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Research Article

Presence of two mitochondrial genomes in the mytilid *Perumytilus purpuratus*: Phylogenetic evidence for doubly uniparental inheritance

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Abstract

This study presents evidence, using sequences of ribosomal 16S and COI mtDNA, for the presence of two mitochondrial genomes in *Perumytilus purpuratus*. This may be considered evidence of doubly uniparental mtDNA inheritance. The presence of the two types of mitochondrial genomes differentiates females from males. The F genome was found in the somatic and gonadal tissues of females and in the somatic tissues of males; the M genome was found in the gonads and mantle of males only. For the mitochondrial 16S region, ten haplotypes were found for the F genome (nucleotide diversity 0.004), and 7 haplotypes for the M genome (nucleotide diversity 0.001), with a distance Dxy of 0.125 and divergence Kxy of 60.33%. For the COI gene 17 haplotypes were found for the F genome (nucleotide diversity 0.009), and 10 haplotypes for the M genome (nucleotide diversity 0.010), with a genetic distance Dxy of 0.184 and divergence Kxy of 99.97%. Our results report the presence of two well-differentiated, sex-specific types of mitochondrial genome (one present in the male gonad, the other in the female gonad), implying the presence of DUI in *P. purpuratus*. These results indicate that care must be taken in phylogenetic comparisons using mtDNA sequences of *P. purpuratus* without considering the sex of the individuals.

Keywords: Mytilidae, 16S, COI, *Perumytilus*, DUI.

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Introduction

In most animal species, mitochondrial DNA (mtDNA) is inherited maternally (Avise *et al.*, 1987; Birky, 1995). Nevertheless, a different inheritance mode has been described in bivalves, known as doubly uniparental inheritance or DUI, (Skibinski *et al.*, 1994; Zouros *et al.*, 1994a; Zouros, 2000, 2013; Passamonti and Ghiselli, 2009; Breton *et al.*, 2007). This type of mitochondrial inheritance has been found in seven families of bivalves (Theologidis *et al.*, 2008), and in five of the 33 genera of the Mytilidae family (Teske *et al.*, 2012), including *Mytilus*, *Geukensia*, *Musculista* and *Brachidontes* (Theologidis *et al.*, 2008). Among Mytilidae, the DUI mechanism has been well studied in *Mytilus* (Hoeh *et al.*, 1997; Quesada *et al.*, 1999; Zbawicka *et al.*, 2003). This inheritance is characterized by the presence of two highly divergent mtDNAs, known as F (Female) and M (Male) mitochondrial genomes (Fisher and Skibinski, 1990; Hoeh *et al.*, 1991; Skibinski *et al.*, 1994; Zouros *et al.*, 1994b).

In DUI species, the F genome is transmitted by females to their male and female offspring, whereas the M genome is transmitted by males, and generally only to male offspring. Consequently, females are homoplasmic for the F genome and males contain both genomes (F and M), although their spermatozoa may be homoplasmic for the M genome (Venetis *et al.*, 2006; Ghiselli *et al.*, 2011). In females, the M genome is generally lost after successive cell divisions, although it can be detected in small quantities in somatic tissues in adult females (Stewart *et al.*, 1995; Garrido-Ramos *et al.*, 1998; Dalziel and Stewart, 2002; Ghiselli *et al.*, 2011; Zouros, 2013). The distribution of mitochondria inherited from sperm shows two different patterns in embryos. In the case of male embryos, mitochondria are aggregated in a single blastomere, which is the precursor of the male germ lineage, while in female embryos, mitochondria inherited from males are dispersed and disaggregated (Cao *et al.*, 2004; Obata and Komaru, 2005; Cogswell *et al.*, 2006; Milani *et al.*, 2011, 2012), meaning that usually only the F genome is present in their somatic tissues. It has also been proposed that the mechanism is due to the presence of a factor known as Z, controlled by a nuclear locus with two alleles, *Z* and *z* (Kenchington *et al.*, 2002). Females with the *Z* allele produce eggs that allow the retention of sperm mitochondria and their aggregation in the germ line of embryos that will become male. Females with the *zz*

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genotype produce eggs without the Z factor; as a result, the mitochondria of the fertilizing spermatozoon are dispersed or lost, and the embryos will all be female (see reviews by Kenchington *et al.*, 2002, 2009; Passamonti and Ghiselli, 2009; Zouros, 2013).

Theologidis *et al.* (2008) cite 36 bivalve species known at that date to have DUI, all of North Atlantic origin; however Boyle and Etter (2013) reported DUI in a cosmopolitan species recorded in the South Pacific. Nevertheless there are few studies which report this process in species distributed in the southern hemisphere. The aim of this study was therefore to determine whether the mytilid *Perumytilus purpuratus* (Mytilidae), an endemic species of the southern cone of South America, displays DUI. This species is of ecological importance, shaping community structure and acting as a bioengineer in the rocky intertidal; its geographical distribution ranges from the Pacific (Ecuador to Chile) to the Western Atlantic, as far north as La Lobería, Argentina (Guiñez and Castilla, 1999; Lancellotti and Vasquez, 2000; Prado and Castilla, 2006; Acevedo *et al.*, 2010; Caro *et al.*, 2011). The species belongs to a monospecific genus, phylogenetically close to the genus *Brachidontes* (Aguirre *et al.*, 2006; Trovant *et al.*, 2013). In this study, fragments of the mitochondrial genes 16S and COI were sequenced from various female and male adult tissues of *P. purpuratus.* The 16S and COI mtDNA were used as molecular markers representing the mitochondrial genome.

Materials and Methods

Sampling area

Adults of both sexes of *Perumytilus purpuratus* were collected from the rocky intertidal of Pelluco (41°12'S; 72°53'W; Puerto Montt, Chile) during the spring (November and December). Each individual was sexed by observation of its gonads under a stereo microscope, and tissue samples were taken from the mantle and gonads of sexually mature adults. Samples were labeled and conserved in ethanol (95%) at 4°C.

DNA extraction

Total DNA was extracted from 30 mg of mantle and gonad tissues from adults using the standard phenol method (Doyle and Doyle, 1987). The quality and quantity of DNA were assessed by electrophoresis in 1% agarose gels stained with SYBR safe (Invitrogen).

PCR amplification

The 16S region of mtDNA was amplified with the universal primer pair 16S-AR and 16S-BR (Palumbi *et al.*, 1991). The cytochrome oxidase subunit I (COI) was amplified using the primers COIaF and COIaR designed by Trovant *et al.* (2013). These amplifications were carried out in a final volume of 30 μL of solution containing: 50 ng DNA template for each individual adult, 2 μL of 10X PCR Rxn Buffer, 0.2 mM of dNTPs, 2.5 mM of $MgCl_2$, 0.2 μM of each primer and 1 U of *Taq* DNA polymerase. The amplification protocol consisted of an initial denaturation at 95 °C for 9 min, followed by 35 cycles of 95 °C for 1 min, 40 °C for 1 min (for the 16S gene) or 45 °C for 1 min (for the COI gene) and 72 °C for 1 min, followed by a final extension at 72 °C for 9 min. The amplified products were visualized under UV light with SYBR safe dye in 1.5% agarose gels. The PCR products were purified with the Purelink PCR purification kit (Life Technologies) and sequenced using an automatic ABI Prism 377 sequencer (Applied Biosystems). Different annealing conditions were evaluated to facilitate amplification and obtain different mitochondrial genomes.

Sequence analysis

The sequences obtained were edited using the BLAST-2 and BIOEDIT 5.0.9 softwares (Hall, 1999), and multiple alignment was carried out with the CLUSTAL X program (Thompson *et al.*, 1994).

The number of polymorphic sites, number of haplotypes, haplotype diversity, nucleotide diversity and the average number of different nucleotides were estimated using the DnaSP software version 5.53 (Librado and Rozas, 2009).

To detect differences in the number of mutations accumulated between sample types, and so establish the expansion history of each genome, an analysis of mutation frequency between sequence pairs (mismatch distribution) (Rogers and Harpending, 1992) was applied using the DnaSP software version 5.53 (Librado and Rozas, 2009).

The Neighbor Joining method (Saitou and Nei, 1987) implemented in MEGA 5 software (Tamura *et al.*, 2011) was used to represent the degree of similarity of sequences. For the 16S sequences, a *Brachidontes* sequence retrieved from GenBank (accession n° DQ836016) was used as external group. To determine the distance between female and male sequences, we calculated the number of base substitutions per site by averaging all sequence pairs between groups with standard error using the Maximum Composite Likelihood model and 1000 bootstrap replicates. The variation rate among sites was modeled with a gamma distribution (shape parameter = 1).

The DNA divergence between F and M genomes was estimated by using the average number of nucleotide substitutions per site between genomes (Dxy) (Nei, 1987) with chi-square test (haplotype data) (Nei, 1987; Hudson *et al.*, 1992) and a permutation test (Hudson *et al.*, 1992). The average number of nucleotide differences between populations (Kxy) was estimated using the DnaSP software. The neutrality of each genome was evaluated with Fu and Li's Test, implemented in DnaSP software version 5.53 (Librado and Rozas, 2009).

To evaluate the origin of DUI and the complex relationships between the F and the M mitochondrial molecules, two phylogenies were constructed based on 16S and COI sequences obtained for *P. purpuratus* and sequences of F and M genomes from *Brachidontes variabilis*, *Mytilus edulis, M, californianus* and *M. galloprovincialis.*

Results

A total of 105 sequences, from both tissues and both mitochondrial markers, were obtained from 35 samples of *P. purpuratus*: 14 females and 21 males (details in Tables 1 and 2). Somatic and gonadal tissue for each individual was analyzed. A total of 65 sequences of 486 bp from the 16S region of mtDNA in *P. purpuratus* were obtained, representing the somatic tissues of 14 females and 20 males, and the gonadal tissues of 13 females and 18 males. For the COI gene, a total of 40 sequences of 540 bp were analyzed, corresponding to the somatic tissues and the gonadal tissues of 10 females and 10 males. Only in two cases both genomes were amplified in a single individual and unfortunately these sequences were impossible to read (Phenograms in Figure S1). All the sequences of the 16S region were deposited in GenBank under accession numbers KF159809 to KF159878 and KF661909 to KF661918 and all the sequences of COI gene under accession numbers KF661919 to KF661973 (sequence alignments are shown in Figure S2).

Two mitochondrial genomes were obtained in *P. purpuratus* mussels. In females only one type of genome was amplified (F genome) for both mitochondrial genes, in both somatic and gonadal tissues. In males, two kinds of genomes were found (F and M) for both mitochondrial genes. Specifically, in the gonadal tissues of males only the M genome was found, for both mitochondrial genes, while somatic tissue of males either the F or the M genome was found for 16S, and only the F genome was obtained with the COI primers used. In male mantles, 16S primers amplified the M genome in 15 samples and the F genome in five others (Figure 1); the COI primers amplified only the F genome in all the samples of male mantle tissues analyzed, while the M genome was amplified in all the male gonadal tissues.

A total of 65 sequences were analyzed for the 16S region; 17 haplotypes were identified, showing 67 polymorphic sites (S) with haplotype diversity h = 0.856; nucleotide diversity was π or Pi = 0.064. For the F genome, 32 sequences were obtained and 10 haplotypes were identified, with nine polymorphic sites; haplotype diversity was h = 0.833 and nucleotide diversity Pi = 0.004. For the M genome, 33 sequences were obtained and seven haplotypes were identified, with seven polymorphic sites; haplotype diversity was h = 0.589 and nucleotide diversity Pi = 0.001 (Table 1). In total, nine haplotypes were obtained in female individuals and 10 in males; there are therefore two haplo-

Table 1 - Indices of genetic variability based on mtDNA (16S) sequences F and M genomes, for females and males (gonadic and somatic tissues together) and total in the mussel *P. purpuratus.*

	Genome F	Genome M	Females	Males	Total
Number of sequences	32	33	27	38	65
Sequence length (bp)	486	486	486	486	486
S (polymorphic sites)	9	7	8	67	67
Number of haplotypes	10	7	9	10	17
Haplotype diversity ± sd	0.83 ± 0.04	0.59 ± 0.1	0.84 ± 0.04	0.69 ± 0.08	0.86 ± 0.03
Nucleotide diversity P	0.004	0.001	0.004	0.031	0.064
N° different nucleotides K	2.046	0.818	2.148	15.065	31.327

Table 2 - Indices of genetic variability based on mtDNA (COI) sequences for F and M genome, females and males (gonadic and somatic tissues together) and total in the mussel *P. purpuratus.*

	Genome F	Genome M	Females	Males	Total
Number of sequences	30	10	20	20	40
Sequence length (bp)	542	542	542	542	542
S (polymorphic sites)	27	18	21	116	118
Number of haplotypes	17	10	12	20	27
Haplotype diversity ± sd	0.97 ± 0.01	1 ± 0.05	0.95 ± 0.03	1 ± 0.02	0.98 ± 0.01
Nucleotide diversity P	0.009	0.010	0.009	0.102	0.076
N° different nucleotides K	5.074	5.911	5.021	55.484	41.621

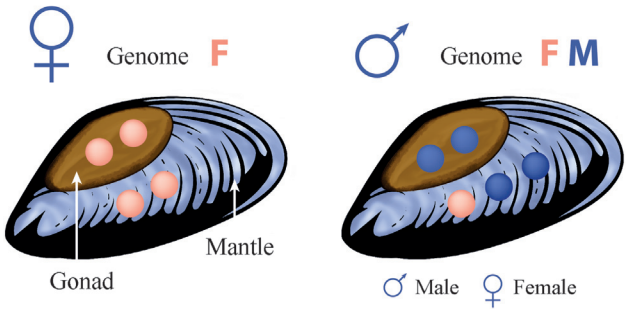

Figure 1 - Distribution of mitochondrial genomes by tissue-type and sex in the Chilean mussel *Perumytilus purpuratus* evaluated with sequences of the 16S region. F: F-genome; M: M-genome.

types shared between males and females, which are present in four out of five males that presented the F genome in mantle tissue.

A total of 40 sequences were analyzed for the COI gene; 27 haplotypes were identified with 118 polymorphic sites (S); haplotype diversity was h = 0.981 and nucleotide diversity Pi = 0.076. In the F genome, 30 sequences were obtained and 17 haplotypes were identified, with 27 polymorphic sites; haplotype diversity was h = 0.966 and nucleotide diversity Pi = 0.009. In the M genome, 10 sequences were obtained and 10 haplotypes were identified, with 18 polymorphic sites; haplotype diversity was h = 0.997 and nucleotide diversity Pi = 0.010 (Table 2). In total, 12 haplotypes were found in female individuals and 20 haplotypes in males; there are therefore five haplotypes shared between males and females, which are present in five out of 10 males that presented the F genome in mantle tissue.

The F genome (found in females and males) showed higher genetic diversity than the M genome (found in males), for both mitochondrial genes (16S and COI) mainly in polymorphic loci, the number of haplotypes, haplotype diversity and different nucleotides (Table 1 for 16S region; Table 2 for COI gene).

Distribution of pairwise differences between haplotypes (mismatch distributions) was used to estimate past population expansions by haplotypes. In this case, the F genome of *P. purpuratus* displayed a larger number of accumulated mutations than the M genome in its 16S sequences. However this pattern was less evident in the COI gene sequences (Figures 2A and 2B, respectively).

Based on a high bootstrap value for both genes analyzed, the sequences were segregated into two well-defined and well-supported clades (Figure 3). One of the clades grouped female haplotypes (F) and somatic tissue of five males together, while the other clade grouped male haplotypes (M) alone, with no differences with regard to tissue type (somatic or gonadal). A significant genetic divergence was detected between F and M haplotypes: in the 16S region sequences the genetic distance was Dxy = 0.125 and genetic differentiation Kxy was 60.33%, with high significance value ($p < 0.001$), estimated by permutations test. The corresponding values for the COI gene sequences were Dxy = 0.184 and genetic differentiation Kxy was 99.97% ($p < 0.001$).

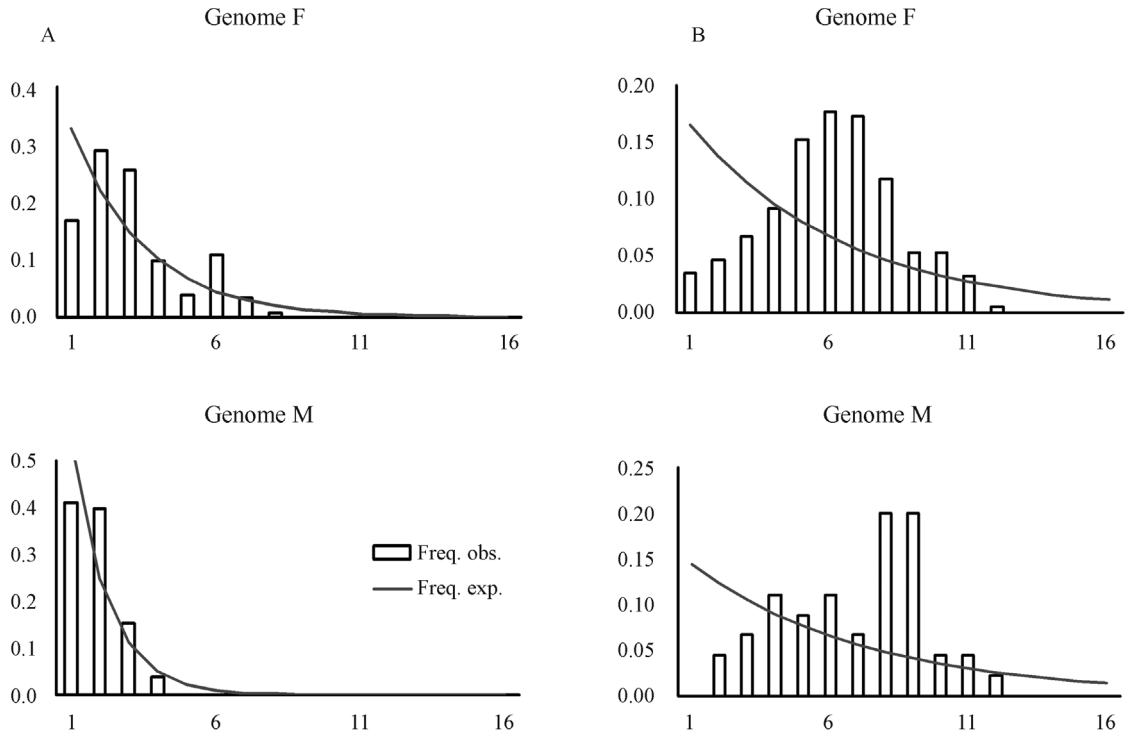

Figure 2 - Frequency distribution graphs of the number of mutations between pairs of sequences (mismatch distribution), obtained from the 16S mitochondrial gene (A) and COI gene (B) for the two genome types, F and M.

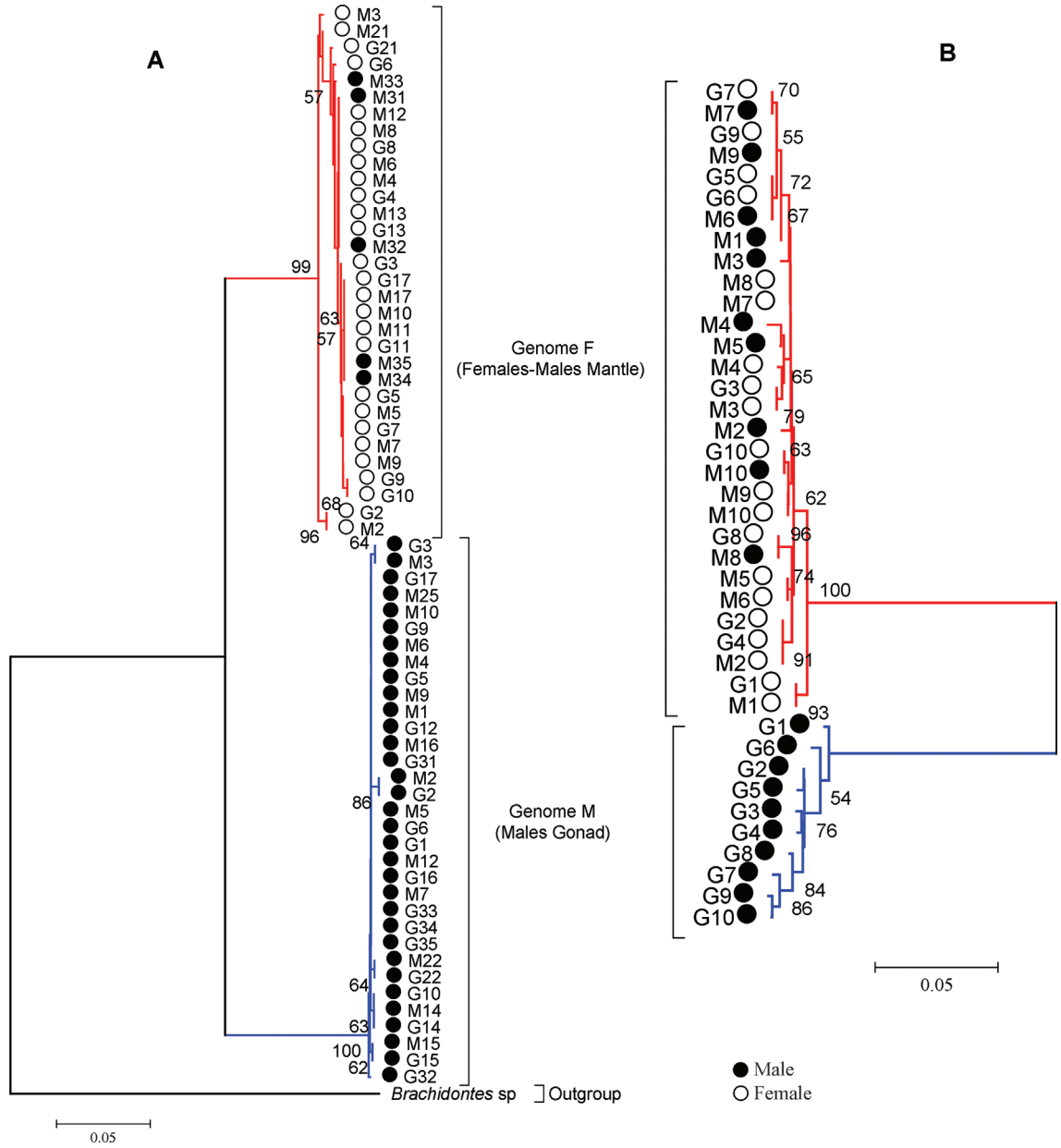

Figure 3 - Neighbour-joining tree of mitochondrial haplotypes in *P. purpuratus*, based on (A) the mitochondrial gene 16S and (B) COI gene. M: mantle tissue; G: gonadal tissue.

Finally, in order to understand the origin of DUI, we analyzed both genomes (F and M haplotypes) from *P. purpuratus* in comparison with F and M haplotypes from other DUI species. The haplotypes were segregated by species of origin (taxon-joining pattern by Zouros, 2013), and a single clade was observed for each of the *P. purpuratus* mitochondrial genes (Figure 4). Phylogenetic reconstruction showed that species formation predated the differentiation of the mitochondrial genomes (gender-joining pattern) in *P. purpuratus*. This was most obvious from the COI data (Figure 4 B).

Discussion

The phylogenetic reconstruction from both genes distinguishes two clades: one specific to M haplotypes, obtained from males, present in 100% of the gonadal tissue samples for both genes and in somatic tissues for 16S; the other specific to F haplotypes, obtained from female somatic and gonadal tissues and male somatic tissues. This conclusion is supported by high values of consistency and significant genetic divergence, displaying robust separation between female (F) and male (M) haplotypes.

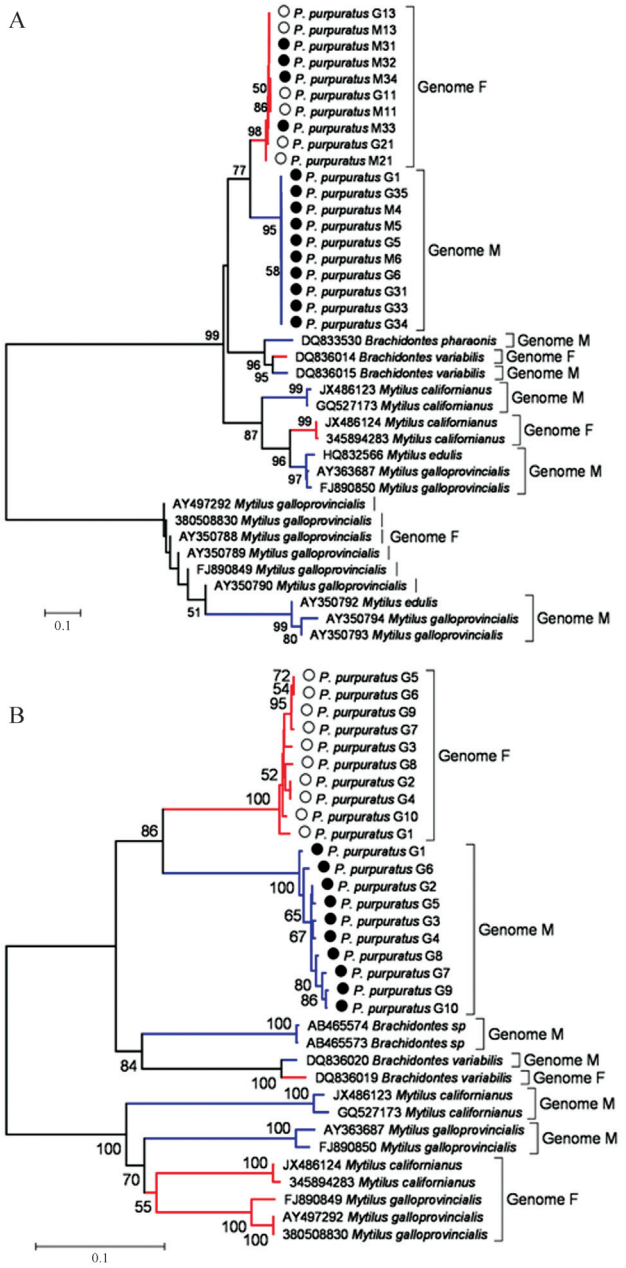

Figure 4 - Neighbour-joining tree for evolutionary relationships of the F and M genomes of *P. purpuratus* and other species of *Mytilus*, based on (A) the mitochondrial gene 16S and (B) gene COI genome. M: mantle tissue; G: gonadal tissue.

This indicates the presence of DUI in *P. purpuratus*. As expected for DUI species, males are heteroplasmic for both F and M genomes, and females are homoplasmic for the F genome only (Hoeh *et al.*, 1996, 1997, 2002; Quesada *et al.*, 1999; Zbawicka *et al.*, 2003). Each sex presents only the sex-specific genome in its gonad. In general, differentiation at the mitochondrial genome level between F and M has been estimated at up to 20% in the Mytilidae family (Passamonti *et al.*, 2003; Cao *et al.*, 2004; Mizi *et al.*, 2005; Breton *et al.*, 2006; Theologidis *et al.*, 2008), and as high as 50% in fresh water species of the Unionidae family (Doucet-Beaupré *et al.,* 2010). In *P. purpuratus,* this value was found to be between 60% and 99%, depending on the mitochondrial gene. This is higher than has been observed for other species with DUI, and may result from the lower occurrence of the F genome detected in the male mantle with 16S primers, and non-detection of the M genome in the male mantle for the COI gene. Detection may be improved in the future by the design of specific genome primers.

Terranova *et al.* (2007) observed intraspecific variability in *Brachidontes variabilis* for differentiation between F and M haplotypes, and therefore in DUI ocurrence. This variability resulted from differences observed between F and M haplotypes present in females and males respectively, similar to *P. purpuratus*. However this pattern was only observed in samples collected in the Indian Ocean, and was not apparent in samples collected in either the Pacific, the Red Sea or the Mediterranean (Terranova *et al.*, 2007). The authors argued that this may be due to the presence of three cryptic species with allopatric distribution in each location.

Moreover, *P. purpuratus* displayed greater variability in 16S F haplotypes than in M haplotypes, whether measured by the number of accumulated mutations in its genome over time (Figure 2) or by genetic variability indicators (Tables 1 and 2). Similar observations are reported in the mytilid *Musculista senhousia* (Passamonti, 2007), which may be explained by an older evolutionary history of the F genome than the M genome, with the latter appearing at a more recent date. However this deviation may also be due to the lower frequency with which the M genome is observed, meaning that its diversity may be underestimated. The pattern observed for the COI gene was more complex, since some indicators showed the same pattern, while others presented greater diversity in the M genome. The explanation suggested for *M. senhousia* is that this mechanism has evolved to protect mtDNA in females (*e.g.* antioxidant gene complexes) while selection could be relaxed in males (Passamonti, 2007; Zouros, 2013). In other mytilid species, however, it has been observed that M genomes are more variable than F genomes (Rawson and Hilbish, 1995; Stewart *et al.*, 1995; Zouros, 2013). Greater genetic diversity of the M genome may be due to several mechanisms, such as greater M mitochondrial replication rates during the early development of male embryos, differences in selection pressures, or the result of different effective sizes between genomes, as proposed by some authors (Skibinski *et al.*, 1994; Stewart *et al.*, 1996; Schmidt *et al.*, 1997; Hasegawa *et al.*, 1998; Ballard, 2000a,b).

The results obtained from the mismatch distribution indicate that the F genome has a longer history than the M genome, based on the greater number of accumulated mutations. This is more evident in the results for the 16S gene than the COI gene, as shown in the mismatch distribution

graphs (Figure 2). An empirical mismatch distribution that does not deviate from a unimodal distribution of pairwise differences among haplotypes and presents smooth distribution (Harpending, 1994) suggests recent expansion (Rogers and Harpending, 1992).

In addition to the evidence mentioned above, which indicates the presence of two mitochondrial genomes and the presence of DUI, the heteroplasmy (two genomes in the same individual) found in five males for the 16S region and 10 males for the COI gene confirms this mode of inheritance in *P. purpuratus*. The presence of M haplotypes in male tissues only (gonadal and somatic), and F haplotypes in both female tissue types and in male somatic tissues, confirms the existence of heteroplasmy in males of *P. purpuratus,* as expected for a DUI species (Mizi *et al.*, 2005; Breton *et al.*, 2006; Venetis *et al.*, 2006; Theologidis *et al.*, 2008; Cao *et al.*, 2009; Passamonti and Ghiselli, 2009). However the M haplotype was more often amplified from gonadal tissues of *P. purpuratus* males with 16S and COI primers (100%) and less often from somatic tissues of males with 16S primers (75.0%). This has previously been observed by Passamonti and Scali (2001) and Ghiselli *et al.* (2011) using cloning and Real-Time qPCR, respectively, and in the results reported by Terranova *et al.* (2007), who detected evidence for DUI only with 16S-rDNA but not with COI. Previous studies have found evidence for such situations, where somatic tissues in males can be dominated by the F genome, with the occasional presence of small quantities of the M genome (Garrido-Ramos *et al.*, 1998; Dalziel and Stewart, 2002; Ghiselli *et al.*, 2011). This different pattern may be tentatively related to the specific segregation mechanism of sperm mitochondria during male embryo development in DUI species (Cao *et al.*, 2004; Obata and Komaru, 2005; Cogswell *et al.*, 2006; Milani *et al.*, 2011, 2012), which drives sperm-derived mitochondria into the primordial mesodermal fate blastomeres (from which the adductor derives), whereas it allows only stochastic leakage into ectodermal fate blastomeres (from which the mantle originates). Our data for the M genome detected in the mantles of some males but not in others, may tentatively be explained by such stochastic events. In future work we propose to design new M- and F-specific primers to establish the degree of heteroplasmy with frequency estimators in this species.

Theologidis *et al.* (2007) demonstrated that in the original study by Saavedra *et al.* (1997), detection of the M genome in males of *Mytilus galloprovincialis* was impossible due to problems linked to mutations in primer annealing sites of the M genome. The fact that a greater number of both genomes was observed using 16S amplification than with COI primers may therefore result from a similar problem to that encountered by Saavedra *et al.* (1997). For example, in our results, the M genome was amplified in males 1, 5, 6 and 9 using 16S primers, but not using COI primers (Figure 3). The next step required is to develop a research strategy that will enable us to perform more detailed and in depth analyses of DUI in *P. purpuratus*.

Phylogenetic reconstruction carried out in order to understand the timing of *P. purpuratus* species formation *vs.* the separation between the two genomes and the origin of DUI showed a taxon-joining pattern. This may indicate that species formation predated genome differentiation in *P. purpuratus*. Nonetheless, the loss of closely related species may in some way hide the presence of a gender-joining pattern, according to which DUI origin would be the first event. This differs from the pattern observed by Rawson and Hilbish (1995) and Zouros (2013) in *Mytilus*, where a gender-joining pattern was found. The hypothesis proposed is that DUI in the genus *Mytilus* had a single origin. In the case of the monospecific genus *Perumytilus*, the absence of closely related species makes it difficult to evaluate whether the process of speciation was earlier or later than differentiation of the inheritance of mitochondrial genomes.

To conclude, the evidence presented here reveals the presence of two mitochondrial genomes that differentiate females (F haplotypes) from males (M and F haplotypes). These results not only confirmed the existence of two mitochondrial genomes in this species, but also enabled us to detect the presence of sex-specific genomes in gonadal tissue of each type of sample (males and females). This indicates that caution must be taken when phylogenetic and phylogeographic comparisons are done using mtDNA sequences of *Perumytilus purpuratus* without considering the sex of the individuals.

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Supplementary Material

The following online material is available for this article:

Figure S1 - Phenograms showing amplification of two genomes from one individual.

Figure S2 - Alignment of all sequences used for phylogenetic reconstruction.

This material is available as part of the online article from http://www.scielo.br/gmb.

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