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ARTICLE

Detection of mitochondrial DNA heteroplasmy suggests a doubly uniparental inheritance pattern in the mussel *Mytella charruana*

Francisco Arimatéia dos Santos Alves[1], Colin Robert Beasley[2], Walter Randolph Hoeh[3], Rossineide Martins da Rocha[4], Luiz Ricardo Lopes de Simone[5] and Claudia Helena Tagliaro[1*]

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ABSTRACT: (Detection of mitochondrial DNA heteroplasmy suggests a doubly uniparental inheritance pattern in the mussel *Mytella charruana*). The unusual type of mitochondrial DNA (mtDNA) transmission in which females pass on their mtDNA F to both sons and daughters, and males pass on their mtDNA M to their sons, is termed Doubly Uniparental Inheritance (DUI) and has been observed in several bivalve mollusk families, including the Mytilidae. The present study found mitochondrial heteroplasmy in *Mytella charruana*, a tropical estuarine mytilid, which is most likely due to the occurrence of DUI. Cytochrome oxidase c subunit I (COI) sequences provide evidence for the presence of mtDNA M in the gonad and adductor muscle of male *M. charruana*. Intraspecific COI mtDNA F and mtDNA M mitotype divergence (p-distances) in *M. charruana* ranged from 20.5% to 20.8%. COI phylogenetic trees suggest that mtDNA M arose before *M. charruana, Perna perna* and *Mytella guyanensis* split, though independently of the DUI event that occurred in the genera *Mytilus* and *Brachidontes*. The comparison of COI gene products of mtDNA M among *M. charruana* and other bivalves, including mytilids, has shown that they all have important differences, suggesting independent evolutionary changes in their male COI lineages.
Key words: DUI, COI, estuarine, Mytilidae, Bivalvia.

RESUMO: (Detecção de heteroplasmia no DNA mitocondrial sugere um padrão de herança dupla uniparental em mexilhões *Mytella charruana*). Um tipo de transmissão não usual de herança do DNA mitocondrial (mtDNA), na qual as fêmeas passam os seus mtDNA F aos filhos e às filhas e os machos passam os seus mtDNA M para os seus filhos machos, é conhecido por herança dupla-uniparental e tem sido observada em várias famílias de moluscos bivalves, incluindo a Mytilidae. O presente estudo mostrou a ocorrência de heteroplasmia mitocondrial em *Mytella charruana*, um mitilídeo estuarino tropical, provavelmente devido a herança dupla uniparental. Sequências da citocromo oxidase c subunidade I (COI) mostraram que o mtDNA M está presente nas gônadas e nos músculos adutores dos machos de *M. charruana*. As divergências intraespecíficas (distância-p) entre as sequências de COI dos mitótipos mitocondriais mtDNA F e mtDNA M em *M. charruana* variaram de 20.5% a 20.8%. As árvores filogenéticas baseadas em COI sugerem que o mtDNA M surgiu antes da separação das espécies *M. charruana, Perna perna* e *Mytella guyanensis,* independentemente do evento DUI que ocorreu nos gêneros *Mytilus* e *Brachidontes*. A comparação dos produtos do gene mtDNA M de *M. charruana* e de outros bivalves, incluindo mitilídeos, mostrou que eles têm importantes diferenças, sugerindo mudanças evolucionárias independentes em suas linhagens masculinas de COI.
Palavras-chave: DUI, COI, estuarino, Mytilidae, Bivalvia.

INTRODUCTION

Metazoan mitochondrial DNA (mtDNA) is usually transmitted by matrilinear inheritance (Rawson & Hilbish 1995). However, patrilinear transmission of mtDNA has been documented in a small number of vertebrates and invertebrates (Gyllensten *et al.* 1991, Kondo *et al.* 1992, Magoulas & Zouros 1993). A high level of gender-associated heteroplasmy has already been observed in seven bivalve mollusk families (Passamonti & Scali 2001, Soroka 2008, Theologidis *et al.* 2008, Plazzi & Passamonti 2010), including the Mytilidae (Zouros *et al.* 1992). In species with this type of mtDNA inheritance, there are distinct female (mtDNA F) and male (mtDNA M) transmitted genomes. Typically, females are homoplasmic for mtDNA F and pass on their mtDNA F to both sons and daughters, whereas males are heteroplasmic carrying both mtDNA F and mtDNA M. However, males do not transmit mtDNA F and pass on their mtDNA M only to their sons (Hoeh *et al.* 1996, Hoeh *et al.* 2002). This type of inheritance is termed Doubly Uniparental Inheritance or DUI (Zouros *et al.* 1994, Hoeh *et al.* 2002).

Maternal and paternal lineages of mtDNA evolve independently (Hoeh *et al.* 1996, 2002, Breton *et al.* 2007), often producing two parallel but distinct evolu-

1. Laboratório de Conservação e Biologia Evolutiva, Instituto de Estudos Costeiros, Campus Universitário de Bragança, Universidade Federal do Pará. Alameda Leandro Ribeiro, s/n, CEP 68600-000, Bragança, PA, Brazil.
2. Laboratório de Moluscos, Instituto de Estudos Costeiros, Campus Universitário de Bragança, Universidade Federal do Pará. Alameda Leandro Ribeiro, s/n, CEP 68600-000, Bragança, PA, Brazil.
3. Department of Biological Sciences, Kent State University, Cunningham Hall. Summit Street, Kent, OH 44242, USA.
4. Laboratório de Histologia e Ultraestrutura, Instituto de Ciências Biológicas, Campus do Guamá, Universidade Federal do Pará. CEP 66075-900, Belém, PA, Brazil.
5. Museu de Zoologia da Universidade de São Paulo. Cx. Postal 42494, CEP 04218-970, São Paulo, SP, Brazil.
*Author for correspondence. E-mail: tagliaro@ufpa.br

tionary trees (Rawson & Hilbish 1995, Stewart *et al.* 1995, Hoeh *et al.* 1996). Studies of mtDNA regions of *Mytilus* spp. have shown that the two sex-specific standard nucleotide sequences differ by 25-30% and that the mtDNA M evolves faster (Hoeh *et al.* 1997, Passamonti & Ghiselli 2009), probably due to relaxed selection against the M type (Stewart *et al.* 1996). Mizi *et al.* (2005) studied the complete mitochondrial genome sequence of *Mytilus galloprovincialis* (Mediterranean mussel) and showed that maternal mtDNA F and paternal mtDNA M retain identical gene content, gene arrangement, similar nucleotide composition and codon usage bias. Both these lineages of mtDNA have diverged by about 20% in nucleotide sequences although the divergence rate was unequal among different regions of the mtDNA (Cao *et al.* 2004a, Mizi *et al.* 2005, Cao *et al.* 2009). On the other hand, a masculinization event (or a role-reversal) of the mtDNA has been seen in some male mytilids where loss of the original male mtDNA M was followed by its subsequent replacement by that lineage's female mtDNA F, which begins to be transmitted via spermatoza (Hoeh *et al.* 1997). Multiple masculinization events have been inferred during the evolutionary history of mytilids (Hoeh *et al.* 2002). According to Breton *et al*. (2007), DUI of mitochondria would be both an elegant strategy to avoid sex-specific constraints associated with maternal mtDNA transmission and an opportunity for mitochondria to evolve adaptively for male function. The independent evolution of the two mitochondrial DNA lineages can be easily seen in the best tree topology obtained by Hoeh *et al.* (2002) from a maximum-likelihood (ML) analysis of 619 bp of cytochrome oxidase c subunit I (COI). In their phylogenetic tree (Hoeh *et al.* 2002), the unionoidean bivalve mtDNA F and mtDNA M are located in distinct clades within which the mitotype relationships are seen to be largely congruent. Hence, the lineage of mtDNA being considered for taxonomic evolutionary inference needs to be correctly identified.

In most *Mytilus* specimens studied, analysis of mtDNA extracted from different tissues provides evidence that mtDNA M predominates in male gonads whereas mtDNA F is most frequent in female gonads (Fisher & Skibinski 1990). Moreover, mtDNA F predominates in somatic tissues of individuals of both sexes (Stewart *et al.* 1995, Garrido-Ramos *et al.* 1998, Dalziel & Stewart 2002). However, in the Manila clam (*Venerupis philippinarum*), most male somatic tissues investigated by Ghiselli *et al*. (2011) showed predominance of the mtDNA M, suggesting that the processes separating sex-linked mtDNAs in somatic tissues of this clam are less precise than in other DUI species.

Within Mytilidae, DUI has been described for *Mytilus* (Breton *et al.* 2007) and *Brachidontes* (Lee & Ó Foighil 2004). On the other hand, there was no evidence for DUI in three species of *Perna* (Wood *et al.* 2007), nor in two species of *Mytella* (Gillis *et al.* 2009), including *Mytella charruana* (d´Orbigny, 1842). In contrast to the latter study, Alves (2008) obtained COI sequences from 171 *M. charruana*, from three localities in the state of Pará and one in the state of Alagoas (Brazil), and detected the male lineage in all four populations, totaling 64 sequences with a predominance of mtDNA M. Thus, the aim of the present study was to investigate the possibility of DUI being the cause of the high level of divergence observed in COI nucleotide sequences of *M. charruana*, a tropical estuarine mytilid. Additionally, this research compares the amino acid changes in COI of several patriarcal mtDNA lineages from bivalves with the same pattern of inheritance, in order to determine possible evolutionary relationships among them.

MATERIAL AND METHODS

Specimens of *Perna perna* (Linnaeus, 1758), *M. charruana,* and *Mytella guyanensis* (Lamarck, 1819) were morphologically identified by L. R. L. Simone, and *M. charruana* shells were deposited (MZUSP 99629) in the malacological collection of the Museu de Zoologia at the Universidade de São Paulo, Brazil. Specimens of *M. charruana* and *M. guyanensis,* shown in Table 1, were collected in Bragança (Pará state, Brazil) and those of *P. perna* in Búzios (Rio de Janeiro state, Brazil).

In order to identify gender-associated heteroplasmy, specimens of *M. charruana* were sexed, and gonad samples from six *M. charruana* specimens (Table 1) were removed and fixed in Davidson's solution immediately after collection and processed using standard histological procedures. Tissues were included in paraffin, from which thin sections (7 µm) were taken and stained with haematoxilin and eosin. Some specimens were very small and the entire gonad was used in the histological procedure. Sexes were identified by observation of the gonad tissue sections under a light microscope (200X).

Total DNA was isolated from adductor muscle and/or gonads of *M. charruana, M. guyanensis* and *P. perna* (Table 1) using the phenol-chloroform protocol of Sambrook *et al.* (1989). Additionally, amplicons of partial cytochrome oxidase c subunit I (COI) mitochondrial genes and from a nuclear DNA region (nDNA), composed of partial 18S rDNA and the internal transcribed spacer 1 (18S-ITS1), were also obtained. COI sequences of other bivalve species were obtained from GenBank (Table 1). Amplification of partial COI genes was carried out with the primers LCOCI 1490 5' GGT CAA CAA ATC ATA AAG ATA TTG G 3' and HCOCI 2198 5' TAA ACT TCA GGG TGA CCA AAA AAT CA 3', designed by Folmer *et al.* (1994), using the following protocol: an initial denaturing step at 95 ºC for 3 min, 35 cycles of denaturing at 95 ºC for 1 min, annealing at 45.5 ºC for 1 min, extension at 72 °C for 1.5 min, followed by a final extension at 72 ºC for 7 min. The 18S-ITS1 nuclear DNA fragment was amplified and sequenced and the primers used to amplify this region were: ITS1A-sal 5'-AAA AAG CTT

Table 1. Species identification, sex, tissue type, mtDNA lineages, and GenBank accession numbers of specimens used in analyses.

Species	Code	Sex (Histology)	Tissue	mtDNA	COI GenBank accession number or reference	18S-ITS1 GenBank accession number
Mytella charruana	1F	Female	muscle gonad	F F	JQ685156	JQ734971
Mytella charruana	2F	Female	muscle	F	JQ685156	JQ734971
Mytella charruana	3F	Female	muscle	F	JQ685157	JQ734971
Mytella charruana	4M	Male	muscle	M	JQ685158	JQ734970
Mytella charruana	5M	Male	muscle gonad	M M	JQ685159	JQ734970
Mytella charruana	6F	Female	muscle	F	JQ685156	JQ734971
Mytella guyanensis	1	-	muscle	-	JQ685160	JQ734973
Mytella guyanensis	2	-	muscle	-	JQ685161	JQ734973
Perna perna	1	-	-	-	JQ685162	JQ734972
Perna perna	2	-	-	-	JQ685163	JQ734972
Mytilus edulis	1F	Female	-	F	Hoeh *et al.* 1996	-
Mytilus edulis	2F	Female	-	F	AY484747	-
Mytilus edulis	3F	Female	-	F	NC006161	-
Mytilus edulis	1M	Male	-	M	AY823623	-
Mytilus edulis	2M	Male	-	M	AY823624	-
Mytilus trossulus	F	Female	-	F	DQ198231	-
Mytilus trossulus	M	Male	-	M	DQ198225	-
Mytilus galloprovincialis	F	Female	-	F	NC006886	-
Mytilus galloprovincialis	M	Male	-	M	AY363687	-
Hormomya exustus (Brachidontes exustus)	F	Female	-	F	AY621835	-
Hormomya exustus (Brachidontes exustus)	M	Male	-	M	AY621936	-
Modiolus modiolus	-	-	-	-	U56848	-
*Corbicula fluminea**	-	-	-	-	U47467	-
*Anomalocardia brasiliana**	-	-	-	-	FJ481182	-
*Crassostrea gasar**	-	-	-	-	HM003499	-
*Neotrigonia margaritacea**	-	-	-	-	U56850	-

* Outgroup for COI analysis

TTG TAC ACA CCG CCC GTC GC- 3' and ITS1B-sal 5'- AGC TTG CTG CGT TCT TCA TCG A- 3' (Pleyte *et al.* 1992). The amplification reactions for 18S-ITS1 fragments were performed using the following schedule: initial denaturing at 94 °C for 5 min, 35 cycles of denaturing at 94 °C at 1.5 min, annealing at 65.2 °C per 1 min, extension at 72 °C for 2 min, and a final extension cycle at 72 °C for 7 min. Sequences of the nuclear region 18S-ITS1 were obtained in order to determine if the distinct mitotypes detected were due to the presence of two cryptic species or to DUI. This region has been shown to be very useful in distinguishing between different mytilid species; see Lee & Ó Foighil (2004), Santaclara *et al.* (2006) and Wood *et al*. (2007). When DUI is present, the population generally presents a single lineage of nuclear genes (18S-ITS1) and two highly divergent lineages of mtDNA genes (COI). On the other hand, in the absence of DUI, and when cryptic species are found, both nuclear and mitochondrial DNA lineages generally show the same evolutionary history in the phylogenetic tree.

Amplification protocols for COI and for 18S-ITS1 were performed in 25 µL reaction volumes and contained 0.25 µL (200 ng/µL) of each primer, 0.75 µL (25 mM) $MgCl_2$, 4 µL (1.25 mM) of each dNTP, 2.5 µL (10X) of amplification buffer, 1 U Taq polymerase (Amersham Pharmacia Biotech), 1.0-2.5 µL of total genomic DNA, and purified water to complete the final volume. A volume of 2.5 µL of each amplified product was purified using ExoSAP-IT (Amersham Pharmacia Biotech) and sequenced by the chain-termination method (Sanger *et al.* 1977) using an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction 'Big Dye Kit' (Applied Biosystems), following the manufacturer's instructions. The products were separated by electrophoresis (3.5 h at 2.400 V) and the sequences collected using an ABI Prism 377 automatic sequencer.

Sequences were aligned using BioEdit version 7.0.5.3 (Hall 1999) and Clustal X (Thompson *et al.* 1997) and nucleotide sequence identities were calculated using the former software. Nucleotide and amino acid composition, the number of variable sites, p-distances and checking for termination codons from the Mytilidae sequences were carried out using Mega version 5.05 (Tamura *et al.* 2011). Nucleotide saturation tests were carried out using DAMBE (Xia & Xie 2001, Xia *et al.* 2003). Comparisons among COI amino acid sequences from male lineages and the calculation of amino acid p-distances were also carried out using Mega 5.05 and with the following data obtained from GenBank: Unionoida (AB055624, FJ809752, FJ809755, FJ809751), Veneroida (AB065374) and Mytiloida (AY823623, AY823624, DQ198225, AY363687).

In order to verify molecular relationships (nucleotide

sequences) between species of Mytilidae and to check for the presence of both F and M mtDNA lineages in *M. charruana*, maximum parsimony (MP), neighbor--joining (NJ) and maximum likelihood (ML) analyzes were carried out using an heuristic search in PAUP 4.0 (Swofford 2003). Gaps were treated as missing data in all analyzes. Divergence matrices and the NJ and ML trees were generated based on the model established by jModeltest version 0.1.1 (Guindon & Gascuel 2003, Posada 2008) using Akaike's Information Criterion (AIC, Posada & Buckley 2004). A maximum likelihood tree, based on COI amino acid sequences (ML_{aa}), was constructed using Mega version 5.05 (Tamura *et al.* 2011) and the choice of the evolutionary model for amino acid data was previously performed using the same program. The significance of the evolutionary hypotheses were tested by bootstrapping (Felsenstein 1985) using 2000 pseudoreplicates for the MP, NJ and ML_{aa} analyzes and 1000 for ML. The criterion adopted to evaluate robustness was to consider nodes with bootstrap values equal or superior to 90% as well supported. *Crassostrea gasar*, *Anomalocardia brasiliana*, *Corbicula fluminea* and *Neotrigonia margaritacea* were used as outgroups (Table 1). No outgroup was used for 18S-ITS1 trees, which were based on sequences of *M. charruana* (males and females), *M. guyanensis* and *P. perna* (Table 1).

RESULTS

Of the six sexed *M. charruana*, two were male and four were female (Table 1). The polymerase chain reaction produced COI amplicons with about 680 bp for six *M. charruana,* two *M. guyanensis* and two *P. perna.* Only parts of the amplicons that were reliable in both DNA strands were considered and, after alignment with COI sequences from other bivalve species from GenBank (Table 1), the final alignment was composed of 542 sites. Due to the small amount of soft parts in most specimens, identification of the sex and extraction of DNA from the gonads was only possible in two *M. charruana* (Table 1). In the female *M. charruana* 1F, the gonad COI sequence was identical to that of the adductor muscle, and the male *M. charruana* 5M gonad had only one lineage (mtDNA M) whereas the muscle showed both lineages with predominance of mtDNA M (Figure 1). Furthermore, comparison of individual *M. charruana* COI sequences, showed that the two main mtDNA lineages are present (Table 2), where all four females presented only two very similar sequences from the same (mtDNA F) lineage and both males showed two closely related sequences from the other lineage (mtDNA M). Sequences of both lineages (mtDNA M and F) were 79.2-79.5% similar, differing in at least 111 nucleotides, 68 transitions and 43 transversions, which coded for 13 distinct amino-acids (Table 3). No termination codon was detected. On the other hand, comparison of *M. charruana* mtDNA F with those of *M. guyanensis* and *P. perna*, revealed similarities of 83.4 to 83.6% and

Table 2. COI mtDNA M and F variable sites from *Mytella charruana* obtained from gonad and/or adductor muscle.

Species, Code, Tissue, Sex	Nucleotide variable sites											
			1111111	1111111111	1122222222	2222222222	2223333333	3333333334	4444444444	4444444444	4444444455	555
	1112223334	5555667778	8990123455	6666677888	9900111222	3555677788	9992223344	5555688990	1122233344	4556666678	8888899902	233
	2581473692	1467194580	1395199706	2356824039	2518069258	1256103923	1474570935	1457327398	4767967801	4360125870	3467956913	514
M. charruana,1, M, Female	AGAGCCCGTT	AAATCCGCGA	TATTGTTTAG	ACTCTCACTC	TGCCTTGGCG	GAAAAATAAG	GAAGTGCTGA	TCTGTCCATT	ACAGTACACT	AAGTCAGGAA	TATGATGCGC	TTA
M. charruana, 1, G, Female	....	....	....	....	....	....	....	....	....	....	....	...
M. charruana, 2, M, Female	....	....	....	....	....	....	....	....	....	....	....	...
M. charruana, 3, M, Female	....	....	....	....	....	....	....	....	....	....	..C.......	...
M. charruana, 5, G, Male	....	....	....	....	....	....	....	....	....	....	....	...
M. charruana, 4, M, Male	TATATTTAAC	TTGATTATAT	CTAAAGCGTT	GTGTGTGTGT	CATTACAATA	AGGGGGAGGT	TGGCCTTGTG	CTCTATGGAC	TAGA.GTTAC	GGACAGATGT	CT.ATGTTAT	AAT
M. charruana, 6, M, Female	TATATTTAAC	TTGATTATAT	CTAAAGCGTT	GTGTGTGTGT	CATTACAATA	AGGGGGAGGT	TGGCCTTGTG	CTCTATGGAC	TAGACGTTAC	GGACAGATGT	CT.ATGTTAT	AAT
M. charruana, 5, M, Male	TATATTTAAC	TTGATTATAT	CTAAAGCGTT	GTGTGTGTGT	CATTACAATA	AGGGGGAGGT	TGGCCTTGTG	CTCTATGGAC	TAGACGTTAC	GGACAGATGT	CT.ATGTTAT	AAT

M= adductor muscle; G= gonad

Table 3. Comparison of amino acid substitutions among mtDNA M and F lineages from *Mytella-Perna* and *Mytilus* clusters based on COI sequences.

Cluster or Species	Sex	mtDNA lineage	Variable amino acid sites
Mytella-Perna cluster			1 1 1 1 1 1 1 1 1 1 2 2 8 9 0 1 4 4 4 5 6 6 6 9 5 7 6 5 8 5 3 6 7 4 2 3 6
Mytella charruana 1F	Female	F	N S Y I A S A D T T S T V V
Mytella guyanensis 1	-	-	. . . .
Perna perna 1	-	-	S . . . . G . N . V . . . .
Mytella charruana 5M	Male	M	S N F V S . S N V N Q S I F
Mytilus cluster			1 1 1 1 1 1 1 2 3 4 0 0 2 2 4 6 6 7 7 9 1 7 2 8 6 3 6
Mytilus edulis 1F	Female	F	Y M G I V S L V V V
Mytilus edulis 2F	Female	F	. . E . . . .
Mytillus trossulus F	Female	F	. . . .
Mytillus galloprovincialis F	Female	F	. . . .
Mytillus edulis 1M	Male	M	F . . . S A V A I A
Mytillus edulis 2M	Male	M	F . . V S A V A I A
Mytilus trossulus M	Male	M	F . . V S A . A I A
Mytillus galloprovincialis M	Male	M	F V . V S A V A . .

78.6 to 79.7%, respectively. The minimum number of nucleotide substitutions was 89 (60 transitions and 29 transversions) in the former interspecific comparison and 110 (67 transitions and 43 transversions) in the latter. No amino acid change was detected between *M. charruana* mtDNA F and *M. guyanensis* and only four were observed between *M. charruana* mtDNA F and *Perna perna* (Table 3). Among *Mytilus* species, seven to eight amino acid substitutions were detected among COI products from mtDNA F and M (Table 3). The average nucleotide composition of *M. charruana* specimens was 0.409 (T), 0.136 (C), 0.267 (A) and 0.188 (G) for COI mtDNA M, and 0.387 (T), 0.155 (C), 0.275 (A) and 0.183 (G) for COI mtDNA F. For comparison with the results of Hoeh *et al.* (1996), p-distances were calculated between both COI nucleotide mtDNA lineages of *M. charruana*, which ranged from 20.5% to 20.8%.

The comparison of COI mtDNA M amino acid sequences among male bivalves of the Unionoida, Veneroida and Mytiloida can be seen in Table 4. The amino acid (male lineage) p-distance among *M. charruana* and Mytiloida species ranged from 27.4% to 35.8%, and from 44.1% to 48.0% among *M. charruana* and other bivalve orders (Veneroida and Unionoida).

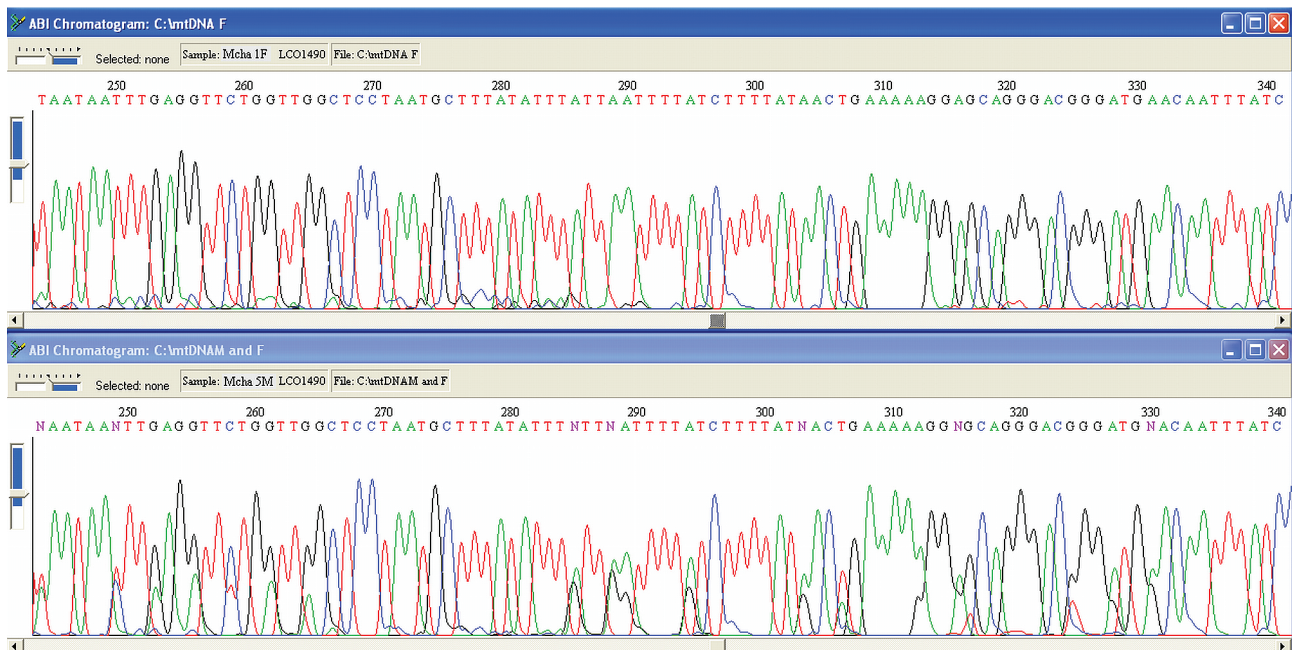

Figure 1. Partial sequences of cytochrome oxidase c subunit I (COI) from a female (above) and a male (below) *Mytella charruana*, extracted from adductor muscle.

Table 4. Comparison of amino acid differences among several bivalve species (Unionoida, Veneroida and Mytiloida) with DUI, based on amino acids coded for by the mtDNA M COI gene.

Species	GenBank number	Amino acid and site position												
								111111	1111111111	1111111111	1111111111	1111111111	1111111111	1111
		1111	1222222222	2333333444	4555556666	6666777777	8888888899	9999000011	1111111222	2223333333	4444444444	5555555555	6666666677	7777
		1345781245	8012345678	9013567267	8016890123	5678035789	0123567901	2357178901	2345689125	6891234589	0123456789	0123456789	0123568901	2689
Mytella charruana 4M	JQ685158	MSSLLIIQSH	GFLKNENLFN	VVVTALVALL	IAFLLCIGGC	LIFPLLFLAP	NALYLVLFMT	EKSTISSLYH	TGPSVILTSL	ILSLLGSIVS	TNKNMPVNKM	KGEKQELYLW	SISIGFLIIS	
Mytilus edulis	AY823623	FA...MM.G.	A...SDWF..	......M.I.	...I.LV..K	M.Y.M.Y.S.	.....M..S.	D.VA..YP..	S...MV.VA.	AV..V.A.A.	......ALE.	...RA...VL	....AA....	IG..
Mytilus trossulus	DQ198225	FA...MM.G.	A...SDWF..	......M.I.	...I.LV..K	M.Y.M.Y.S.	.....M..S.	D.VAV.YP..	S...MV.VA.	A...V.A.A.	......ALE.	...RA...VL	....AA....	IG..
Mytilus galloprovincialis	AY363687	FA...MM.G.	A...SDWF..	........I.	...I.LV..K	M.Y.M.Y.S.	.....M..S.	D.VAV.YP..	S...MV.VA.	AV..V.A.A.	......ALE.	...RA...VL	...VAV....	IG..
Brachidontes exustus	AB055624	VL.YMLLHM.	NL.RS.S.Y.	......I...	...I.FL.AM	.A..IF.IL.	S....L.GYI	.EV..TIEF	SS.AMLAL..	ASG.M.A.LV	S...L..D..	...RSV..I.	..TVA..LM.	L.A.
Inversidens japanensis	FJ809752	VL..VVAEG.	.LMH.DQ.YY	S..A.F.VAM	MGIVIML.CP	MA...V..LA	G.GF.GWMFV	.GC....AF.	S.V....F..	A..I...LIT	.ILG.RSDIL	RA.RMT.LV.	.VLCAGIVA.	F.A.
Venustaconcha ellipsiformis	FJ809755	.TG.I.MEAM	KM.EDGQ.Y.	L..AG..LMM	.G.I.MLKIP	MA.AM...LM	GSMI.LGTYV	DGA...IG..	S.RA.F.F.A	GV.I.A..LI	.TAL.RTGV.	LILRSSMLS	CLGVS..VVA	M..L
Pyganodon grandis	FJ809751	IL...LVEG.	.VIRDDQ.YY	SI.S.FILAM	MG.VIML.IP	MS...V..LV	GSGLMGWIIV	.SC..NKV..	SSVC.V.F..	AS.IM..VIV	.IL..R.GVL	YL.RMS.FV.	.VLCAGVLL.	F.A.
Quadrula quadrula	AB065374	VL..M.VE.C	.VVAH.QVYY	S.IA.F..AM	MGLV.ML.CP	MA...V..LI	SSGF.GWMIL	.SC..NKIFY	S.TA.VVF..	AV.I...LIT	.IL..R.EV.	QV.RMP.FV.	.VLCAGVLV.	F.A.
Venerupis philippinarum	AB065374	VL..VLVEG.	.VMR..Q.YY	SI.A.F.VAM	MGMVIML.SP	MS...V..LM	GSGL.GWMFV	.SC...VG..	S.VCM.VF..	AA.I..ALIT	.IL..R.EAL	RV.RMT.FT.	.VLSAGVLL.	F.A.

Little substitution saturation was detected for COI nucleotide sequences (Iss = 0.4250; Issc = 0.7138; P < 0.0001). The evolutionary model chosen was the General Time Reversible model (GTR, Lanave *et al.* 1984, Rodriguez *et al.* 1990), which takes into account gamma distributed rate variation across sites and allows for six different rates of change between bases. The settings for the best-fit model selected were: base frequencies (A = 0.2764, C = 0.1172, G = 0.1931, T = 0.4133), gamma distribution shape parameter (α = 0.5290), substitution model rate matrix (Rmat; A-C = 1.3904, A-G = 10.1478, A-T = 1.6016, C-G = 7.1627, C-T = 20.0930, G-T = 1.0000) and proportion of invariant sites equal to 0.1710. The distance (d) matrix obtained using the GTR method with the above evolutionary model parameters showed that COI nucleotide sequences from *M. charruana* mtDNA F were more closely related to those from *M. guyanensis* (d = 0.192 to 0.195) than to those from *M. charruana* mtDNA M (d = 0.248 to 0.251). Two slightly different COI sequences were observed for both mtDNA M and mtDNA F and distances ranged from 0 to 0.002. Distances among the *M. charruana - M. guyanensis - P. perna* group and the *Mytilus* cluster ranged from 0.392 to 0.517.

The evolutionary model chosen for amino acid data was the General Reversible Mitochondrial model (mtREV; Adachi & Hasegawa 1996) and a discrete gamma distribution was used to model evolutionary rate differences among sites (*G* parameter = 0.6928). The analysis involved 26 amino acid sequences.

ML, MP, NJ and ML_{aa} trees for COI showed very similar arrangements (Fig. 2) and all COI nucleotide (mtDNA M and F) or amino acid sequences of *Mytilus* species grouped together (ML, MP, NJ, ML_{aa} = 100%). On the other hand, *M. charruana* (mtDNA M and F), *M. guyanensis* and *Perna perna* COI sequences also formed a group (ML = 100%, MP = 94%, NJ = 100%, ML_{aa} = 98%), which joined that of *Mytilus* with significant bootstrap values (ML = 95%, MP = 96%, NJ = 100%, ML_{aa} = 93%).

The nuclear 18S-ITS1 fragment amplified and sequenced for *M. charruana*, *M. guyanensis* and *P. perna* ranged from 505 to 545 bp. However, unambiguously aligned, the final sequences of these three species were composed of 529, 491 and 492 bp, respectively, and after alignment, the database contained a total of 544 sites, due to the presence of indels. At the intraespecific level, 18S-ITS1 sequences were identical for *M. charruana* (N = 6), *M. guyanensis* (N = 2) and *P. perna* (N = 2), therefore all six *M. charruana* specimens, either male or female, had the same sequences. However, interspecific comparisons revealed large numbers of variable sites (68 to 97) between the three species. The results indicated little saturation (Iss = 0.2643; Issc = 0.8022; P < 0.00001) for 18S-ITS1 nucleotides in this database. The settings selected by jModeltest 0.1.1 for 18S-ITS1 were: base frequencies (A = 0.2627, C = 0.2586, G = 0.2649, T = 0.2138), gamma distribution shape parameter (α =

0.3660), substitution model rate matrix (Rmat; A-C = 3.1061, A-G = 6.2137, A-T = 3.1061, C-G = 1.0000, C-T = 6.2137, G-T = 1.0000) and proportion of invariant sites equal to 0. The genetic distances were obtained using the General Time-Reversible model (GTR; Lanave *et al.* 1984, Rodriguez *et al.* 1990). The intraspecific distances were zero for *M. guyanensis*, *P. perna*; and among the six male and female specimens of *M. charruana*. The interspecific distances ranged from 0.158 to 0.243 among *M. guyanensis*, *P. perna* and *M. charruana*. ML, MP and NJ trees for 18S-ITS1 joined all *M. charruana* together (bootstrap: 100%), which were separate from *M. guyanensis* and *P. perna* (Fig. 3).

DISCUSSION

Stenyakina *et al*. (2010) suggested that low food availability may cause sex reversal in *M. charruana*. Our mussels were immediately processed and thus did not suffer from starvation after collection. Besides, COI sequences of the two males and the four females showed mtDNA M and mtDNA F, respectively. Sex reversal is unlikely to have occurred in the *M. charruana* sampled by us. Even if some individuals were to change sex in their natural habitat, both lineages of mtDNA would remain as they are distributed during embryogenesis (Garrido-Ramos *et al.* 1998).

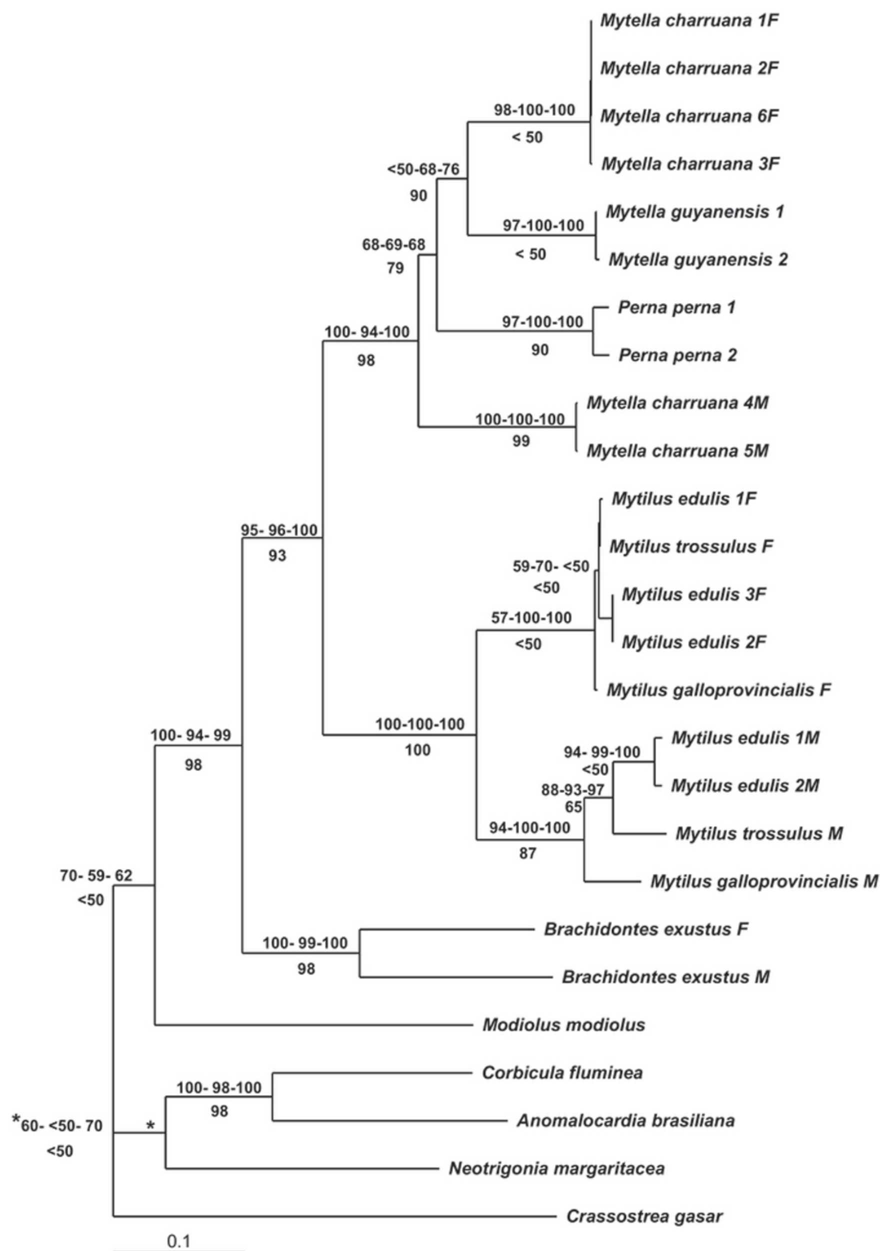

Figure 2. Neighbor Joining tree based on bivalve COI nucleotide sequences. Maximum Likelihood, Maximum Parsimony and Neighbor Joining bootstrap results (left, middle and right, respectively) are shown above the branch and the amino acid Maximum Likelihood result below.

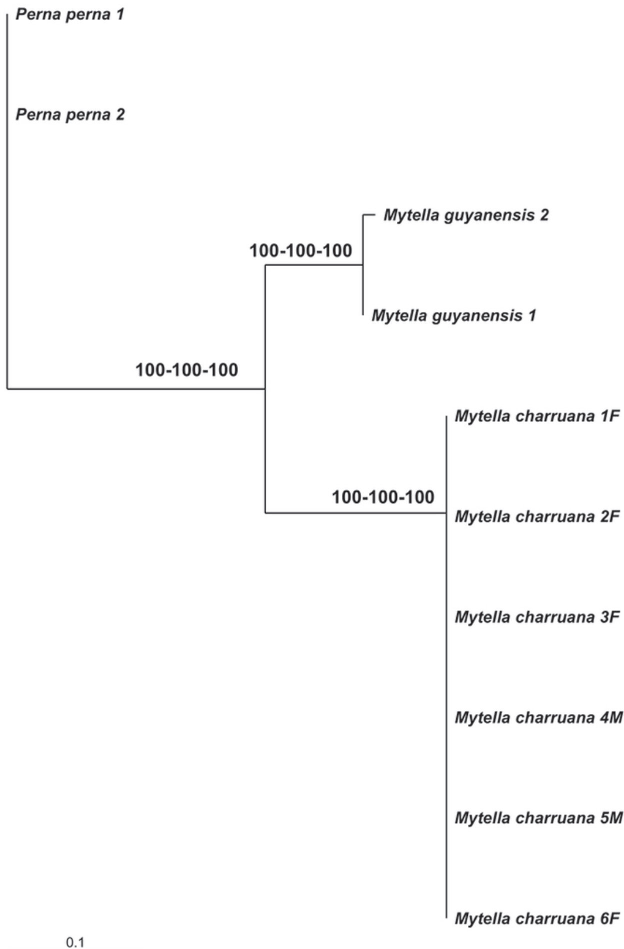

Figure 3. Neighbor Joining tree based on bivalve 18S-ITS1 sequences. Maximum Likelihood, Maximum Parsimony and Neighbor Joining bootstrap results (left, middle and right, respectively) are shown above the branch.

Although it is well known that DUI is found in several mytilid species, the presence of two very distinct COI sequences might also indicate the existence of two cryptic species. The nuclear 18S-ITS1 sequences obtained in the present study along with those of Mytilidae deposited in GenBank (Lee & Ó Foighil ,2004, Santaclara *et al.* 2006, Wood *et al.* 2007) show that this DNA fragment can be used reliably to distinguish species in this family. In the present study, two mtDNA lineages and a single nDNA lineage were consistently found in *M. charruana* specimens sequenced. These results suggest that all our *M. charruana* specimens belong to the same species, which contains both maternal and paternal mtDNA lineages, further supporting the available evidence for DUI in the Mytilidae (Zouros *et al.* 1992).

In the present study, *M. charruana* males were found to have mtDNA M amplicons in the adductor muscle and gonad, whereas females presented only mtDNA F amplicons in both tissues. However, COI sequences obtained from adductor muscle of male *M. charruana* and several more sequenced by Alves (2008) clearly contain both mtDNA F and M (Figure 1). Gillis *et al.* (2009) did not detect distinct mtDNA lineages suggestive of DUI in their samples of *M. charruana*. The forward primer designed by the latter authors (5' GTGTGGGG-CTGGGTTAATAG 3') had a similar sequence to the mtDNA F lineage in our study at this position (5' GTG-TGGGCTGGGTTAATAG 3') but is quite distinct from the sequence of mtDNA M (5' GTGTGAACTGGGC-TAATAG 3'), which suggests that they may have been unable to amplify the male lineage. We were unable to compare the reverse primer due to the shorter length of our fragments.

Obata *et al*. (2008) found that the F type was predominant in adductor muscle of males and females of *M. galloprovincialis*. Garrido-Ramos *et al*. (1998) detected both mitotypes in male *M. edulis* adductor muscle, and showed that the amount of mtDNA M can vary among somatic tissues and individuals. These results were supported by Dalziel & Stewart's (2002) study of *M. edulis* tissues, which detected mtDNA M in all tested gonads and in half of the adductor muscle samples. Moreover, mtDNA F was found in all somatic tissues and the results suggested a more stochastic pattern to the presence and expression of the M type in male somatic tissues. Cao *et al*. (2004b) studying the migration of mitochondria observed that in *M. edulis* embryos from females that produce only daughters, sperm mitochondria are randomly dispersed among blastomeres, but in those from females that produce mostly sons, sperm mitochondria tend to aggregate and possibly end up in the first germ cells. Kakoi *et al*. (2008) explain that the reason for the presence of the M mitotype in these two distinct tissues (gonad and adductor muscle) is due to embryogenesis; adductor muscle cells originate in the anterior mesoderm, whereas germ cells originate from the posterior mesoderm. The latter study supports the earlier conclusion of Garrido-Gomes *et al.* (1998), in that the sperm mitochondrion could migrate to adjacent cell lineages.

In our study, intraspecific divergence between COI mtDNA F and M (p-distances) in *M. charruana* ranged from 20.5% to 20.8%. Similar values were obtained by Hoeh *et al.* (1996) for *M. edulis*, *M. trossulus,* and *Geukensia demissa* (20.4%, 18.4% and 18.4%, respectively). In *M. charruana,* the distances among all mtDNA F were very small, whereas those between mtDNA M and F are of the same order of magnitude as the distances between *M. charruana* and *M. guyanensis*. Similar distances were found among three *Mytilus* species (Breton *et al.* 2006).

Hoeh *et al.* (1996, 1997) presented evidence that M mitotypes in the genus *Mytilus* are derived from multiple ancestral lineages, one of which emerged from an ancestor of *M. edulis* and *M. trossulus.* In another study, Fisher & Skibinski (1990) suggested that the divergence time between the mtDNA F and M might be more ancient than the split between *M. edulis* and *M. galloprovincialis* or that the M genome had been de-

rived through introgression from a related species. In our study, the trees obtained from COI sequences suggest that the M mitotype arose before the split between *M. charruana, M. guyanensis,* and *P. perna,* despite the fact that DUI has not been reported in the latter two (Wood *et al.* 2007, Gillis *et al.* 2009). The presence of two mtDNA lineages in *M. charruana* shows that molecular studies of this species using mtDNA sequences should be preceded by clear identification of the mtDNA M and mtDNA F mitotypes.

The M genome of *M. galloprovincialis* contains a full complement of genes with no premature termination codons, which is evidence against it being a selfish element that rides with the sperm (Mizi *et al.* 2005). The mtDNA M COI sequence from *M. charruana* also does not have a termination codon, suggesting that the COI protein derived from the mtDNA M lineage may still retain some activity. The comparison of COI gene products of mtDNA M among bivalves in the Unionoida, Veneroida and Mytiloida has shown that they are quite distinct, suggesting independent evolutionary changes among groups. In our study with the COI gene, amino acid sequences from mtDNA F of *M. charruana* and *M. guyanensis* were identical, despite the substantial divergence values between the respective nucleotide sequences. On the other hand, there were 13 different amino acids between mtDNA F and mtDNA M from *M. charruana* (Table 3). Stewart *et al.* (1996) proposed that there is lower selective pressure for the mtDNA M in comparison to mtDNA F. This may be explained by the fact that mtDNA M is mainly present in the male gonad and to a lesser extent in somatic tissues, where it sometimes occurs together with the more abundant mtDNA F (Breton *et al.* 2006).

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