No evidence for presence of maternal mitochondrial DNA in the sperm of *Mytilus galloprovincialis* males

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Species of the mussel family Mytilidae have a special mitochondrial DNA (mtDNA) transmission system, known as doubly uniparental inheritance (DUI), which consists of a maternally inherited (F) and a paternally inherited (M) mitochondrial genome. Females are normally homoplasmic for the F genome and males are heteroplasmic mosaics, with their somatic tissues dominated by the maternal and their gonads dominated by the paternal genome. Several studies have indicated that the maternal genome may often be present in the male germ line. Here we report the results from the examination of mtDNA in pure sperm from more than 30 males of *Mytilus galloprovincialis*. In all cases, except one, we detected only the M genome. In the sperm of one male, we detected a paternal genome with an F-like primary sequence that was different from the sequence of the maternal genome in the animal's somatic tissues. We conclude that the male germ line is protected against invasion by the maternal genome. This is important because fidelity of gamete-specific transmission of the two mitochondrial genomes is a basic requirement for the stability of DUI.

Keywords: mitochondrial DNA; heteroplasmy; *Mytilus*; doubly uniparental inheritance

1. INTRODUCTION

The mitochondrial genome of metazoans is a closed circular DNA encoding for two ribosomal RNAs, 22 tRNAs and 12 or 13 proteins, which are all components of the oxidative phosphorylation system that is essential for most cellular functions. One apparently universal feature of the mitochondrial genome in animals is that it is maternally inherited. A notable exception is the phenomenon of doubly uniparental inheritance (DUI) that has been found in species of the molluscan bivalve families Mytilidae (sea mussels—Skibinski *et al.* 1994a,b; Zouros *et al.* 1994a,b) and Unionidae (fresh water mussels—Hoeh *et al.* 1996; Liu *et al.* 1996; Cuore & Kocher 2002; Hoeh *et al.* 2002; Serb & Lydeard 2003) and in at least one species of the family Veneridae (clams—Passamonti & Scali 2001). In these species two mitochondrial genomes exist, one that is transmitted through the eggs (the F genome) and one that is transmitted through the sperm (the M genome). Both female and male individuals start as heteroplasmic, i.e. they contain both genomes (Sutherland *et al.* 1998; Cao *et al.* 2004a; Obata & Komaru 2005), but females become homoplasmic for the F genome, whereas males become heteroplasmic mosaics, with the F genome dominating the somatic tissues and the M genome dominating the gonad.

The tissue distribution pattern of maternal and paternal genomes may have important implications for DUI. In mammals a mature sperm carries approximately 100 copies of mitochondrial DNA (mtDNA), which might be necessary to sustain the sperm's mitochondrial activity (Hecht *et al.* 1984). In contrast, the mammalian oocyte may contain 10^5 – 10^8 copies of mtDNA, thus exceeding the sperm's mtDNA content by a factor of at least 10^3 (Jansen & de Boer 1998; Birky 2001). Recent observations during the first embryonic divisions in mussels of sperm mitochondria stained with mitotracker green, a fluorescent dye that binds non-destructively to the outer mitochondrial surface, suggested that the fate of these mitochondria is different depending on whether the embryo is destined to develop into a female or male individual (Cao *et al.* 2004a; Cogswell *et al.* in press). In female embryos the sperm mitochondria behave passively and are diluted within the much larger pool of egg mitochondria. In male embryos, most sperm mitochondria are found in the larger cell at the two-cell or the four-cell stage. This cell, designated 'CD' in the two-cell stage and 'D' in the four-cell stage (Conklin 1897), is the one that gives origin to the germ line (Verdonk & Van Den Biggelaar 1983). This controlled behaviour of sperm mitochondria in male embryos is, apparently, part of a developmental mechanism through which these mitochondria are delivered to the first germ cells.

It follows from these observations that the presence of a maternal genome in the sperm may have more serious consequences for the stability of DUI than the presence of a paternal genome in eggs. The M genome is either entirely absent from mature females or present as a tiny

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minority. This means that even if the M genome is present in the egg, it will represent a small proportion of the egg's mtDNA pool. Following cell division of the newly formed zygote, the egg's M genome will either be lost stochastically or remain as a minority, whether the zygote develops into a male or female individual. An F genome carried by the sperm will have the same fate in a female embryo as the sperm's M genome, i.e. it will become diluted within the much larger pool of the egg's maternal mtDNA. But in a male embryo a sperm-carried F genome will have a distinctly different fate. It will be directed into the gonad, where it will multiply and will be transmitted by the sperm into the next generation. This will decouple the two genomes from their sex-specific transmission and may eventually lead to the elimination of the M genome.

In view of the above, it is important that we obtain a clear idea as to whether the maternal genome of a male may enter the male's germ line and how often this may occur. The literature is not clear on this point. Early reports on DUI indicated that the phenomenon may break down in interspecific hybrids, so that the paternal genome may be present in females and absent in males (Zouros *et al.* 1994b; Rawson *et al.* 1996). Deviations from the standard tissue distribution of the two genomes were also observed in pure-species individuals (Stewart *et al.* 1995; Garrido-Ramos *et al.* 1998; Dalziel & Stewart 2002), including presence of F-type mtDNA in the sperm (Skibinski *et al.* 1994b; Beagley *et al.* 1997; Saavedra *et al.* 1997). These reports suffer, however, from several difficulties. One is contamination of DNA extracts from different tissues coupled with PCR sensitivity. This problem is particularly serious in males where the F content of the somatic tissues could be easily contaminated by the gonad's M genome and *vice versa*. This cross-contamination is further facilitated by the fact that in mussels the gonad does not form a distinct and anatomically separate organ, but rather is diffused within the animal's mantle. Another difficulty arises from mtDNA recombination, which is common in the gonads of male mussels (Ladoukakis & Zouros 2001). Most previous studies have used the restriction profile or the DNA sequence of a small mtDNA fragment to characterize the genome as F or M. This extension to the whole genome from one of its parts may be misleading because the examined piece might have been transferred from one genome into the other through recombination. A third, and perhaps more serious cause of mistaken presence of maternal genomes in the sperm, is the presence in the population of paternal genomes whose primary sequence resembles more the F than the M genome (Hoeh *et al.* 1997; Ladoukakis *et al.* 2002).

In this study, we have examined the possibility that the maternal genome of a male might be present in the male's sperm. To minimize the probability of sperm contamination by cells or fragments of cells of somatic origin we forced spawned sperm to swim through a solution of Percoll (Pharmacia). We recovered only the M-type mitochondrial genome in total DNA extractions from Percoll-treated sperm. However, in the case of one male, the sperm contained an F-type molecule, which was different from the genome in the male's somatic tissues. This same genome was subsequently found in the gonads of several other males. These two observations strongly suggest that in this male the sperm's mtDNA was also of paternal origin.

2. MATERIAL AND METHODS

Adult mussels (*Mytilus galloprovincialis*) were kindly provided by 'Poseidon Co.—Mussels of New Peramos' (Nea Peramos, Saronikos Gulf, Greece) during the winter, when the gonads were reproductively mature and ready for spawning. Mussels were placed in individual containers filled with seawater at 8 °C and one day later transferred to 20 °C to induce spawning by thermal shock. Spermatozoa were forced to swim through a discontinuous gradient of 45 and 90% Percoll (Pharmacia, obtained from Sigma-Aldrich) by centrifugation at 500g for 20 min (Forster *et al.* 1983). This resulted in the collection of live sperm free of somatic cells or cellular debris, which might have been released during spawning. The spermatozoa from each fraction were collected, washed with phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4) to remove Percoll and used for total DNA extraction. The 45% Percoll fragment was kept for further analysis, but the 90% Percoll fragment was discarded because on several occasions it contained heavy debris of apparent somatic cell origin. DNA extracts from gonadal and somatic tissues of *M. galloprovincialis* males collected in 1999 from the Black Sea (Ladoukakis *et al.* 2002) were also analysed.

Characterization of a genome as F or M was based on a length difference in the first variable domain (VD1) of the main control region (CR) (Cao *et al.* 2004b; figure 1). An F-specific and an M-specific pair of primers were designed for PCR amplification of a part of VD1, whose sequence is highly different between F and M molecules (Cao *et al.* 2004b). The F-specific pair (ssFd11 and QssFd12; table 1) gives no product from the M genome, but it gives a 186 bp product from the F genome, as expected from known F-type sequences (GenBank accession numbers: AF315573, AF188278, AY350784, AY350790, AY350789, AY350788, AY350787, AY350786, AY350785, AY497292 and AY484747). The M-specific pair (QssMdl1 and QssMdl2; table 1) gives no product from the F genome, but it gives a product from the M genome that varies between 206 and 208 bp in published M sequences (GenBank accession numbers: AF188280, AF188279, AY350791, AY350794, AY350793, AY350792, AY823623, AY823624), except in one case (GenBank accession number AY363687) where the length is 191 bp.

One male carried in its sperm an F-like paternal genome (see §3). In order to compare the paternal and maternal mitochondrial genomes of this individual we used DNA isolated from sperm and somatic tissue (gills). We used the primer pair Lola1 and LCOIIIr (Mizi *et al.* 2005; table 1) to amplify a fragment from 16S-rRNA to cytochrome oxidase subunit 3 (COIII). The PCR products were cloned in a TOPO XL PCR cloning vector (Invitrogen) and digested with *EcoRI* and *EcoRI/EcoRV* which do not have recognition sites within the CR. Size differences between the cloned parts from the purified sperm and the gills were visualized through agarose gel electrophoresis (figure 2b). Differences between F-like paternal and maternal genomes were further studied by comparing the sequences of three coding regions of mtDNA isolated from sperm and somatic tissue of this male and two males (BS12, BS81) from the Black Sea. For this we used the following primer pairs: (i) COIII-FOR and COIII-REV, which amplifies a 867 bp fragment in COIII gene, (ii) COI-F and COI-R, which amplifies a 863 bp fragment in the cytochrome oxidase subunit 1

Figure 1. Diagrammatic representation of the circular *M. galloprovincialis* mtDNA showing, in grey, the relative length of the three domains (VD1: variable domain (1); CD: conserved domain; VD2: variable domain (2) of the main control region (CR) and the parts of three protein coding genes used in this analysis. Black areas represent PCR amplified and sequenced regions. Numbers indicate the length of PCR products.

Table 1. PCR primers used and their corresponding position in the complete F-type (GenBank accession number AY497292) and M-type (GenBank accession number AY363687) mitochondrial genomes of *M. galloprovincialis*.

gene, region	code	sequence (5' to 3')	location in AY497292	location in AY363687
VD1 domain of CD	ssFdl1	GGTGATAGGTTGTTAAGYGTGG	28–49	—
	QssFdl2	ACCGTCRCCTTCTCCWCCC	195–213	—
	QssMdl1	GTAARTGAGGTTGGCTATAMGTGT	—	9 ^a
	QssMdl2	CTGGTATACACACGYTACACACC	—	177–199
COIII	COIII-FOR	TATGTACCAGGTCCAAGTCCGTG	6029–6051	5763–5785
	COIII-REV	ATGCTCTTCTTGAATATAAGCGTACC	6870–6895	6604–6629
COI	COI-F	GGTAARGATATAATTTAYCCRCGG	9394–9417	9275–9298
	COI-R	TGTGCTACMACRTARTAAGTATCAT	10232–10256	10113–10137
ND5	ND5-F	CTKCWTTAGTKCAYTCTTCWAC	12439–12460	12320–12341
	ND5-R	CCTCASACCACTARCTGATAAG	13459–13480	13340–13361
16S to COIII (long PCR)	Lola1	ACATGAKCTGAGTTTCAGAMCGGYGTRAGC	16577–16605	16459–16487
	LCOIIIr	CCTACGACRACRTGTATCCCRTGGAATCC	6611–6639	6345–6373

^a The 3' end of the primer does not match with the AY363687, due to a deletion in this particular molecule.

(COI) gene and (iii) ND5-F and ND5-R, which amplifies a 1042 bp fragment in the NADH dehydrogenase subunit 5 (ND5) gene (table 1).

Long-PCR reactions were carried out in 50 µl reaction volumes containing 50–100 ng of template DNA, 300 nM of each primer, 500 nM dNTPs, 3.75 mM MgCl₂ and 0.75 µl of enzyme mix (Roche 'Expand Long Template PCR System') in buffer-2 supplied by the company. Reaction conditions were in accordance with the supplier's recommendations. All other PCR reactions were carried out in 25 µl reaction volumes containing 50–100 ng of template DNA, 500 nM of each primer, 200 nM dNTPs, 3 mM MgCl₂ and one unit of Taq-DNA polymerase (Promega), using the following programme: 2 min at 94 °C, 32 cycles of 20 s at 94 °C, 30 s at 54–58 °C (depending on the optimum annealing temperature of the primer pair) and 1 min at 74 °C and a final step of 5 min at 74 °C. The PCR products were cloned using the TA Cloning Kit (Invitrogen). The cloned fragments from the three coding regions were

sequenced using an ABI 377 automated sequencer with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems), or using a commercial outlet. Sequences were aligned using the CLUSTALX program (Thompson *et al.* 1997) and their phylogenetic relationships were deduced using the neighbour-joining method (MEGA, v. 3.1; Kumar *et al.* 2004). Sequences have been deposited in GenBank and their accession numbers are given in table 2.

3. RESULTS AND DISCUSSION

For 35 out of 36 males we obtained the result shown in figure 2a. When the gonad was used as the source of DNA for the PCR reaction, both sex-specific primers produced a product for the targeted VD1 domain of CR. When the sperm was forced to swim through a solution of Percoll and then used as the source of DNA, only the M-specific primers produced a product. The presence of both

Figure 2. (a) Typical electrophoretic patterns of PCR products from gonad and sperm of *M. galloprovincialis* using the F and M VD1-specific primers. 100 bp ladder: GeneRuler 100 bp DNA Ladder (Fermentas). Zone sizes (208 and 186 bp) were obtained after sequencing the PCR product. (b) Electrophoretic patterns of long-PCR products digested with *EcoRI* (left) and *EcoRI/EcoRV* (right). These enzymes do not cut the CR, therefore different fragment sizes correspond to different CR sizes. F, the mitochondrial genome in gills of the exceptional male; Fc, the mitochondrial genome in the sperm of the exceptional male. PL, TOPO XL PCR cloning vector. 1 kb ladder: GeneRuler 1 kb DNA Ladder (Fermentas). Estimated sizes in base pairs (with corresponding sizes from Mizi *et al.* 2005 in parenthesis): Left panel, 1, 280 (275); 2, 2000 (1955); 3, 4600 (4577); 4, 7000, novel for this genome. Right panel, 1, 280 (275); 2, 1850 (1852); 3, 2000 (1955); 4, 2700 (2725); 5, 4200, novel for this genome.

Table 2. GenBank accession numbers of the sequences obtained in this study.

code in text	description	tissue	gene/region	GenBank accession number
exceptional male	male with F-like paternal mtDNA from Saronikos Gulf	sperm	COI (partial)	DQ469132
			COIII (partial)	DQ469133
			ND5 (partial)	DQ469134
		gills	COI (partial)	DQ403169
			COIII (partial)	DQ403170
			ND5 (partial)	DQ403171
BS12	male #12 from the Black Sea with F-like paternal mtDNA	gonad	COI (partial)	DQ445466
			COIII (partial)	DQ445468
			ND5 (partial)	DQ445467
		soma	COI (partial)	DQ445469
			COIII (partial)	DQ445471
			ND5 (partial)	DQ445470
BS81	male #81 from the Black Sea with F-like paternal mtDNA	gonad	COI (partial)	DQ445472
			COIII (partial)	DQ445474
			ND5 (partial)	DQ445473
		soma	COI (partial)	DQ445475
			COIII (partial)	DQ445477
			ND5 (partial)	DQ445476

genomes in the gonad is the expected pattern for males of species with DUI. About two-thirds of these males (table 3) continued to show presence of the F genome when spawned sperm was used for the mtDNA assay, which suggests that cells—or fragments of cells—of somatic origin are often present in the spawned product. The contribution of this material to the DNA preparation

from non-purified sperm need not be high for our specific primers to produce a detectable PCR product. Non-purified sperm cannot, therefore, be used as material to judge whether a male's maternal genome enters the male's germ line. Our results agree with other studies (Forster *et al.* 1983) that the Percoll treatment is a reliable way for obtaining a pure sperm preparation.

Figure 3. Neighbour-joining tree of the concatenated sequences of the COI, COIII and ND5 fragments totalling 2630 bp. Sperm, gills, mtDNA from the sperm and the gills of the exceptional male; BS12, BS81, gonadal (gonad) and somatic (soma) mtDNA from two males from the Black Sea; F, M, the corresponding parts of the typical F and M *M. galloprovincialis* fully sequenced genomes (Mizi *et al.* 2005). Numbers represent percent of 1000 bootstrap replicates (also see electronic supplementary material).

One male (which we will call the exceptional male) did not follow the pattern described above. This male was exceptional in several aspects. First, it produced an F-type, but not an M-type, pattern when its gonad was used as the DNA source. Thus, it appeared that this male was homoplasmic for the maternal genome. Second, the purified sperm also tested positive for an F-type genome. The question that arises from these observations is whether this male did not receive a mitochondrial genome from its male parent, i.e. whether it was truly homoplasmic or whether its paternal genome could not be distinguished from the maternal genome on the basis of the detection assay of figure 2*a*. This is an important question for the purpose of this paper, because if the former case is true it would mean that a reasonably high proportion of males in the population (roughly 3%) do not receive a mitochondrial genome from the sperm. In such males the maternal genome might become the sole occupant of the gonad and thereafter be transmitted as a paternal genome.

To answer this question we compared four regions of the mtDNA molecule from the Percoll-purified sperm of the exceptional male with the corresponding regions from its somatic tissues (gills). Ladoukakis & Zouros (2001) have shown that mtDNA recombination is common in the gonads of *M. galloprovincialis* males. As explained in §1, this could create a complication if the comparison was restricted to one or two regions. If the regions were identical in the sperm and in the somatic tissues, their identity could be attributed to the presence of one and the same genome in both tissues or to recombination between two genomes, one in the gonad and the other in the soma. The examination of four distantly located regions (figure 1) makes it easy to decide between these two alternatives.

The comparison of the main CR is shown in figure 2*b*. This region is about three times longer in the genome that was isolated from the sperm of the exceptional male than in the typical F or M genome. PCR products from three coding regions (COIII, COI and ND5; figure 1) were obtained from the sperm and the gill of the exceptional male. In all three regions, the sequence was different in the sperm and gill products (figure 3 and electronic supplementary material). It is clear that the mtDNA from the sperm of the exceptional male is closer to the typical F

Table 3. Presence of F- or M-type VD1 region.

number of individuals	gonad	sperm	
		without Percoll fractionation	Percoll, 45% fraction
23	F/M	F/M	M
12	F/M	M	M
1	F	F	F

than to the typical M genome, but different from its somatic mtDNA. Given that under DUI the somatic genome is of maternal origin, the genome in the sperm must represent the male's paternal genome.

To obtain further evidence for the paternal origin of this genome we re-examined a sample of *M. galloprovincialis* males from the Black Sea that were collected in 1999 and sacrificed without spawning. Restriction fragment length polymorphism (RFLP) profiles of gonadal mtDNA for the COIII and the 16S-rRNA genes were obtained for a total of 30 males, as part of a previous study (Ladoukakis *et al.* 2002). Eleven of these males produced profiles that indicated absence of the M genome and presence of two F-type genomes (table 3; class III in Ladoukakis *et al.* 2002). In addition, in all these males the RFLP profile of one of the two F-like genomes was identical to that expected from the sequence of the sperm genome of the exceptional male. The DNA of two of these eleven Black Sea males was exhausted at the time of the present study. For the remaining nine we found that the CR of their gonadal mtDNA was similar to that found in the sperm of the exceptional male (figure 2*b*). In two of these males we sequenced the COI, COIII and the ND5 parts from both somatic and gonadal tissues. The sequences (see electronic supplementary material) were used to produce the phylogenetic tree of figure 3. In addition to the sperm and somatic mtDNA of males with an F-like paternal genome, we give in figure 3 the corresponding parts of the complete sequences of the typical F and M genomes of *M. galloprovincialis* from Mizi *et al.* (2005). It can be seen that the gonadal genomes are nearly identical to the genome isolated from the sperm of the exceptional male. These observations show that the

genome observed in the sperm of the exceptional male is not peculiar to this male, but rather exists in the population as a paternally transmitted mtDNA molecule.

In 35 males that contained a typical maternal F genome and a typical paternal M genome, we failed to detect the F genome in the purified sperm, even though the F genome was detected in 23 of these males before the sperm was passed through Percoll. With this sample size we could have missed the detection of the F genome in the purified sperm with probability 5% even if this genome occurred in the sperm of as many as 10% of the males in the population. Yet, our non-detection of the typical F genome in the male germ line is at variance with previous studies in which presence of the F genome in the male gonad was reported in high frequencies (Skibinski *et al.* 1994b; Beagley *et al.* 1997; Saavedra *et al.* 1997). As suggested by several of these studies, these high frequencies must be gross overestimates owing to the leakage of the maternal genome from somatic cells into the DNA preparations from gonads and sperm. Another source of error is that previous studies did not examine the possibility of different genomes in somatic and gonadal tissues. We would have committed the same mistake if we had not carried out this comparison in the exceptional male. The comparison showed that this male conformed to, rather than violated, the rule that the sperm's mtDNA is of paternal origin.

The observation that the sperm of animals with DUI is free of maternal mtDNA has a direct bearing on the 'masculinization' or 'sex-reversal' (Hoeh *et al.* 1997; Zouros 2000) hypothesis, which suggests that occasionally a maternal genome may invade the male transmission line and become 'masculinized', i.e. become sperm-transmitted from that point on. As noted, several previous studies have reported males that lacked a typical M genome. Many of these males were considered as likely homoplasmic, i.e. as containing only a maternal genome, which, according to the hypothesis, could become sperm-born and, therefore, initiate a new episode of masculinization. Our study shows that no male in our sample lacked a paternal genome and that the maternal genome does not enter the sperm. This suggests that masculinization events are much rarer than implied by previous studies which suggested a frequent invasion of the male germ line by the maternal genome. This agrees with the lack of evidence for masculinization in the family Unionidae, where DUI is apparently present in all species, and it is a very old phenomenon (Hoe *et al.* 1996). It also helps explain the stability of DUI in Mytilidae. If events of masculinization in mytilids were as common as implied by previous studies of presence of the F genome in male gonads and sperm, they would have resulted, eventually, in the removal of M-like genomes from the population. To prevent this, one would have to postulate strong selection against newly masculinized F genomes. This postulation is, however, unnecessary in view of our demonstration that invasion of the sperm by the maternal genome may be an extremely rare event.

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