

# Mitochondrial DNA variation in a species with two mitochondrial genomes: the case of *Mytilus galloprovincialis* from the Atlantic, the Mediterranean and the Black Sea

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## Abstract

We have examined mitochondrial DNA (mtDNA) variation in samples of the mussel *Mytilus galloprovincialis* from the Black Sea, the Mediterranean and the Spanish Atlantic coast by scoring for presence or absence of cleavage at 20 restriction sites of a fragment of the COIII gene and at four restriction sites of the 16S RNA gene. This species contains two types of mtDNA genomes, one that is transmitted maternally (the F type) and one that is transmitted paternally (the M type). The M genome evolves at a higher rate than the F genome. Normally, females are homoplasmic for an F type and males are heteroplasmic for an F and an M type. Occasionally molecules from the F lineage invade the paternal transmission route, resulting in males that carry two F-type mtDNA genomes. These features of the mussel mtDNA system give rise to a new set of questions when using mtDNA variation in population studies and phylogeny. We show here that the two mtDNA types provide different information with regard to amounts of variation and genetic distances among populations. The F genome exhibits higher degrees of diversity within populations, while the M genome produces higher degrees of differentiation among populations. There is a strong differentiation between the Atlantic and the Black Sea. The Mediterranean samples have intermediate haplotype frequencies, yet are much closer to the Black Sea than to the Atlantic. We conclude that in this species gene flow among the three Seas is restricted and not enough to erase the combined effect of mutation and random drift. In one sample, that from the Black Sea, the majority of males did not contain an M mtDNA type. This suggests that a molecule of the maternal lineage has recently invaded the paternal route and has increased its frequency in the population to the point that the present pool of paternally transmitted mtDNA molecules is highly heterogeneous and cannot be used to read the population's history. This liability of the paternal route means that in species with doubly uniparental inheritance, the maternal lineage provides more reliable information for population and phylogenetic studies.

*Keywords:* Doubly Uniparental Inheritance, mtDNA variation, mussels

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## Introduction

Mitochondrial DNA (mtDNA) has become one of the most widely used tools in molecular phylogeny and

phylogeography (Avice 2000) for reasons that are well known: ease of handling, high rate for selectively neutral mutation, uniparental inheritance and lack of recombination. After more than two decades of use of mtDNA in population studies these advantages remain valid, but only as a matter of degree. With the exception of mtDNA deletions that lead to severe defects [e.g. myopathies (Chinnery & Turnbull 1998) and male sterility (Kao *et al.* 1998)], there is no strong evidence that animal mtDNA variants of the kind normally used in population surveys

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are themselves targets of selection. Yet the behaviour of these polymorphisms may not follow the patterns of neutral variation. In particular, these polymorphisms may be under the influence of selective sweeps (Begun & Aquadro 1992) or background selection (Charlesworth *et al.* 1995). Indeed patterns of mtDNA polymorphism that are not compatible with neutrality have been reported by several authors (Rand 1994; review by Ballard & Kreitman 1995). Manifold differences in mtDNA mutation rate are also known among groups of organisms, apparently related to external or internal environmental regimes (Martin & Palumbi 1993). Exceptions to strict maternal inheritance are known in several animals, namely *Drosophila* (Kondo *et al.* 1990), mice (Gyllenstein *et al.* 1991) and anchovy (Magoulas & Zouros 1993). Finally, the controversy of whether animal mtDNA undergoes homologous recombination (Arctander 1999; Merriweather & Kaestle 1999; Wallis 1999; Kivisild *et al.* 2000; Wallis 2000) seems to have been settled in favour of its occurrence (Ladoukakis & Zouros 2001a,b) and shifted to how frequently it may occur.

A major exception to the rule of uniparental transmission of organelle DNA has been described by Skibinski *et al.* (1994a,b) and Zouros *et al.* (1994a,b) in species of the blue mussel family Mytilidae. These species carry two types of mitochondrial genomes, one that is transmitted maternally to offspring of both sexes and another that is transmitted paternally to male progeny only. This system of mtDNA transmission has become known as doubly uniparental inheritance (DUI; Zouros *et al.* 1994b). DUI, which, in addition to blue mussels, is now known to occur in species of the fresh water mussels Unionidae (Liu *et al.* 1996) and the clam *Tapes philippinarum* (Passamonti and Scali 2001), has certain features that set it apart from the standard maternal inheritance system: (i) Males are heteroplasmic for a maternal (F) and a paternal (M) genome (Skibinski *et al.* 1994b; Zouros *et al.* 1994b). But the heteroplasmy is not of the conventional form: somatic tissues are dominated by the maternal genome and the gonad by the paternal genome (Garrido-Ramos *et al.* 1998); (ii) the paternal genome evolves faster than the maternal genome (Hoeh *et al.* 1996a); (iii) occasionally, a maternally transmitted molecule may invade the paternal route, a phenomenon for which the term 'masculinization of the F molecule' has been used (Hoeh *et al.* 1997; Saavedra *et al.* 1997). This creates a heterogeneous pool of paternally transmitted molecules: those that have been following the paternal route even before the splitting of the species from its relatives (Rawson & Hilbish 1996; Stewart *et al.* 1996) and have as a result accumulated a large number of nucleotide differences from the maternally transmitted molecules, and those that have recently entered the paternal route and have nucleotide sequences that resemble the F type more than the M type (Hoeh *et al.* 1997); (iv) studies of pair-matings have shown that the sex

ratio among the offspring of a female mussel may be heavily biased toward either the male or the female sex and that this bias is characteristic of the female parent and independent of the male to which the female is mated (Saavedra *et al.* 1997); (v) finally, Ladoukakis and Zouros (2001a) have shown that homologous mtDNA recombination is common in the male gonad of mussels.

Clearly, these peculiarities of the DUI system give rise to a number of questions that are not encountered when using mtDNA for population studies of species with standard maternal inheritance. Are the frequencies of F haplotypes similar in the female and the male parts of the population? Are the frequencies of F/M combinations found among males simple products of the population frequencies of F and M haplotypes? How different might be the information one gets from the F or the M lineage on matters of population structure, gene flow, selection and phylogeny?

We have attempted to address these questions using the Mediterranean blue mussel, *Mytilus galloprovincialis* as a model. The species distribution extends from the southern coast of England and Ireland to the French and Iberian Atlantic, the Mediterranean and the Black Sea. The species has been reported outside its native area of distribution in California, Australia, New Zealand, Tasmania, Japan, northwards along the East China coast and South Africa (Gosling 1992). We have examined one sample from the Spanish Atlantic coast, five from the Mediterranean (Adriatic, Ionian, southern Aegean, middle Aegean and northern Aegean) and one from the far northern part of the Black Sea. We have used the restriction fragment length polymorphism (RFLP) method to assay mtDNA variation in two regions of the molecule (parts of the COIII and the 16S rRNA genes).

## Materials and methods

### *Populations sampled*

Mussel samples were taken from five localities in the eastern Mediterranean Sea, one from the Atlantic, and one from the Black Sea. The localities sampled in the Mediterranean were the following: Chioggia (CHI), near Venice, Italy (north Adriatic Sea); Cefalonia Island (CEF), Greece (Ionian sea); Faros (FAR), near Heraklion, Greece (south Aegean Sea); Megalo Pefko (MPE), near Athens, Greece (central Aegean Sea); and Halastra (HAL), near Thessaloniki, Greece (north Aegean Sea). The sample from the Black Sea (BS) came from Sebastopol (Ukraine). The sample from the Atlantic (ATL) comprised commercial mussels from Ría de Arousa (Galicia, northwest Spain).

### *Dissection and sexing*

Mussels were opened in the laboratory, and a piece of the gonadal tissue was examined under the microscope to

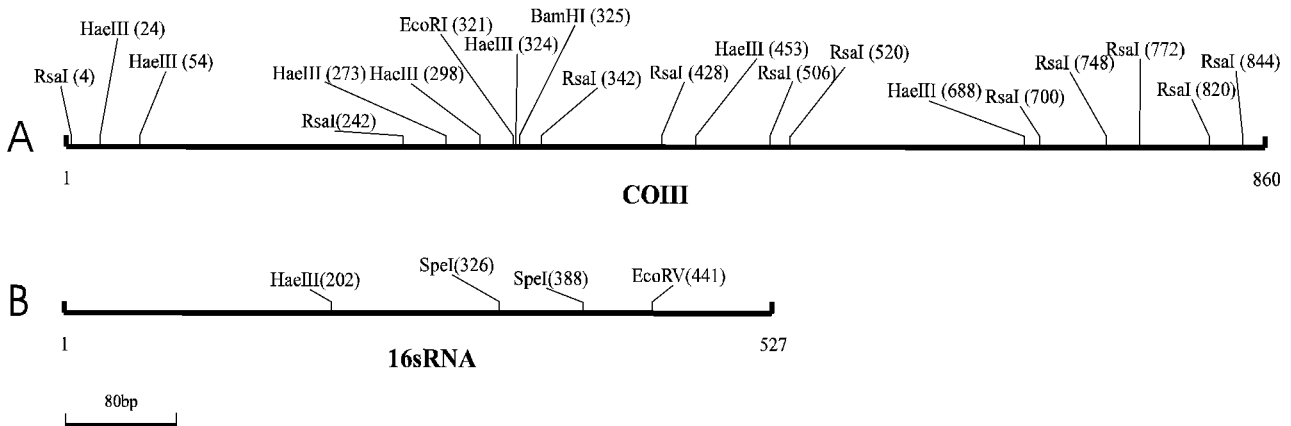


Fig. 1 Restriction maps of two fragments of *Mytilus galloprovincialis* mtDNA (A) COIII, (B) 16S rRNA. Each site is indicated by the enzyme by which it is recognized. Distances (in parenthesis) are in base pairs (bp) from the left end of the amplified region.

establish the sex of the animal by the presence of eggs or sperm. Individuals that showed no gametes were classified as 'not sexed' and were excluded from the analysis. Tissue samples from gonad (attached to the mantle tissue), adductor muscle, gills and digestive gland were taken and kept at  $-20^{\circ}\text{C}$  or  $-80^{\circ}\text{C}$ .

#### Mitochondrial DNA genotyping

Each individual mussel was genotyped for the mitochondrial genome by a polymerase chain reaction (PCR)-RFLP procedure. Total DNA was isolated by a variant of a salt precipitation method (Zouros *et al.* 1992). PCR was first performed on total DNA extracted from the gonadal tissue. In some selected individuals the PCR was repeated on DNA extracted from somatic tissue (gills). Two parts of the mitochondrial genome were assayed. The first was a fragment of 860 base pairs (bp) of the cytochrome oxidase III gene, which was amplified using the primers and the PCR conditions described by Saavedra *et al.* (1997) and digested with the enzymes *EcoRI*, *BamHI*, *HaeIII* and *RsaI*. Scoring of the restriction haplotype of F molecules was straightforward in females, owing to the fact that females were homoplasmic for this molecule. Knowing what F haplotypes were present in the population from their occurrence in females helped in reading the composite (F/M) restriction patterns of males. The fragments that corresponded to the maternal genome were determined first (the F haplotype), and the remaining fragments were assigned to the paternal molecule (the M haplotype), on the condition that they added to 860 bp.

The second fragment corresponded to a 527-bp part of the 16S rRNA gene. It was amplified with the universal primers AR and BR (Palumbi *et al.* 1991), and the PCR product was digested simultaneously with the enzymes *EcoRV*, *HaeIII* and *SpeI*, according to Rawson & Hilbish (1996). In most males, the 16S rRNA primers described

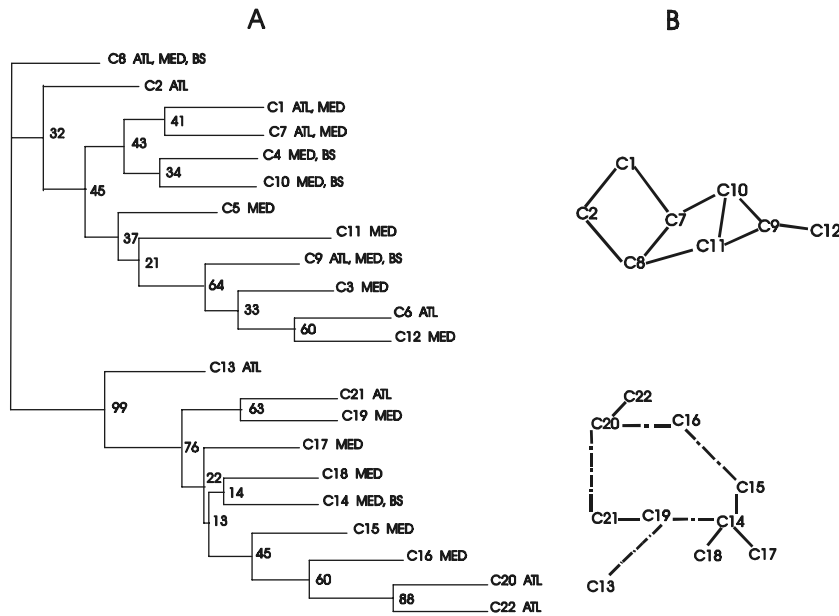
annealed preferentially to the M genome, so that the F genome could not be detected. To detect the F type of these males the AR primer was replaced with the primer PR-19 Rawson *et al.* (1996), which is specific to the F genome, and the obtained fragment of 440 bp was digested with the same three restriction enzymes. In some males it was impossible to detect either the COIII or the 16S rRNA parts of the M genome with the assays described. In these males we were able to recover the fragments of the 16S rRNA gene by using the primers PR17 and PR18 of Rawson & Hilbish (1996), which are specific to the M genome. The 440 bp PCR product was digested with *SpeI* and *EcoRV* to confirm that the restriction pattern agreed to known M genome patterns (Rawson & Hilbish 1996).

#### Restriction maps

The restriction map of the COIII region of the F molecule was constructed using the sequences of Ladoukakis and Zouros (2001a) and that of the COIII of the M molecule used the sequences of Stewart *et al.* (1996). The restriction maps of the 16S rRNA genes of the F and the M molecules were constructed from the data of Rawson & Hilbish (1996). The restriction enzymes used allowed for the scoring of presence or absence of cleavage at 20 sites of the COIII gene and of four sites on the 16S rRNA gene (Fig. 1).

#### Haplotype networks

For any pair of haplotypes of the same mtDNA region one may count differences in restriction sites. Two haplotypes differing by only one site are assumed to be separated by one mutation event. This approach can be used to construct networks of minimum numbers of mutation events that connect the whole set of haplotypes. Because of homoplasy or recurrent mutation there is no single best network of this type nor can there be perfect additivity in the



**Fig. 2** (A) Neighbour-joining unrooted tree of all COIII haplotypes recovered in the study. Numbers indicate bootstrap values and branch lengths are proportional to bootstrap support. Haplotypes C1 to C12 belong to F cluster, haplotypes C13 to C22 to the M cluster. The sample in which each type was found is also given. (B) Networks depicting the minimal number of mutational steps connecting the various haplotypes. The number of line segments between two neighbouring haplotypes is the required number of mutations for the transition from one type to the other.

number of mutation steps connecting distant haplotypes. Networks shown in Fig. 2(B) were obtained manually by trial and error.

#### Data analysis

Nei distances (Tajima & Nei 1984) were used to construct neighbour-joining majority rule consensus trees for restriction haplotypes (PHYLIP; Felsenstein 1995) with 100 bootstrap replicates. This number is sufficient given the small number of surveyed sites ( $n = 18$  for variable sites) and the binary nature of the data (presence/absence of cleavage). Haplotype frequencies were computed and used to estimate haplotype diversities with the program DA in REAP (McElroy *et al.* 1992). Differences in haplotype frequencies in  $2 \times n$  contingency tables were tested by simple  $\chi^2$  tests of homogeneity, even if expectations in some cells were very small (Lewontin & Felsenstein 1965). In  $n \times m$  tables with both  $n$  and  $m$  larger than 2 we used the exact test of Raymond & Rousset (1995a) [program STRUC as implemented in GENEPop; Raymond & Rousset (1995b)]. The sequential Bonferroni test (Sokal & Rohlf 1995) was used to correct for multiple tests.

#### Terminology

The complexity of the doubly uniparental mitochondrial system requires a specific terminology. We first note that the distinction maternally transmitted vs. paternally transmitted genomes is different from the distinction F vs. M genomes. The first refers to the parent from which the genome was inherited, whereas the second has been used in the literature to denote the fact that the mitochondrial

genomes of mussels form, in terms of molecular affinity, two clusters: cluster F which contains molecules found in females and males, and cluster M which contains molecules usually found only in males. The vast majority of female individuals are homoplasmic for an F genome. Such females will be called typical females. Occasionally a female individual may be heteroplasmic for an F and an M genome. These females will be called atypical females. In males the somatic tissues are predominantly homoplasmic for an F genome and the gonad for an M genome. Such males will be called typical males. Quite often genomes that belong to the F cluster are male-transmitted. These are 'recently masculinized F molecules' (Hoeh *et al.* 1997) for which we proposed the symbol  $M^f$  (Ladoukakis & Zouros 2001a). Male individuals that inherit an F molecule from their female parent and an  $M^f$  from their male parent will be characterized as atypical males. When their F and  $M^f$  molecules are distinguishable by the assay in use, the atypical males will be scored as heteroplasmic atypical males, otherwise they will be scored as homoplasmic atypical males. In atypical heteroplasmic males it is possible to identify the maternal genome (and by subtraction the paternal genome) from the fact that it is the dominant molecule in somatic tissues.

#### Notation of mtDNA haplotypes

Traditionally, restriction haplotypes are scored with a string of 1 and 0 indicating presence or absence of cleavage, respectively, in the corresponding site. For example, the first haplotype in Table 1 would be symbolized as 11000101000110010011. We have replaced this system by adopting one number for every distinct restriction

**Table 1** Restriction haplotypes for an 860-bp segment of COIII and a 527-bp fragment of 16S rRNA

Enzymes Sites:	A																				B				
	R	H	H	R	H	H	E	H	B	R	R	H	R	R	H	R	R	R	R	R	H	S	S	EV	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	1	2	3	4	
C1	1	1	0	0	0	1	0	1	0	0	0	1	1	0	0	1	0	0	1	1	RA	1	1	1	0
C2	1	1	0	0	0	1	0	1	0	1	0	1	1	0	0	1	0	0	1	1	RD	1	0	1	0
C3	1	1	0	0	0	1	0	1	0	1	0	1	1	0	0	1	1	0	0	1					
C4	1	1	0	0	0	1	0	1	0	0	0	1	1	0	0	1	1	0	1	1					
C5	1	1	0	0	0	1	0	1	0	1	0	1	1	0	0	1	1	0	1	1					
C6	1	1	0	1	0	1	0	1	0	1	0	1	1	0	0	1	1	0	0	1					
C7	1	1	0	0	0	1	1	1	0	0	0	1	1	0	0	1	0	0	1	1					
C8	1	1	0	0	0	1	1	1	0	1	0	1	1	0	0	1	0	0	1	1					
C9	1	1	0	0	0	1	1	1	0	1	0	1	1	0	0	1	1	0	0	1					
C10	1	1	0	0	0	1	1	1	0	0	0	1	1	0	0	1	1	0	1	1					
C11	1	1	0	0	0	1	1	1	0	1	0	1	1	0	0	1	1	0	1	1					
C12	1	1	0	1	0	1	1	1	0	1	0	1	1	0	0	1	1	0	0	1					
C13	1	1	0	0	0	1	1	1	0	1	1	1	0	0	0	0	0	1	1	0	RC	1	0	0	0
C14	1	0	1	0	0	1	0	1	0	1	1	1	0	1	1	0	0	1	1	1	RE	1	0	0	1
C15	1	0	1	0	0	1	0	1	0	1	1	0	0	1	1	0	0	1	1	1					
C16	1	0	0	0	0	0	0	1	0	1	1	0	0	1	0	0	0	1	1	1					
C17	1	0	0	0	0	1	0	1	0	1	1	1	0	1	1	0	0	1	1	1					
C18	1	0	1	0	1	1	0	1	0	1	1	1	0	1	1	0	0	1	1	1					
C19	1	0	1	0	0	1	0	1	0	1	1	1	0	0	1	0	0	1	1	0					
C20	1	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0	1	1	0					
C21	1	0	1	0	0	1	0	1	1	1	1	1	0	0	1	0	0	1	1	0					
C22	1	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	1	1	0						

Site numbers correspond to sites in Fig. 1 (from left to right). Above the site number appears the enzyme that recognizes the site (R = *RsaI*, H = *HaeIII*, E = *EcoRI*, B = *BamHI*, S = *SpeI*, EV = *EcoRV*). In the main body, designation 1 denotes presence of cleavage and 0 denotes absence of cleavage. Haplotypes are separated into those belonging to cluster F and those belonging to cluster M.

haplotype. We, thus, refer to the above haplotype as C1. The letter 'C' is used to indicate that we refer to the COIII region. Likewise we use the letter 'R' to refer to the 16S rRNA region. For this region we have detected four haplotypes for which Rawson & Hilbish (1996) have used the symbols A, C, D and E. Subsequently, we use the symbols RA, RC, RD and RE for these haplotypes. An issue arises when we want to refer to the restriction pattern of a genome at both surveyed regions. In this case we used the notation C.R., where the dot takes the value of the specific haplotype for the corresponding region. Thus, an mtDNA genome that has the restriction pattern #2 at the COIII region and the type A at the 16S rRNA region (Table 1) will be symbolized as C2RA. A further complication arises when we want to refer to both genomes of a heteroplasmic individual (as in the majority of male mussels). For this, we have adopted the convention that the first genome would be the maternally transmitted one followed by the paternally transmitted, with the two genomes separated by a slash. Thus the notation C3RD/C14RE indicates that this is a heteroplasmic individual, whose maternal genome has the restriction pattern #3 at the COIII region and the D pattern at the 16S rRNA region and whose paternal

genome has the #14 pattern at the COIII region and the E pattern at the 16S rRNA region. In several animals we could not score the paternal genome at the COIII region or at both, the COIII and the 16S rRNA regions. In these cases a dash is used to indicate the missing information. Thus C4RD/-RE indicates that the COIII region of the paternal genome could not be scored, whereas C10RA/- indicates that no information could be gained for either region of the paternal genome.

## Results

### Restriction maps and haplotypes

The restriction maps of the two studied regions, COIII and 16S rRNA, are shown in Fig. 1. Twenty-two different haplotypes were observed among our samples at the COIII region and four at the 16S rRNA region (Table 1). When the COIII haplotypes were used to produce a phylogenetic tree, they formed two distinct clusters (Fig. 2A), the F and the M cluster. Both, the neighbour-joining (shown) and the UPGMA (not shown) methods produced phylogenetic trees in which F and M haplotypes formed distinct clusters.

**Table 2** Numbers of mitochondrial profiles of female individuals found in each sample

	ATL	CHI	CEF	FAR	MP	HAL	Med. pooled	BS	Total
C1RA	8								8
C2RA	2								2
C3RA						1	1		1
C4RA						1	1		1
C7RA	3					3	3		6
C8RA	5					1	1	1	7
C9RA	2	2	7	10	6	12	37	3	42
C10RA		1	1				2	8	10
C12RA		1	1	1			3		3
C13RA	1								1
C9RC								1	1
C5RD						1	1		1
C8RD						1	1		1
C9RD		3	2	1	5		11	19	30
C11RD		1	1	2	1	2	7		7
C12RD								1	1
Total	21	8	12	14	12	22	68	33	122
C9RA/C19RE						1	1		1
C9RD/C14RE			1				1		1
Grand total	21	8	13	14	12	23	70	33	124

C<sub>j</sub> (where j varies from 1 to 22) refers to COIII haplotypes (part A of Table 1); R<sub>i</sub> (where i = A, C, D, or E) refers to 16S rRNA haplotypes (part B of Table 1). The slash separates the two genomes of heteroplasmic individuals.

Most haplotypes of the F cluster were recovered from females or 'typical' males (i.e. males that also contained an M type; see Terminology section in Material and Methods) and the majority of haplotypes of the M cluster from typical males. There were two exceptions to this rule. Haplotype C10 was recovered both from females and from 'atypical' males, i.e. males that did not contain an M type. Molecules of this type, i.e. molecules of type F found in atypical males, represent cases of 'masculinization', i.e. genomes that originated in the maternal lineage and have subsequently invaded the paternal transmission route (Hoeh *et al.* 1997). The second exception is haplotype C13, which clusters with the M haplotypes, but was recovered from a female individual. In five restriction sites C13 has a state found only in M-type molecules and in two sites it has a state found only in F-type molecules. There are two possibilities for the placement of this molecule in the M cluster: it may represent a case of 'feminization', i.e. a case of a molecule that originated in the paternal lineage and has subsequently invaded the maternal transmission route, or it may represent a case of recombination at the COIII region between an F and an M molecule. The latter alternative is more likely given that the female from which C13 was taken contains at the 16S rRNA gene a haplotype that is typical of an F molecule (Table 2).

Figure 2(A) shows also the populations in which each haplotype was found. In the same figure (Fig. 2B) we

provide the most parsimonious network of mutational descent of haplotypes. The multiple connections of some haplotypes with others and the circular patterns in parts of the networks indicate convergence through homoplasy or recurrent mutation. As a result, there are several equally probable possibilities of haplotype descent. Four F haplotypes (C3, C4, C5 and C6) could not be placed in the network in a way that would be consistent with their restriction differences from other haplotypes. Interestingly, they are also the four rarest haplotypes in the entire collection of samples: C3, C4 and C5 were each found twice and C6 was found once. This suggests that these haplotypes might be the products of multiple mutational hits, which will make them rare in the population and at the same time will make it difficult to embed them within the mutational network.

The first observation from comparing the phylogenetic tree with the mutational networks is that the tree may not depict accurately the mutational history of haplotypes. For example, C14 is placed closer to C18 than to C15 or C17, even though it is separated from both by one restriction difference and also co-occurs with both in the same geographical area. The second observation is that in both networks the haplotype with the larger number of connections (C9 for the F cluster and C14 for the M) is also the most common haplotype in the collection of samples (Table 4). The third observation is that the networks of descent are in

good agreement with the geographical origin of haplotypes. The high majority (89%) of all F-type molecules in the Atlantic sample is made of C1, C2, C7 and C8 which are tightly connected in the network of descent. The same applies to the Black Sea sample where 97% of the F molecules are made of C9 (by far the most common haplotype), and of C12 and C10, which are one and two steps, respectively, away from C9. All eight haplotypes of the F network were found in the Mediterranean, in agreement with the hypothesis that there must be a certain amount of gene exchange between the Mediterranean and the other two seas (see below). With regard to the M types, all three haplotypes found in the Atlantic sample (C22, C21 and C20) are interconnected in the network. The C14 was the only haplotype found in the Black Sea, and it is also the most common haplotype in the Mediterranean. This haplotype, together with C15, C17, C18 and C19 (all of which are tightly connected with C14), comprises 97% of the Mediterranean collection. The fourth observation is that the F haplotypes are more tightly interconnected than are the M haplotypes. Taking all pairwise combinations of haplotypes and counting the mutation steps that separate one member of the pair from the other (using always the shorter path), we obtain a mean distance of 2.17 mutations for the eight haplotypes of the F network and 4.64 for the 10 haplotypes of the M network. This is strong evidence for a higher turnover rate of M types in populations of mussels (see Discussion).

#### *Animals with typical and atypical mtDNA genotypes*

The composite haplotypes (i.e. the restriction patterns for the COIII and the 16S rRNA regions) of females from all seven samples are given in Table 2. We have observed only two atypical females among 124 examined (1.6%). These females contained an F and an M type and are therefore assumed to have inherited mtDNA from both parents. They represent an exception to DUI. Heteroplasmic females of this kind have been observed in previous studies, in some cases in high frequencies (Fisher & Skibinski 1990; Rawson *et al.* 1996).

Composite haplotypes for males are given in Table 3. Genotypes were grouped into four classes. Classes I and II include typical males. In class I the paternal genome could be scored for both mtDNA regions. In class II the paternal genome could be scored only for the 16S rRNA gene. There can be two explanations for the failure to score the paternal COIII region in males of class II. One is that the PCR primers we have used could not recognize the paternal COIII sequence in these males. The second is that in these males the paternal genome is in low amounts. This latter hypothesis coupled with the observation (Saavedra, unpublished) that the used PCR primers bind preferentially to the COIII of the F genome, would lead to the conclusion that the PCR

failed to produce a scorable COIII amount from these males. A previous study by Saavedra *et al.* (1997) supports this interpretation. These authors observed that one of the male parents used in laboratory crosses (male # 70) gave sons in which the COIII assay failed to produce evidence of presence of the paternal genome, yet the paternal COIII product could be detected in the father's gonad and sperm, thus eliminating the possibility of primer failure. When sons from the same sibships were scored at an older age, they were found to be positive for the paternal genome on the basis of the 16S rRNA assay (this assay was not performed on young males from the same sibship) and some were also positive for the COIII assay (Saavedra *et al.* unpublished results).

Classes III and IV contain atypical males, i.e. males that did not carry a molecule that in terms of nucleotide sequence belonged to the M cluster. In class III we could recognize two molecules both of type F: one that was dominant in the somatic tissues, and should therefore be the maternal molecule, and one that was dominant in the gonad, and should thus be the paternal molecule. In class IV males appear homoplasmic for an F-type molecule. There exist several possibilities for these males: (i) They carried a typical M molecule, but our assay failed to detect both the COIII and the 16S rRNA parts of these molecules. This explanation is unlikely on two grounds. First all males in our samples were large and with ripe gonads suggesting that age could not be a limiting factor in the detection of the M molecule. Second, even though we have noted in previous studies that COIII is more difficult to detect in juvenile than adult males no such difference was noted for the 16S rRNA region, which produces a scorable PCR product from young and old males that carry a typical M molecule; (ii) these males carry two molecules whose sequences may be different, yet they produce the same restriction profile at both scored regions; (iii) they have inherited truly identical molecules from each of their parents; (iv) they failed to inherit an mtDNA genome from their male parent. Atypical males of either class II or IV have been observed in previous studies by other authors (Fisher & Skibinski 1990; Rawson *et al.* 1996).

Unlike atypical females, whose rarity does not allow us to speak about differences in their geographical distribution, the distribution of the four classes of males differs strongly among samples. All Atlantic males belonged to class I. Almost all males of class II (30 out of 32) were found in the Mediterranean, and within the Mediterranean they were present in all five samples. We failed to observe males of class III or IV in the sample from the Atlantic, but this may simply be due to the limited size of this sample. The sporadic occurrence of males of this type in the Mediterranean samples is in line with what we would expect from previous population studies (Quesada *et al.* 1995). The comparison of the pooled Mediterranean frequency (8 out

**Table 3** Mitochondrial genotypes of male individuals found in each sample

	ATL	CHI	CEF	FAR	MP	HAL	Med. pooled	BS	Total
Class I									
C9RA/C15RE			2		1	2	5		5
C9RA/C14RE		1	6		2	6	15		15
C9RA/C19RE			1	1		3	5		5
C7RA/C18RE						1	1		1
C1RA/C15RE						1	1		1
C10RA/C14RE			2		1		3		3
C10RA/C15RE			1				1		1
C10RA/C18RE		1					1		1
C10RA/C19RE				1			1		1
C8RA/C20RE			1				1		1
C8RA/C19RE			1				1		1
C7RA/C19RE		1					1		1
C12RA/C14RE		1					1		1
C12RA/C15RE		1			1		2		2
C11RA/C14RE					1	1	2		2
C9RD/C14RE		4	3	4	1	1	13	6	19
C9RD/C19RE			2			1	3		3
C4RA/C14RE								1	1
C11RD/C18RE			1				1		1
C11RD/C17RE						1	1		1
C10RD/C17RE					1		1		1
C9RC/C14RE								1	1
C11RD/C16RE			1				1		1
C1RA/C22RC	2								2
C2RA/C22RC	2								2
C7RA/C22RC	2								2
C8RA/C21RC	1								1
C6RA/C20RC	1								1
Total	8	9	21	6	8	17	61	8	77
Class II									
C7RA/-RE				1	1		2		2
C8RA/-RE				1	1		2		2
C9RA/-RE		4	2	4			10		10
C10RA/-RE			1	1			2		2
C12RA/-RE		1					1		1
C1RD/-RE		1					1		1
C3RD/-RE		1					1		1
C5RD/-RE				1			1		1
C9RD/-RE		4	3			1	8	2	10
C10RD/-RE		1					1		1
C11RD/-RE				1			1		1
Total	0	12	6	9	2	1	30	2	32
Class III									
C9RA/C10RD								7	7
C9RC/C10RA								1	1
C9RD/C10RD								3	3
Total	0	0	0	0	0	0	0	11	11
Class IV									
C10RA/-			1				1	5	6
C9RD/-		2			2	1	5	4	9
C10RD/-		1					1		1
C11RD/-			1				1		1
Total	0	3	2	0	2	1	8	9	17
Grand Total	8	24	29	15	12	19	99	30	137

Symbolism as in Table 2. In class II no reading of the COIII haplotype of the paternal molecule was possible. In class IV only one molecule could be read.

of 99) with that of the Atlantic (0 out of 8) is not significant ( $P = 0.525$ , by Fisher's exact test).

In contrast to the sporadic presence of atypical males in the Atlantic and the Mediterranean samples, the frequency of these males in the Black Sea sample is exceptionally high, comprising two-thirds of all males. Eleven of the atypical males of the Black Sea sample belong to class III, which makes it possible to identify their paternal and maternal genomes through use of somatic tissue. In 10 of these males the paternal molecule has the restriction pattern C10RD and in one it has the pattern C10RA. C10RD was not found among females, so we may conclude that this haplotype is always paternally transmitted, even though on the basis of restriction pattern at both the COIII and the 16S rRNA regions it belongs to the F family. This cannot be said for the C10RA pattern that was also found among females, typical males and atypical class IV males.

#### *Distribution of maternal types among typical female and typical male individuals*

Given that typical males contain an easily identifiable maternal genome, one can ask whether maternal genomes found among males have the same frequency distribution as those found among females. When this test was carried out within each sample separately, it was found to be significant only in one Mediterranean sample (FAR: d.f. = 4,  $\chi^2 = 11.861$ ,  $P = 0.018$ ). This is not significant after the Bonferroni correction (5% critical value  $\alpha = 0.007$ ). Thus there is no reason to reject the null hypothesis that maternally transmitted genomes have an equal probability of being found among female or male individuals.

Another question is whether pairs of maternal and paternal haplotypes found among males are random combinations of the maternal and paternal haplotypes of the population as a whole. The question can be answered by using only males of class I. In class II the only scored part of the molecule, the 16S rRNA, was monomorphic and in classes III and IV both genomes were of the F type. The Black Sea sample does not provide information because all males contain the same paternal haplotype (C14RE). For the remaining six samples, the Raymond and Rousset test was barely significant in two Mediterranean samples (CEF,  $P = 0.057$ ; FAR,  $P = 0.065$ ) and significant for the Atlantic sample ( $P = 0.032$ ). Again, the latter result is not significant after the Bonferroni correction.

#### *Haplotype variation and geographical differentiation*

The observation that within each sample F types have similar distributions among female and male individuals allows the pooling of F genomes from both genders to form one distribution of F-type haplotypes. Table 4 gives the

numbers of F haplotypes and M haplotypes observed in each sample. In the same table we provide haplotype diversities. One noteworthy observation is that maternal molecules are, with one exception, more diverse than paternal molecules. This difference between the two types of genomes takes its extreme form in the BS sample, where there is no polymorphism for the M genome. This observation suggests that effective population sizes are larger for the F genome. We may use the method of Zouros (1979) to estimate the ratio of the two effective sizes in each population. Assuming neutrality, the expected effective number of alleles ( $n_e$ ) in the population is given by  $n_e = Cn_{e,u} + 1$  (Crow & Kimura 1970). The constant  $C$  varies on whether one assumes an infinite or a step-wise model of mutation and on whether one deals with nuclear or mitochondrial genomes. From this we can see that

$$N_{e,F}/N_{e,M} = (n_{e,F} - 1)/(n_{e,M} - 1)$$

where  $N_{e,F}$ ,  $N_{e,M}$ ,  $n_{e,F}$  and  $n_{e,M}$  are the effective population size and the effective number of alleles for the F and M genomes, respectively. An estimate of  $n_e$  is obtained from

$$n_e = 1/\Sigma p_i^2 = 1/(1 - D)$$

where  $D$  is the mitotype diversity (Table 4). The ratio of  $N_{e,F} : N_{e,M}$  is given in Table 4. No statistical testing can be done on these ratios, which are expected to have large standard errors given the small number of genomes on which they were estimated, but it is clear that the effective population size for the F genome exceeds that for the M genome, perhaps by a factor of two or more.

The distribution of the haplotypes of the F genome (Table 4) was significantly different when all seven samples were considered together ( $P = 0$  to the fifth decimal point by Raymond and Rousset's test). All pairwise comparisons of the Atlantic sample with the Mediterranean and the BS samples were also highly significant and so was the comparison of Atlantic vs. 'Mediterranean', i.e. the combined five Mediterranean samples. The comparison of BS with the Mediterranean samples, either separately or with their mean, produced the same result. For the five Mediterranean samples the same tests yield  $P = 0.036$ . A closer look at these samples reveals that the three samples from the Aegean (HAL, MP, FAR) are homogeneous (HAL/MP,  $P = 0.208$ ; HAL/FAR,  $P = 0.858$ ; MP/FAR,  $P = 0.810$ ). The Adriatic sample was different for the Ionian sample (CHI/CEF,  $P = 0.028$ ) and from the two Aegean samples (CHI/HAL,  $P = 0$ ; CHI/MP,  $P = 0.830$ ; CHI/FAR,  $P = 0.053$ ). The Ionian sample was different from two Aegean samples (CEF/HAL,  $P = 0.084$ ; CEF/MP,  $P = 0.003$ ; CEF/FAR,  $P = 0$ ). Thus, there is an indication that samples from the Aegean form a more homogeneous collection than the Mediterranean as a whole.

**Table 4** Frequencies of F-type and M-type in each sample, with corresponding haplotype diversities

	ATL	CHI	CEF	FAR	MP	HAL	Med. pooled	BS
F types								
C1RA	10					1	1	
C2RA	4							
C3RA						1	1	
C4RA						1	1	1
C6RA	1							
C7RA	5	1		1	1	4	7	
C8RA	6		2	1	1	1	5	1
C9RA	2	7	18	15	9	24	73	10
C10RA		2	6	2	1		11	14
C11RA					1	1	2	
C12RA		4	1	1	1		7	
C9RC								3
C1RD		1					1	
C3RD		1					1	
C5RD				1		1	2	
C8RD						1	1	
C9RD		13	11	5	8	4	41	34
C10RD		2			1		3	10
C11RD		1	4	3	1	3	12	
C12RD								1
Total	28	32	42	29	24	42	169	74
Haplotype diversity	0.824	0.784	0.734	0.683	0.768	0.662	0.745	0.648
M types								
C13RA	1							
C14RE		6	12	4	5	8	35	8
C15RE		1	3		2	3	9	
C16RE			1				10	
C17RE					1	1	20	
C18RE		1	1			1	30	
C19RE		1	4	2		5	12	
C20RE			1				10	
C20RC	1							
C21RC	1							
C22RC	6							
Total	9	9	22	6	8	18	63	8
Haplotype diversity	0.583	0.583	0.675	0.533	0.607	0.732	0.641	0

See text for the calculation of the ratio of the effective population sizes for the two types of genomes.

The comparison of the distribution of M haplotypes (Table 4) shows a much stronger degree of geographical differentiation. The Atlantic sample consists of four types, none of which was found in either the Mediterranean or the Black Sea to five decimal points in each sample, so there is complete differentiation between this sample and the rest. The Black Sea sample is fixed for haplotype C14RE. All Mediterranean samples contain two or more haplotypes, but haplotype C14RE is also the most common in all of them. Tests of heterogeneity are not very informative owing to small sample size of M genomes for most samples, but a comparison of the Black Sea sample with the combined Mediterranean sample is highly significant

( $P = 0$  to fifth decimal point from the Raymond and Rousset test).

The between sample Nei haplotype distances parallel the results from the homogeneity tests (Tables 5 and 6). The distance matrix illustrates clearly the fact that populations are more differentiated for the M than the F genome. The M distance is higher than the F in any pairwise comparison. The distances of the Atlantic sample for the M genome are on average nine times as large as that for the F genome. Likewise, the mean distance of the Black Sea sample from the Mediterranean samples is on average seven times as high for the M as for the F genome. The same table provides distances and  $F_{ST}$  values for the three seas. For the

**Table 5** Nei distances for pair-wise combinations of samples

	ATL	CHI	CEF	FAR	MP	HAL	BS
ATL	—	0.133	0.141	0.137	0.137	0.108	0.142
CHI	1.355	—	0.002	0.002	0.002	0.004	0.002
CEF	1.012	0.008	—	0.000	0.000	0.003	0.001
FAR	1.118	0.017	0.014	—	0.000	0.002	0.002
MP	1.318	0.009	0.015	0.041	—	0.002	0.002
HAL	1.115	0.008	0.005	0.005	0.024	—	0.004
BS	1.520	0.005	0.019	0.028	0.009	0.022	—

F genomes above the diagonal; M genomes below the diagonal.

**Table 6** Nei distances and  $F_{ST}$  values for the three major Seas for both types of molecules (MED is the average for the five Mediterranean samples)

	ATL/MED	MED/BS	ATL/BS
Nei distances			
F	0.131	0.002	0.142
M	1.184	0.017	1.520
$F_{ST}$ values			
F	0.196	0.098	0.235
M	0.378	0.133	0.694

Mediterranean the given values are the means of the five samples from the ATL and the BS samples. Some Mediterranean samples are marginally closer to BS than to other Mediterranean samples and it is only on posterior averaging that the Mediterranean samples appear to be a more homogeneous collection (average distances among Mediterranean samples 0.0017 and 0.0146 for the F and M genomes, respectively, compared to 0.0022 and 0.0166 which are the average distances of the five Mediterranean samples from BS). The  $F_{ST}$  values produce the same qualitative result.

## Discussion

The mere presence of two independently evolving mitochondrial lineages within the same species raises new opportunities but also calls for special attention when using mtDNA variation to study population structure and phylogeny in species with DUI. Here we have attempted to demonstrate these opportunities and difficulties in the mussel *Mytilus galloprovincialis*. A recent report of DUI in a species of the clam family Veneridae (Passamonti and Scali 2001) suggests that DUI may be more widespread than expected, at least within bivalves, so that our conclusions may have implications for a large number of species.

Lack of awareness that the species under study may have the DUI system of mtDNA may lead to false interpretations of population data. For example presence in a population of individuals that are heteroplasmic for two highly divergent mtDNA molecules could be the result of contact between two hybridizing yet sufficiently differentiated subspecies (Rand & Harrison 1989), or it could be the result of DUI (Hoeh *et al.* 1991). An unusually high degree of mtDNA variation among conspecific individuals from the same population should be taken as a first sign of DUI, but it cannot by itself establish the presence of DUI. For this, further evidence is needed, such as gender-specific differences in mtDNA sequence and male-specific heteroplasmy.

Another complication comes from the observation in mussels that not all individuals in a population conform to the rules of DUI. In this study the vast majority of females produced the typical DUI pattern, i.e. they were homoplasmic for the maternal genome. However, we have clearly identified two females that contained a maternal and a paternal genome. These females appear to violate the hypothesis of sex determination in mussels proposed by Saavedra *et al.* (1997). In an attempt to explain the tight correlation between maleness and presence of paternal mtDNA and the observation that in pair-matings of mussels the sex ratio varies widely and is under the control of the female parent, these authors suggested that in mussels the female is the default sex and that maleness is induced by a factor carried by sperm mitochondria. The mother's nuclear genotype determines whether the egg will abort the sperm mitochondria (and therefore develop into a female) or will retain the sperm mitochondria (and therefore develop into a male). The two atypical females suggest that maleness may not depend on the mere presence of a sperm mitochondrial factor in the early stages of development, but on the delivery of this factor in the primordial germ cells, a postulate made by Saavedra *et al.* (1997).

The majority of males in the Atlantic and the Mediterranean samples were also typical for a species with DUI. Atypical males (i.e. males lacking a typical M genome) in these two seas appeared with a frequency that is typical to most natural populations of mussels (8/107, or about 7%). In the Black Sea sample 20 of the 30 males were atypical. Eleven of these were clearly heteroplasmic for two slightly different F molecules. By using pure somatic tissues we could identify which of these molecules was inherited from the mother and which from the father. Given that mtDNA recombination is occurring in mussels (Ladoukakis & Zouros, 2001a), the possibility exists that the mtDNA molecule that dominates the gonads of these males is in fact of the M type, and that simply the COIII region of it has incorporated an F-like sequence through recombination between an F and an M molecule. This seems unlikely given that these same molecules have also an F-like

sequence at the 16S rRNA gene, which is located at a distant part of the mtDNA molecule. We conclude that the whole paternal molecule in these 11 males is of the F type, yet in terms of function and mode of transmission this molecule behaves as an M molecule. This is one type of evidence used by Hoeh *et al.* (1996b, 1997) to propose the 'masculinization' or 'role reversal' hypothesis according to which a maternally transmitted genome may revert into a paternally transmitted one. The other type of evidence comes from the pair-mating study of Saavedra *et al.* (1997), who observed that sons from a specific male produced sons with no or abnormally small amounts of M genome.

A large fraction of atypical males in the sample from the Black Sea were scored as homoplasmic for an F molecule (class IV, Table 3). We have examined several hypotheses about how this class of males can be explained (see Results). Apparent male homoplasmy is compatible, indeed expected, under the phenomenon of masculinization, which we have outlined above. This will happen when the paternal and maternal molecules in a male individual cannot be diagnosed as different with the assay in use. Taken together classes III and IV raise the question why these atypical males predominate in the BS sample, while they are rather rare in the other samples. The most likely explanation is that in the sampled Black Sea population there has been a recent invasion of the paternal route by a maternally inherited molecule and that this 'recently masculinized' molecule has increased its frequency either by random drift or selection. Ignoring class IV, in which the paternal molecule cannot be distinguished from the maternal one, we may recognize two types of masculinized molecules: C10RD and C10RA (class III, Table 3) of which the latter was found only once. It is difficult to claim that these molecules resulted from two separate masculinization events, since RA differs from RD by one restriction site and could have come about by mutation or recombination.

The question of whether the masculinized molecule owes its high frequency to selection or random drift is of high importance for the phenomenon of DUI, but we do not have at present direct evidence that bears on this issue. However, the phenomenon of replacement of M molecules by F molecules appears to have occurred very often over evolutionary time. Such events provide the best explanation for the mitochondrial phylogenies between the families Mytilidae and Unionidae (Hoeh *et al.* 1996b), between the genera *Gukencia* and *Mytilus* of Mytilidae (Hoeh *et al.* 1996b) and between *Mytilus californianus* and the *Mytilus edulis* species complex of the genus *Mytilus* (Hoeh *et al.* 1997). It was also suggested as the more likely explanation for the introgression of F-type sequences from *Mytilus edulis* into the paternally transmitted molecules of *Mytilus trossulus* of the Baltic Sea (Quesada *et al.* 1999). We may consider the Black Sea population as a case where we have an in-progress intraspecific replacement of the M lineage

by a molecule (and its mutational and/or recombinational derivatives) that has invaded the paternal route from the maternal route. If so, in due time the entire pool of maternal molecules of this population will resemble the maternal pool of this and other conspecific populations. Presence of 'typical' M molecules in this population will only depend on gene flow from other populations. The independent segregation of maternal from paternal genomes and of both these two molecules from the nuclear genes makes it impossible to decide whether the typical M molecules found in the Black Sea sample are indigenous to this population or have been brought in by migration. Regardless of the usual difficulties in attributing the increase of a molecular variant to selection or drift or in assigning its presence to indigenous origin or migration, the fact remains that the phenomenon of role reversal may lead to completely erroneous results when using the pool of paternally inherited molecules to draw conclusions about biogeography and phylogeny. Our awareness of the phenomenon allowed the assignment of F-like paternal molecules to the F cluster of molecular phylogeny, so that evolutionary distances among samples were separately inferred from each of these clusters. Treating all paternal molecules of the BS sample as one (even though highly heterogeneous) lineage would have created difficulties at arriving in a consistent *M. galloprovincialis* phylogeography and would have necessitated appeals to macro-evolutionary events, such as speciation and interspecific hybridization.

Once the occurrence of DUI itself is recognized and the complications from the associated events of role reversal are appreciated, the analysis of population data of mtDNA variation may proceed by examining the F-cluster and the M-cluster separately. Three related differences between the two genomes are worth noting. Firstly, the connectedness of the F haplotype mutational network is much tighter than the M network (Fig. 2B), suggesting an overall higher turnover rate for the M genome. Secondly, the within-sample variation (and therefore the estimated effective population size) is smaller for the M genome (Table 4); and thirdly, the among-sample differentiation is larger for the M genome (Table 6). Several authors have made the observation that the M genome evolves faster than the F (Skibinski *et al.* 1994b; Hoeh *et al.* 1996a; Rawson & Hilbish 1996; Stewart *et al.* 1996) and proposed explanations for it. One explanation is that the F genome has a larger effective population size by virtue of its occurrence in both sexes, whereas the M genome is restricted to males. This explanation ignores the fact that a mutation that occurs in an F molecule during its residence in a male individual will not pass on to the next generation, thus males are evolutionary dead ends for the F genome. But this argument assumes no free recombination between F and M molecules, for which we have no evidence one way or the other [the recombination

observed by Ladoukakis and Zouros (2001a) was between true F and masculinized F molecules]. Another hypothesis is that the M genome is under strong directional selection imposed from a presumed specialized role for high sperm performance (Skibinski *et al.* 1994b). Such a selection would generate successive waves of selective sweeps (Begun & Aquadro 1992) for the entire molecule (again assuming no or minimal recombination) and would explain all three differences between the two genomes mentioned above. A third hypothesis makes the contrary assumption that the M genome is under relaxed selection owing to its limited function in the male germ line, whereas the F genome's role is to support the female germ line and the soma of both males and females. The two competing hypotheses were addressed by Stewart *et al.* (1996) who used data for the COIII gene from mollusc species other than *Mytilus* to identify highly constrained amino acid sites (those that did not vary among species) and less-constrained sites (those that varied). The *Mytilus* data were then used to show that the faster replacement rate of the M genome was confined to the less-constrained sites, whereas the rate was the same at highly constrained sites. This finding is compatible with the hypothesis of relaxed constraint for the M genome, i.e. there were many more sites at which mutation would be nearly neutral or slightly deleterious when occurring in the M genome, but highly deleterious when occurring in the F genome. This hypothesis will explain the higher evolutionary rate of the M molecule. It would also explain the lower standing variation within a population and the high rate of turnover and intersample differentiation by accumulation of mild deleterious mutations (Ohta 1976) or background selection (Charlesworth *et al.* 1995).

The faster evolution and turn-over rate of the M lineage combined with its liability to invasion from the F lineage further suggest that this lineage is not reliable for phylogenetic and biogeography studies of species with DUI. Liu *et al.* (1996) have shown that among three types of genetic information of population differentiation (nuclear allozymes, F mtDNA lineage and M mtDNA lineage) the M lineage was the one that produced the larger degrees of differentiation. This is clearly the case in our study, where the M genome completely differentiates the Atlantic sample from both the Mediterranean and the Black Sea. It also differentiates the Mediterranean from the Black Sea, even though this is because the Black Sea is fixed for a haplotype that is one among several present in the Mediterranean (Table 4).

From the point of biogeography of *Mytilus galloprovincialis* our study suggests that in spite of the species' inherent ability to disperse through its planktonic larval stage, geographical differentiation can accumulate. This can be seen even within the Mediterranean, where the Adriatic, the Ionian and the Aegean Seas are different from each other even at the 'normally' behaving F molecule, and from the

fact that no such differences can be seen among the three Aegean samples. At a macro-geographical scale, the Atlantic is placed at one end of differentiation, on account of both the M and the F genomes. This observation was made previously (Quesada *et al.* 1995) for the Atlantic/Mediterranean comparison and is attributed to the Almeria–Oran hydrographic barrier, which is known to affect the gene flow of other species as well (Ramon & Castro 1997; Naciri *et al.* 1999; Zane *et al.* 2000). The differentiation of the Mediterranean and the Black Sea is significant but much smaller than that between each of these seas from the Atlantic. Contrary to the Atlantic, the Black Sea sample contained no haplotype, either F or M that was not found in the Mediterranean. This might appear as unexpected given the water straits and hydrographic barriers that may restrict migration between the Mediterranean and the Black Sea. In anchovies mtDNA haplotype variation suggested a predominantly one-way gene flow from the Black Sea to the Aegean (Magoulas *et al.* 1996), but this pattern is not obvious in *M. galloprovincialis* from this study, as evidenced from failure to observe an increased presence of F-type paternal molecules in the Aegean Sea. This, however, might be attributed to the large geographical distance of the examined BS sample from the corridor of communication between the two seas. We note, however, that our results regarding the degree of population differentiation within the Mediterranean and the Black Sea, as well as among the three major Seas, are based on small samples sizes. This is particularly true for the Black Sea, from where we were able to secure only one sample. Obviously, a more elaborate sampling scheme is needed before we could have a good idea of the gene flow of *M. galloprovincialis* between these three large water bodies.

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