; Saavedra et al. 1996). Furthermore, many males appear to be homoplasmic, and observed instances of male heteroplasmy are restricted to genomes differing in size which are closely related to F-type sequences (Wenne and Skibinski 1995; Quesada et al. 1999). Here we ask how prevalent is the introgression of the *M. edulis* M genome into European *M. trossulus* populations, and whether genomes that were formerly maternally inherited have invaded the male germ line and replaced the previously existing paternally transmitted genomes. We attempt to address these questions by assessing mtDNA heteroplasmy and phylogenetic relationships among paternal and maternal genomes in European *M. trossulus* and in control *M. edulis* individuals.

## Materials and Methods

### *Specimen Collection and DNA Extraction*

Adult mussels were collected from two European localities where, on the basis of allozyme data, only *M. trossulus* (Gdansk, Southern Baltic) or *M. edulis* (Swansea, South Wales) are known to occur (Skibinski et al. 1983; Väinolä and Hvilson 1991; Wenne and Skibinski 1995). These two sampling localities are geographically distant from regions where intermixing occurs with other *Mytilus* taxa (Skibinski et al. 1983; Väinolä and Hvilson 1991). The specimens were sexed by microscopic analysis of the gonads whenever they had attained reproductive maturity. DNA preparations enriched in mtDNA were prepared from whole body tissue using a CTAB procedure as described previously (Fisher and Skibinski 1990). Genomic DNA was also extracted from gill tissue following Skibinski et al. (1994b).

### *Genome-Specific Methods for Detection of M and F mtDNA*

M and F mtDNA types similar in sequence to those found in *M. edulis* were detected by the PCR technique using the F genome-specific primers FOR1 and REV1, and the M genome-specific primers FOR2 and REV2 (Skibinski et al. 1994b; Quesada et al. 1998). These two sets of primers have been tested in European populations of *M. edulis* and *M. galloprovincialis* and found to amplify fragments covering parts of the *ND2* and *COIII* genes of 1.5-Kb (M type) and 1.3-Kb (F type) (Skibinski et al. 1994b; Quesada et al. 1998). PCR conditions were as described by Quesada et al. (1998).

The PCR approach described above is not sensitive in detecting M and F mtDNA types in which the M and F specific priming sites tested might be absent. To overcome this difficulty, we also performed a dot-blot experiment. Total DNA (20 μg) from highly enriched mtDNA extractions was digested with *Eco*RI, separated on 1% agarose gels, and blotted on a negatively charged nylon membrane (Bio Rad) using a BIO-Dot SF microfiltration apparatus. Cloned inserts obtained by Skibinski et al. (1994b) and covering different genes of the *M. edulis* F mtDNA type (clone 48: srRNA, lrRNA, and *Cytb*) and M mtDNA type (clones 32 + 35: *COI*, *ATPase6*, *ND4L*, *ND5*, *ND6*, and srRNA) were used as hybridization probes. These genome-specific probes demonstrated to successfully discriminate the M and F mtDNA types of European populations of *M. edulis* and *M. galloprovincialis* (Skibinski et al. 1994b; Quesada et al. 1995a). Digoxigenin labeling of mtDNA probes by random primed synthesis and hybridization were carried out using colorimetric protocols and kits supplied by Boehringer-Mannheim. Clones 48 and 35 were also used as internal controls against scoring an individual as lacking the M or F mtDNA type when in fact neither the F nor the M clones could be detected due to technical error. Washing after hybridization was carried out under conditions denoted as high stringency following Fisher and Skibinski (1990).

### *lrRNA Characterization of M and F mtDNA*

The triple restriction digest approach of Rawson and Hilbish (1995a, b) to characterize M and F mtDNA types in European and American *Mytilus* taxa was used in this study. For this procedure, a 527-bp fragment of the large subunit ribosomal RNA (lrRNA) gene was amplified using the universal lrRNA primers AR and BR (Palumbi et al. 1991). The PCR reaction, the PCR cycling conditions and the digestion of PCR products with *Spe*I, *Hae*III, and *Eco*RV were as in Rawson and Hilbish (1995a). Digestions were separated on 6% polyacrylamide gels and visualized with silver-staining. This lrRNA RFLP assay was repeated twice for each individual to eliminate the possibility of misscoring due to partial digestions.

At least one gene fragment of every haplotype detected in each species using the lrRNA triple digestion approach was sequenced. Two separate sequencing reactions were performed using independently obtained lrRNA PCR products to sequence one strand using the Thermo Sequenase cycle sequencing kit (Amersham) on an ALF automated sequencer (Pharmacia). DNA from heteroplasmic males

**Table 1.** Hybridization and PCR analyses using genome-specific methods (for F and M) in two European populations

| Taxa | Sex | Hybridization | | | PCR | | |
|---|---|---|---|---|---|---|---|
| | | F | M | $n^a$ | F | M | $n$ |
| *M. trossulus* | Male | + | – | 8 | + | – | 19 |
| | Female | + | – | 6 | + | – | 19 |
| | Unsexed | + | – | 15 | + | – | 15 |
| *M. edulis* | Male | + | + | 5 | + | + | 10 |
| | Female | + | – | 5 | + | – | 10 |

[a] $n$ is the number of mussels showing the patterns represented by the + (present) and – (absent) signs. For hybridization, only a subset of the sample was assayed, and M signal was considered as absent if not present or weak.

could not be amplified and sequenced directly because of the presence of a second mtDNA type. To overcome this difficulty, two different approaches were used. First, a nested PCR method was used to sequence the male-specific haplotype from individuals heteroplasmic for two different genomes. In this procedure, DNA extracted from male mussels was amplified using lrRNA primers as described above. The lrRNA PCR product was subsequently double digested with *Spe*I and *Eco*RV. These enzymes did not cut the male-specific fragment but did cut the other fragments (Rawson and Hilbish 1995a). After separation of the digest on 6% polyacrylamide gels and ethidium bromide staining, the large uncut PCR product was gel purified and used as a source for a new lrRNA PCR amplification. The reamplified PCR product was then sequenced after confirming its restriction pattern by the lrRNA triple digestion approach. Second, a single stranded conformation polymorphism (SSCP) approach was used to sequence haplotypes in males that were found to be heteroplasmic for three different genomes. In this method, PCR products were denatured at 95°C for 10 min, chilled on ice for 10 min, and loaded onto 10% vertical polyacrylamide gels cooled at 10°C with a circulator bath (Hoefer RCB 500). Gels were run at 300 V for 24 h with TBE pH 8.3 buffer (0.09 M trizama base, 0.09 M boric acid, 0.002 M EDTA), and then the resulting bands were gel purified. Each band elution from the same individual was reamplified using lrRNA primers and sequenced after checking its RFLP pattern using the lrRNA triple digestion approach.

### *Mitochondrial Origin of PCR-Based lrRNA Haplotypes*

Nuclear copies of mtDNA sequences (Numts) may complicate the interpretation of mtDNA data by contaminating PCR-based mitochondrial assays, although no evidence exists for their occurrence in Mollusca (Bensasson et al. 2001). Numts come in many sizes, but they are mostly fragments of genes often less than 150 bp (Blanchard and Lynch 2000). The lrRNA gene contains an internal *Hae*III cut site that is common to all haplotypes (Rawson and Hilbish 1995a) and that is flanked by upstream and downstream *Hae*III cut sites in tRNA mitochondrial genes located at distances of 1.2 kb and 1.6 kb, respectively (Hoffmann et al. 1992). Thus, digestion of genomic DNA with *Hae*III should produce, amongst others, only two mtDNA fragments covering parts of the lrRNA gene and of a size of 1.2 kb and 1.6 kb. For this analysis, *Hae*III restriction digests of 20 μg of the purified genomic DNA were made according to the manufacturer's instructions. The fragments were subsequently separated in a 1% agarose gel and were transferred to Hybond N+ nylon membrane (Amersham Pharmacia) following manufacturer's instructions. Probes of lrRNA were generated by PCR following the above amplification protocol and $^{32}P$ labeled using the Rediprime II Kit (Amersham Pharmacia). Hybridization of filters was with Church and Gilbert (1984) hybridization buffer at 68°C for 16 h. Filters were subsequently washed in 25 m*M* phosphate buffer pH 7.2 and 0.7% SDS at 65°C before exposure to X-ray film.

### *DNA Analysis*

The nine newly obtained lrRNA sequences were aligned using the Clustal W program (Higgins et al. 1996) with lrRNA sequences previously obtained by Rawson and Hilbish (1995a, 1998) of European and American populations of *M. trossulus*, and American *M. edulis*. Only published sequences from pure specimens of each taxa were included in this analysis. An additional European *M. edulis* lrRNA sequence was obtained from Hoffmann et al. (1992). A neighbor-joining tree was generated from a matrix of genetic distances based on Jukes-Cantor correction for multiple hits using MEGA version 2.1 (Kumar et al. 2001). A maximum parsimony tree was also constructed using a heuristic search with simple stepwise addition of taxa and tree-bisection reconnection branch swapping option in effect, as implemented in PAUP 3.1.1 (Swofford 1993). The level of support for branching patterns in phylogenetic trees was determined by 1000 bootstrap replications of the original data. The published lrRNA mtDNA sequence data of *Perna canaliculata* was used as an outgroup because the dichotomy between M and F mtDNA does not exist in *Perna* (Rawson and Hilbish 1995a). The association between gender and lrRNA RFLP genotypes was tested with the Monte Carlo method of Roff and Bentzen (1989).

## Results

### *No Detection of Introgressed M mtDNA Type in European* M. trossulus

Slot blots of highly enriched mtDNA extractions produced the expected strong signal for the occurrence of the F mtDNA type in all the assayed males and females of *M. edulis* and *M. trossulus*, but *M. edulis* males alone gave a strong signal for the occurrence of the M mtDNA type (Table 1 and Fig. 1). Some *M. trossulus* males gave a faint M-type signal, but of a lower intensity to that obtained in some females of *M. trossulus* and *M. edulis*. Accordingly, some F clones gave a faint M-type signal (Fig. 1). Thus, these weak M-type signals were considered as the result of cross-hybridization, as reported elsewhere (Skibinski et al. 1994b). In line with the slot blot analysis, *M. trossulus* males gave no M-type PCR signal, *M. edulis* males gave a strong M-type signal, and the F-type signal was strong in males and females of both taxa. No exceptions to this PCR pattern were observed in any of the 73 European specimens analyzed.

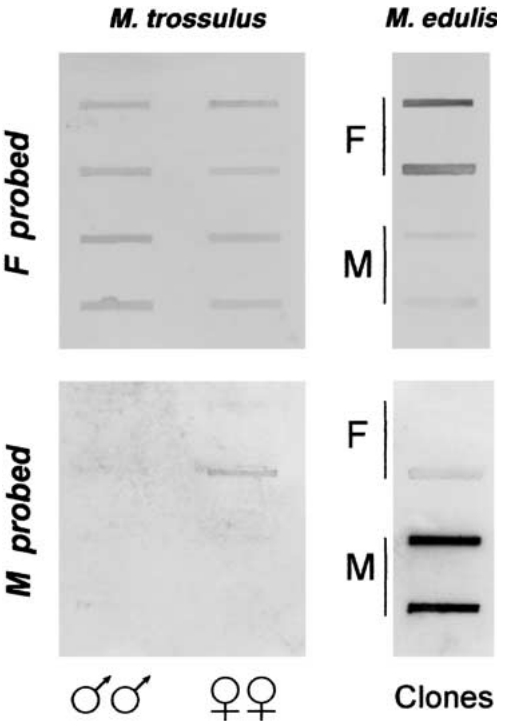


**Fig. 1.** Slot blots of highly enriched mtDNA extractions from *M. trossulus* males and females (left panels). The blots were probed with digoxigenein labeled DNA from *M. edulis* cloned fragments of F genome (upper panels) and M genome (lower panels). The same clones used as probes were blotted as a control (right panels).

Two additional assays were performed to test further the possibility that the lack of an M-type signal in European *M. trossulus* could be due to the fact that this genome occurs at a very low dose and thus could remain undetected. First, different dilutions of a subset of ten PCR products obtained from the highly enriched male mtDNA extractions were reamplified using M genome-specific primers. Second, for the same ten *M. trossulus* males, PCR was repeated for each individual using serial dilutions of gill tissue DNA extractions ranging from 1 ng to 1000 ng. Serially diluted DNA samples purified from gill tissue have shown that the sensitivity of our genome-specific PCR approach is such as to be able to detect *M. edulis* M-type genome in 100 pg of genomic DNA (Skibinski et al. 1994b). Both analyses provided no evidence of M-type signal on silver stained polyacrylamide gels. In summary, these genome-specific assays indicate that introgressed *M. edulis* M mtDNA type does not exist or is very rare in European *M. trossulus*, and that very different paternally transmitted mtDNA types occur in European populations of both taxa.

*Sex-Biased lrRNA Haplotypes in* M. trossulus

Restriction digestion of lrRNA PCR products obtained from highly enriched mtDNA extractions of all the sexed *M. trossulus* mussels and a subset of 14 sexed *M. edulis* specimens used as controls produced four main distinct restriction patterns that were labeled A through D (Fig. 2), according to Rawson and Hilbish (1995a). Haplotypes B, C, C2, and D were found exclusively in *M. trossulus*, haplotype C1 was exclusive to *M. edulis*, and haplotype A was found in both species (Table 2). Haplotypes C1 and C2 lack the single *Sac*I restriction site present in haplotype C (Rawson and Hilbish 1995b). The largest bands in C1 and C2 also display a slightly lower electrophoretic mobility than the largest band in C (Fig. 2) even though both uncut PCR products and sequencing reveal no size differences between these haplotypes. However, there are sequence differences between them, suggesting conformational shape differences among fragments (e.g., Singh et al. 1987; Vigilant et al. 1988). Previous studies in world-wide *Mytilus* populations did not assess the possibility for Numts using lrRNA primers (Rawson and Hilbish 1995a, b, 1998; Rawson et al. 1996, 1999; Hoeh et al. 1997; Hilbish et al. 2000; Ladoukakis et al. 2002). In this work, several observations fail to provide evidence of Numts for the lrRNA gene, thus it seems most unlikely that they could contaminate accidentally lrRNA PCR-based amplifications from highly enriched mtDNA extractions. First, no ghost bands were found in PCR amplifications from both genomic and highly enriched mtDNA extractions. Second, no indels were encountered when sequencing the lrRNA haplotypes. Third, no extra bands were observed in the restriction profiles of any of the 24 *M. trossulus* and *M. edulis* individuals tested in southern blots of genomic digestions with *Hae*III. Furthermore, *Hae*III genomic digestions produced, in all cases, the double-band restriction pattern of 1.2 kb and 1.6 kb predicted from the complete mtDNA sequence (Hoffmann et al. 1992), as opposed to what would be expected if one or two of the flanking tRNA *Hae*III cut sites were lost as a result of a nuclear insertion of the lrRNA gene (Fig. 3).

Gender and lrRNA haplotype combinations are strongly and significantly associated in both species as revealed by a contingency table of independence (genotype × sex) when using the Monte Carlo method and a total of 10,000 permutations per test ($p < 0.001$; Table 2). Within *M. trossulus*, haplotypes C and D are found only in heteroplasmic males and display a significant male association when tested against all other pooled (PL) haplotypes in a 2 × 2 (haplotype × sex) contingency table (data for 55 *M. trossulus* haplotypes: $[C+D]_{male} = 10$; $[C+D]_{female} = 0$; $PL_{male} = 25$; $PL_{female} = 20$; $p < 0.01$). In *M. trossulus*, haplotype A appears in the homoplasmic condition in males and females, whereas haplotype B appears in the homoplasmic condition only in females [data for 25 homoplasmic (H) *M. trossulus* individuals: $HA_{male} = 7$; $HA_{female} = 10$; $HB_{male} = 0$;

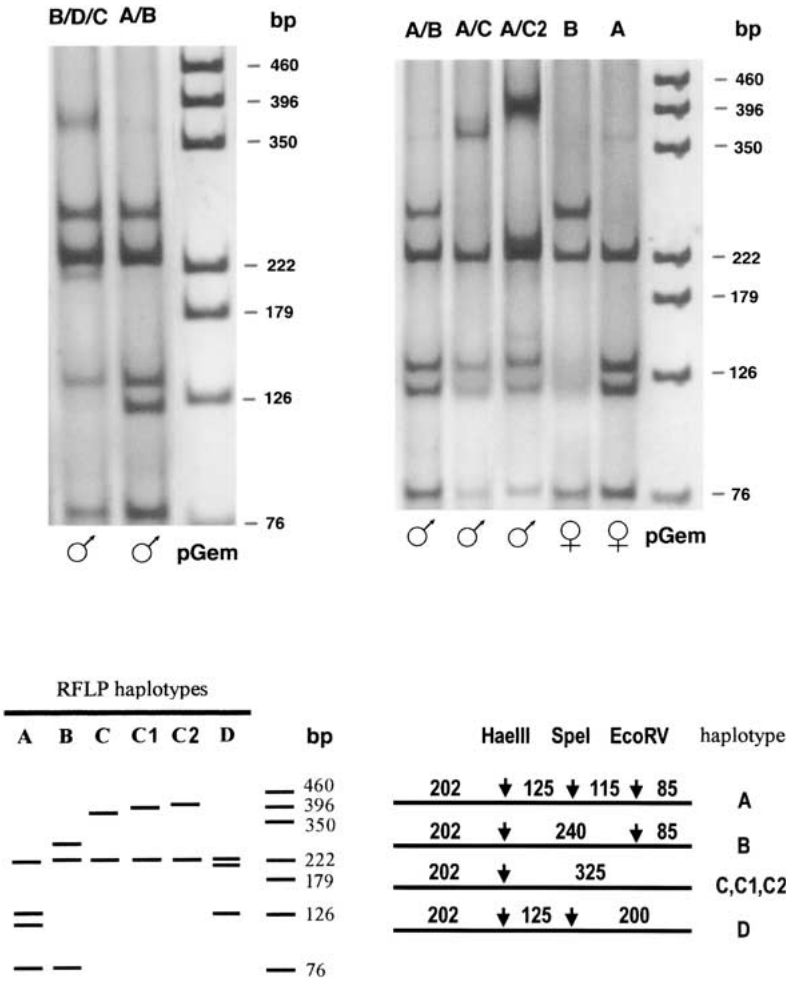


**Fig. 2.** Restriction fragment analysis of lrRNA PCR products when digested with the restriction enzymes *Hae*III, *Spe*I, and *Eco*RV. Fragments were separated on 6% polyacrylamide gels and visualized with silver staining. The RFLP haplotype is indicated on each lane. The molecular standard PGEM is used as a fragment marker. Upper panels, heteroplasmic males and homoplasmic females of *M. trossulus*. Lower panel (left), schematic representation of the restriction profiles. Lower panel (right), restriction sites for each haplotype as proposed by Rawson and Hilbish (1995a). The restriction sites were confirmed in the European samples analyzed by sequencing.

**Table 2.** Frequency of lrRNA RFLP genotypes for European *M. trossulus* and control *M. edulis* mussels

| | *M. trossulus* | | *M. edulis* | |
|---|---|---|---|---|
| | Male | Female | Male | Female |
| A | 7 | 10 | 0 | 8 |
| B | 0 | 8 | 0 | 0 |
| A/B | 5 | 1 | 0 | 0 |
| A/C | 2 | 0 | 0 | 0 |
| A/C1 | 0 | 0 | 6 | 0 |
| A/C2 | 1 | 0 | 0 | 0 |
| B/D/C | 4 | 0 | 0 | 0 |
| $p^a$ | $< 0.001$ | | $< 0.001$ | |
| Homoplasmic | 7 | 18 | 0 | 8 |
| Heteroplasmic | 12 | 1 | 6 | 0 |
| $p^a$ | $< 0.001$ | | $< 0.001$ | |

[a] Probabilities of (genotype × sex) contingency tables (Roff and Bentzen 1989).

$HA_{female} = 8$; $p < 0.05$]. In *M. edulis*, haplotype C1 is male biased (data for 20 *M. edulis* haplotypes: $C1_{male} = 6$; $C1_{female} = 0$; $PL_{male} = 6$; $PL_{female} = 8$; $p < 0.05$), whereas haplotype A is found in both sexes but only in the homoplasmic condition in females ($p < 0.001$; Table 2). Given that females lack the C (C, C1, C2) and D haplotypes, males will inherit them from their fathers. We conclude from these data that haplotypes C (C, C1, C2) and D correspond to paternally transmitted haplotypes, whereas haplotype B corresponds to the maternally transmitted haplotype. Haplotype A is associated with females in *M. edulis* indicating that it is always maternally transmitted in this taxon. This cannot be concluded

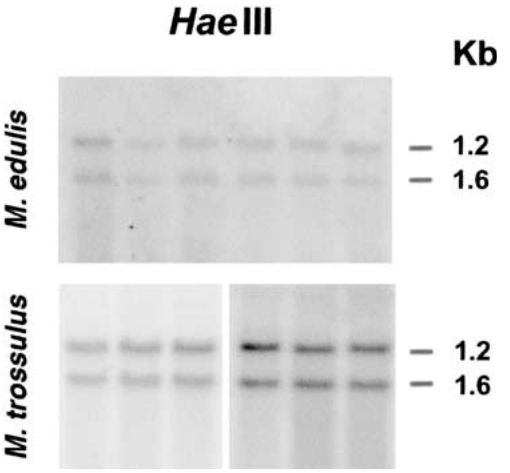


**Fig. 3.** Southern hybridization patterns of *M. trossulus* and *M. edulis* genomic DNA digested with *Hae*III and detected with a $^{32}P$ labeled lrRNA probe. Only the double-banded pattern of 1.2 kb and 1.6 kb expected from the complete mitochondrial DNA sequence was observed in all the 24 individuals tested.

in *M. trossulus*, where it appears associated with both males and females, consistent with a recent masculinization event in this species (Quesada et al. 1999).

### *Phylogenetic Relationships Among lrRNA Haplotypes*

In total, we compared 35 lrRNA sequences from American and European populations of *Mytilus trossulus* and *M. edulis* for 416 nucleotides. The nine sequences generated in this study are available from the EMBL nucleotide sequence database under accession numbers AJ293730–AJ293738. Similar topologies were obtained using the neighbor-joining and parsimony methods, thus for brevity the neighbor-joining method is presented (Fig. 4). As expected, lrRNA sequences were grouped into an F and an M cluster. However, a number of unusual features are observed for European *M. trossulus* sequences. First, within the F cluster, maternal sequences of European *M. trossulus* are closely related with maternal sequences of European and American *M. edulis*, but are grouped separately from maternal sequences of American *M. trossulus*. This is consistent with asymmetric introgression of maternal mtDNA from *M. edulis* to *M. trossulus* in Europe but not in America, as discussed elsewhere (Quesada et al. 1999). Second and most importantly, European *M. trossulus* sequences corresponding to the paternally transmitted haplotypes D (T-2) and C (T-3, T-4) cluster within the F clade instead of within the M clade. This indicates that these two haplotypes correspond to masculinized genomes, i.e., maternally transmitted molecules that have invaded the paternal lineage. Their sequence divergence with other F-type sequences ranges from 0.2% to 2.5% (Table 3), in

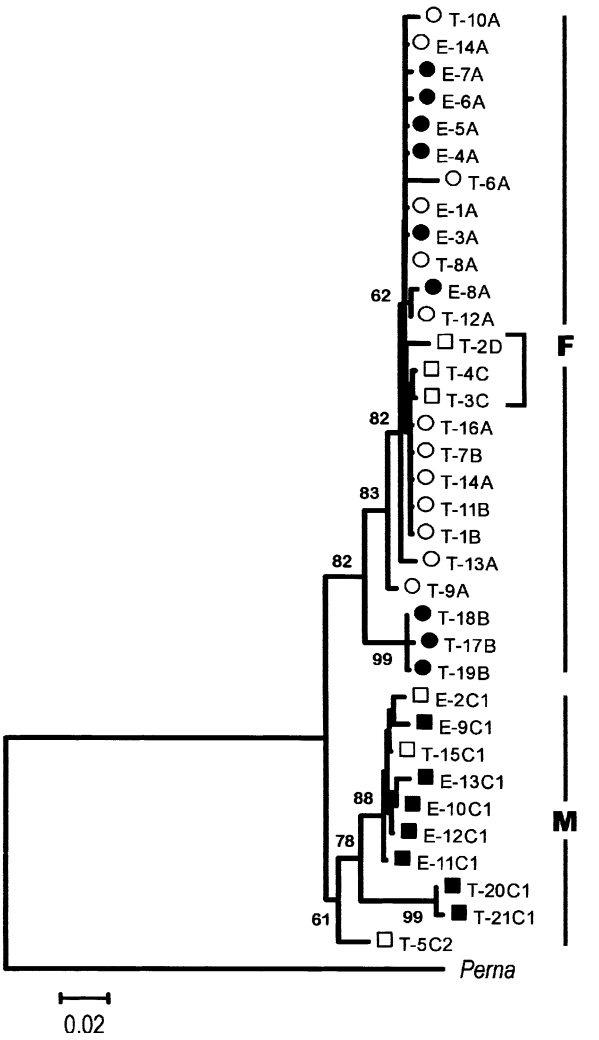


**Fig. 4.** Neighbor-joining phylogeny of European and American lrRNA mtDNA sequences of *Mytilus trossulus* and *Mytilus edulis*. The tree is based on 416 nucleotides and is rooted using *Perna* as an outgroup. Only bootstrap values higher than 60% are presented. Sequences are indicated by an alphanumeric code indicating the taxa (T, *M. trossulus*; E, *M. edulis*), the number of the sequence, and the corresponding RFLP haplotype (A, B, D, C, C1, and C2). The correspondence between European RFLP pattern and lrRNA sequence is as in Table 3. Maternal types are depicted as circles, and paternal types are depicted as squares. European sequences appear as open, and American sequences appear as solid. The bracket indicates the location of the newly masculinized genomes. Sequences T-8 to T-21 and E-3 to E-13 are from Rawson and Hilbish (1995a, 1998). Sequence E-14 is from Hoffmann et al. (1992).

contrast with the 8% average divergence among paternally and maternally transmitted genomes of conspecific American *M. trossulus* populations (Rawson and Hilbish 1995a). In European *M. trossulus*, the only paternally transmitted haplotypes that are grouped within the highly diverged M cluster are C2 (T-5) and the C1 haplotype (T-15) previously detected by Rawson and Hilbish (1998) in one male from an Eastern Baltic population (Aland Islans).

**Table 3.** Jukes-Cantor corrected estimates of genetic distances (below diagonal, standard errors above diagonal) between the European lrRNA RFLP patterns for which the DNA sequence was obtained

| RFLP | Gender assoc. | Sequence | T-1 | T-2 | T-3 | T-4 | T-5 | T-6 | T-7 | E-1 | E-2 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| B | fem. | T-1* | | 0.006 | 0.003 | 0.003 | 0.012 | 0.007 | 0.002 | 0.004 | 0.015 |
| D | male | T-2* | 0.012[a] | | 0.006 | 0.005 | 0.011 | 0.008 | 0.006 | 0.006 | 0.015 |
| C | male | T-3* | 0.005 | 0.012 | | 0.002 | 0.012 | 0.007 | 0.002 | 0.004 | 0.015 |
| C | male | T-4 | 0.005 | 0.010 | 0.002 | | 0.011 | 0.007 | 0.002 | 0.004 | 0.014 |
| C2 | male | T-5 | 0.055 | 0.051 | 0.053 | 0.050 | | 0.013 | 0.012 | 0.012 | 0.010 |
| A | fem./male | T-6 | 0.020 | 0.025 | 0.020 | 0.020 | 0.067 | | 0.007 | 0.006 | 0.016 |
| B | fem. | T-7 | 0.002 | 0.012 | 0.002 | 0.002 | 0.053 | 0.018 | | 0.003 | 0.015 |
| A | fem. | E-1 | 0.007 | 0.012 | 0.007 | 0.007 | 0.053 | 0.015 | 0.005 | | 0.014 |
| C1 | male | E-2 | 0.084 | 0.080 | 0.082 | 0.079 | 0.042 | 0.092 | 0.082 | 0.079 | |

* T1, T2, and T3 were isolated from the same heteroplasmic male.
T, *M. trossulus*; E, *M. edulis*.
[a] Comparisons of newly masculinized D and C types with *M. trossulus* female types are underlined.

However, whereas the C1 haplotype is closely affiliated with the *M. edulis* paternal sequences, the C2 haplotype clusters into a separate and ancestral paternal clade, although this clade is poorly supported by bootstrap (Fig. 4). Sequences represented by haplotypes A (T-6 and E-1) and B (T-1, T-7) were grouped within the F clade. In conclusion, heteroplasmy for two or more highly similar genomes is a prevalent feature of most European *M. trossulus* males (Tables 2 and 3 and Fig. 4). Therefore, when a highly diverged M-type is not detected in a heteroplasmic male, then the male carries a second genome that is similar in sequence to the maternally transmitted F-type of another species, *M. edulis*.

## Discussion

### *Replacement of Old Paternal mtDNA Lineages by Introgressed Maternal Genomes That Have Recently Invaded the Paternal Route*

The data summarized here provide clear evidence for the widespread occurrence of gender-associated haplotypes and sex-biased heteroplasmy in European *M. trossulus* (Table 2), as expected from the existence of separate paternal and maternal transmission routes. If male- and female-specific haplotypes are demonstrated by population data, then the need for crosses to support separate paternal and maternal inheritance is redundant in the absence of a plausible alternative hypothesis to DUI (Skibinski et al. 1994b; Ladoukakis et al. 2002). Moreover, population data allows assessing the generality of the phenomenon within the population under study. This work confirms that the native European *M. trossulus* F genome has recently been replaced by the *M. edulis* F genome (Quesada et al. 1999) and, most importantly, demonstrates that most of the European *M. trossulus* M genome has also been replaced by F molecules of an introgressed maternal *M. edulis* origin. This is supported by phylogenetic trees indicating that, in European *M. trossulus*, all maternal haplotypes and the majority of male-specific haplotypes are very similar to, and cluster together with, F *M. edulis* haplotypes, whereas they cluster separately from conspecific F or M American *M. trossulus* haplotypes that are not disturbed by introgressive hybridization (Fig. 4). This pattern resembles that expected from a masculinization event, and suggests that introgressed molecules of the F lineage invaded the paternal transmission route resulting in males that carry two F-type molecules. The replacement appears to be in progress, since highly diverged M types can still be found. The rare and highly diverged C2 paternal haplotype (T-5; Fig. 4) corresponds most likely to the native and ancestral (Väinolä and Hvilson 1991) European *M. trossulus* M genome, whereas the single *M. trossulus* paternal haplotype (T-15; Fig. 4) reported by Rawson and Hilbish (1998) is an introgressed *M. edulis* male haplotype as indicated by its *M. edulis* phylogenetic affiliation. This polymorphism within the M lineage suggests that the replacement probably started before introgression could complete the substitution of native *M. trossulus* male mtDNA by introgressed *M. edulis* male mtDNA.

Some *M. trossulus* males did not conform to the DUI pattern. These males appear to be homoplasmic for an introgressed F-type *M. edulis* A haplotype (Table 2). If DUI is disrupted in such males as a result of introgression and hybridization anomalies (Saavedra et al. 1996; Rawson et al. 1996), then they would be unable to transmit their A haplotype to their sons. Indeed, evidence does exist for Atlantic *M. edulis* nuclear alleles introgressing into *M. trossulus* far beyond the narrow hybrid zone at the mouth of the Baltic (Borsa et al. 1999; Riginos et al. 2002). However, heteroplasmic A/B males most likely in-

herit the A haplotype from a homoplasmic rather than a heteroplasmic male parent, given that heteroplasmic A/C and A/C2 males should transmit only to their sons the C or C2 haplotype that they received from their own fathers. An alternative possibility, however, is that such homoplasmic males failed to inherit a mtDNA genome from their fathers. Nevertheless, the frequency of such males in European *M. trossulus* (37%) largely exceeds the low rate (2.5%) of spontaneous failure of paternal transmission observed in laboratory crosses (Saavedra et al. 1997). Masculinization events appear to provide the best explanation for this observation, implying that these males are heteroplasmic for a masculinized genome that has not diverged, or that has diverged very little, from the F genome from which it arose yet sharing the same restriction profile. In conclusion, these mtDNA data show a high lability within the paternal route. That this feature can be extended to other taxa is indicated by the observation of a qualitatively similar mtDNA heteroplasmy for two conspecific F-type molecules within males of the mussel *M. galloprovincialis* (Ladoukakis et al. 2002).

### *Co-transmission of Multiple Paternally Inherited Genomes*

This study provides evidence of male heteroplasmy for three different genomes in European *M. trossulus* (Table 2). Hoeh et al. (1991) noted previously a low incidence of heteroplasmy for more than two genomes in a world-wide sample of several *Mytilus* taxa using a whole mitochondrial RFLP analysis, although they did not determine the sex of individuals. In our results this phenomenon is restricted to males. These males were heteroplasmic for the same three haplotypes (B/D/C), thus indicating that conditions leading to heteroplasmy for three genomes are not met in all matings as noted previously (Hoeh et al. 1991). If females lack the D and C haplotypes (Table 2), males will inherit them from their fathers. It follows that both sexes will inherit the B haplotype from their mothers. This suggests that the two male-specific haplotypes (D/C) are co-transmitted through the paternal route.

Several alternative hypotheses fail to account for the observed heteroplasmy for three different genomes. Fortuitous paternal leakage of the F genome within a single or a few generations rather than DUI ensuring paternal co-transmission predicts that the most frequent component haplotypes in heteroplasmic individuals should be common in both males and females. This is not consistent with the observation that C and D haplotypes were not found in females (Table 2). Tissue-specific mtDNA methylation is also an unlikely explanation. We tested this possibility by sequencing the three different lrRNA RFLP haplotypes from a single male. The three haplotypes (B, D and C) differed in sequence (Table 3), and cleavage sites were consistent with the expected restriction pattern using the lrRNA triple digestion approach. Mutational events would imply a unrealistically high mutation rate and would not explain how the same three haplotypes arose in several males. A *Taq* polymerase error is also an unlikey explanation. Haplotype D differed by five nucleotide changes from both B and C, whereas haplotypes B and C differed by another two nucleotides. This would imply a unusually high rate of *Taq* polymerase errors among haplotypes in different individuals, and would not explain the association between the three haplotypes in several males. Homologous recombination (Ladoukakis and Zouros 2001) or PCR chimeric molecules resulting from strand switching during the coamplification of mixed mitochondrial genomes (e.g., Wang and Wang 1997) are not consistent with the data. This is because it is not possible to reconstruct any haplotype sequence from any combination of the other two present in a heteroplasmic male even when removing singleton sites. The possibility of contamination by an external source or some endosymbiotic organism or parasite can be excluded given the high sequence similarity of all three haplotypes (Table 3 and Fig. 4). In conclusion, although data from crosses are not yet available, it is difficult to identify any alternative explanation in agreement with our population data. This is the first report of evidence consistent with the co-transmission of two mtDNA genomes in the sperm of *Mytilus* or indeed any other animal.

Cytological studies of fertilization in *Mytilus* are consistent with a paternal co-transmission hypothesis. In *Mytilus*, mitochondria of the early spermatid are fused to form five large mitochondria that occupy the midpiece of the spermatozoon and that enter into the egg (Longo and Dornfeld 1967). This would provide ample opportunity for the physical coexistence of two mtDNA types within the same mitochondrion and for their joint paternal transmission. The alternative hypothesis that C and D haplotypes are co-transmitted in different mitochondria appears unlikely given that a specific mechanism setting apart each mtDNA type and ensuring their occurrence in the spermatozoon has to be invoked. Analysis of single sperm mitochondria will provide the definitive experimental evidence to distinguish between these two competing hypotheses.

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