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The quest for Doubly Uniparental Inheritance in heterodont bivalves and its detection in *Meretrix lamarckii* (Veneridae: Meretricinae)

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Abstract

Doubly Uniparental Inheritance (DUI) is possibly the most striking exception to the well known maternal inheritance of mitochondria. It poses several stimulating questions concerning among others the function of these organelles, sex determination, embryonic development, and evolutionary conse quences. At present, DUI has been found in few species of bivalve molluscs, but more research is necessary to obtain a clearer picture of its distribu tion within the group, a picture that is mandatory to make any reasonable inference about its origin and evolutionary meaning. The debate about a single evolutionary origin of DUI versus multiple origins is still open. In this manuscript, we investigated seven species of heterodont bivalves and provide evidence for the presence of DUI in the venerid *Meretrix lamarckii*.

Key words: *Meretrix lamarckii* Doubly Uniparental Inheritance mitochondria Heterodonta mitochondrial inheritance

Introduction

The phenomenon of Doubly Uniparental Inheritance or DUI (Zouros 1994a,b), which is known only from bivalves, constitutes by far the most striking exception to the well known rule of maternal inheritance of mitochondria in animals. Briefly, species with DUI possess two separate mitochondrial lineages, termed M (male) and F (female), being sex linked: the F mitochondrial DNA (mtDNA) is passed by mother to the offspring irrespective of their sex, while the M mtDNA is passed by fathers to the male offspring, where it tends to localize in the germline (Breton et al. 2007; Passamonti and Ghiselli 2009; Passamonti et al. 2011).

Interest is growing in determining the exact distribution of DUI among bivalves, because this issue triggers further discussion about the origin of this peculiar mitochondrial feature, as shown by the pioneering works of Theologidis et al. (2008) and Doucet Beaupré et al. (2010). Autobranchiate bivalves are basi cally split into Palaeoheterodonta and Amarsipobranchia *sensu* Plazzi et al. (2011). Two main clusters are nested within Amar sipobranchia and both contain DUI species (Fig. 1): Pteriomor phia and Heterodonta.

Doubly Uniparental Inheritance (DUI) appears to be wide spread and somewhat conserved in the large, mainly freshwater superfamily Unionoidea (Palaeoheterodonta; Hoeh et al. 2002; Curole and Kocher 2005; Walker et al. 2006; Breton et al. 2009; Doucet Beaupré et al. 2010; and reference therein) and in mytilids (Pteriomorphia), especially in the genera *Mytilus* Linnaeus, 1758 (Skibinski et al. 1994a,b; Zouros 1994a,b) and *Musculista* Yamamoto and Habe, 1958 (Passamonti et al. 2011).

The phenomenon of DUI also appears among other bivalve families. For instance, it was detected and thoroughly characterized in the Manila clam *Venerupis philippinarum* (Passamonti and Scali 2001; Passamonti et al. 2003; Ghiselli et al. 2011; Milani et al. 2011). The presence of two different, sex linked, mitochondrial genomes was also reported for *Donax trunculus* Linnaeus, 1758 (Theologidis et al. 2008) and *Ledella ultima* (E. A. Smith, 1885) (Boyle and Etter 2013). Moreover, data in GenBank suggest the possible occurrence of DUI in the species *Solen grandis* Dunker, 1862 (GenBank accession numbers AB064985 and AB064983) and *Cyclina sinensis* (Gmelin, 1791) (GenBank accession numbers AB040833, AB040834 and AB040835).

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Until recently, a single appearance at the root of bivalves, or at least at the root of autobranchiate bivalves, was taken as most probable (see, e.g. Theologidis et al. 2008; Doucet Beaupré et al. 2010; Zouros 2013; and references therein). After the general pattern of mitochondrial inheritance through two distinct lineages, many similarities were evidenced between distantly related DUI species, like mytilids and venerids, from the female driven sex ratio bias to the different behaviour of sperm mitochondria in embryos of either sex (see Zouros 2013; and references therein). As a consequence, researchers generally considered as unlikely the idea of multiple origins of this complex phenomenon.

However, recent insights in supranumerary ORFs (Milani et al. 2013), which are sometimes found in mitochondrial genomes (see, e.g. Gissi et al. 2008; Breton et al. 2009; Pont Kingdon et al. 1998; Shao et al. 2006; Plazzi et al. 2013), suggest a possible viral origin of these sequences. If, as supposed (Milani et al. 2013; and reference therein), such sequences play a fundamental role in the onset of DUI, the idea of multiple origins of DUI, related to multiple infection horizontal transfer events, would become much more conceivable.

How is it possible to detect the presence of DUI in a given species? The first clue typically consists in mitochondrial heteroplasmy. If a significant divergence can be found and repeatedly confirmed between mitochondrial sequences obtained from sperm and eggs, this can be taken as an evidence of two separate mitochondrial lineages. In most cases, DUI was firstly detected using this method, as e.g. in *V. philippinarum* (*rrnL* gene; Passamonti and Scali 2001), *Musculista senhousia* (*cox1*, *cytb*, and *rrnL* genes; Passamonti 2007), *Donax trunculus* (*cytb* and *rrnL* genes; Theologidis et al. 2008), and *Ledella ultima* (*cytb* and *rrnL* genes; Boyle and Etter 2013).

In this manuscript, we describe results of our quest for DUI in heterodont bivalves and report our findings about two different, sex linked mtDNAs (and therefore the possible presence of DUI) in the venerid species *Meretrix lamarckii* Deshayes, 1853.

Materials and Methods

Specimens' collection

For this work, we examined specimens of the following species: *Callista chione* (Linnaeus, 1758) (Veneridae: Pitarinae), *Ensis siliqua*

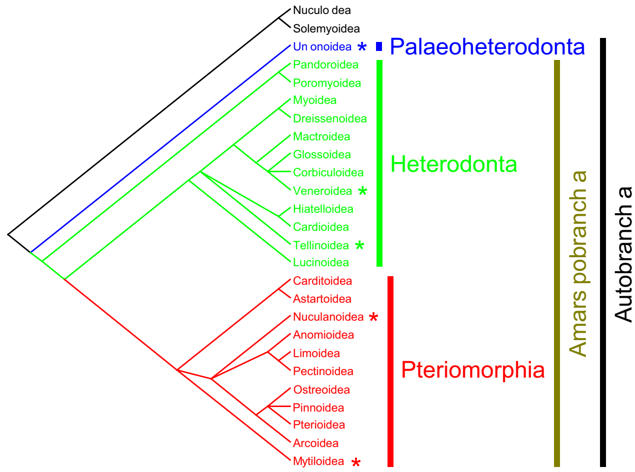

Fig. 1. Phylogeny of bivalves redrawn after Plazzi et al. (2011). For the sake of simplicity, superfamilies Pandoroidea and Poromyoidea were included within Heterodonta and superfamilies Carditoidea and Astartoi dea were included within Pteriomoprhia. For further details on these rela tionships, see Plazzi et al. (2011; and reference therein). Asterisks show superfamilies where Doubly Uniparental Inheritance (DUI) has been detected. Palaeoheterodonta, Heterodonta, Pteriomorphia, Amarsipobran chia *sensu* Plazzi et al. (2011), and Autobranchia are also shown.

minor (Chenu, 1843) (Cultellidae), *Meretrix lamarckii* Deshayes, 1853 (Veneridae: Meretricinae), *Mercenaria mercenaria* (Linnaeus, 1758) (Veneridae: Chioninae), *Mya arenaria* Linnaeus, 1758 (Myidae), *Rudi tapes decussatus* (Linnaeus, 1758) (Veneridae: Tapetinae), and *Venus verrucosa* Linnaeus, 1758 (Veneridae: Venerinae). They were commercially purchased in Bologna, Italy, with the exception of *M. arenaria* and *R. decussatus* which were kindly provided by Prof. Edoardo Turolla from Goro, Italy and *M. mercenaria* and *M. lamarckii* which were purchased at the Tsukiji Wholesale Fish Market (Tokyo, Japan). Most species were sampled in March 2011 (*C. chione*, *E. siliqua minor*, and *V. verrucosa*) and June 2011/2012 (*M. mercenaria*, *M. lamarckii*, and *R. decussatus*), while *M. arenaria* was sampled in May 2011. All individuals were screened alive by microscopic inspection of gonad content to check sexual maturity and to determine the sex of each spec imen. Number of specimens analysed for each species is shown in Table S1.

DNA extraction, PCR amplification, and sequencing

Sperm and eggs were extracted from gonads using capillary tubes. DNA extraction was carried out either with a standard phenol:chloroform protocol (see Sambrook and Russell 2006) or through the MasterPure™ Complete DNA and RNA Purification Kit (Epicentre, Madison, WI, USA), following manufacturer's instructions. PCR ampli fication of cytochrome c oxidase subunit I (*cox1*), large subunit ribo somal RNA (*rrnL*), and small subunit ribosomal RNA (*rrnS*) genes was performed with GoTaq® Flexi DNA Polymerase (Promega, Madison, WI, USA), as follows: 10 µl 5× Green GoTaq® Flexi Buffer, $MgCl_2$ 3 mM, nucleotides 800 µM each, primers 500 nM each, 1.25 U GoTaq® DNA Polymerase, 5 µl template DNA, ddH_2O up to 50 µl. Primers are listed in Table S2 along with their working condi tions (annealing temperature/time); length of amplicons was 371 1009 bp for *cox1*, 322 366 bp for *rrnL*, and 451 663 bp for *rrnS* (see Table S3 for details) (see Folmer et al. 1994; Palumbi et al. 1996; Matsumoto 2003; Simon et al. 2006). PCR cycle was set following manufacturer's instructions, with extension time ranging from 1′ to 1′30″. PCR results were visualized onto a 1% electrophoresis agarose gel stained with ethidium bromide and purified through a standard isopropanol protocol, the Wizard® SV Gel and PCR Clean Up System (Promega), or an optimized version of the PEG protocol of Lis and Schleif (1975). Suitable amplicons were sequenced through the Macro gen Europe (Amsterdam, The Netherland) facility.

Data analysis

Electropherograms and sequences were edited with the software MEGA 5.03 (Tamura et al. 2011), which was also used to computed *p* distances and standard errors with pairwise deletion of gaps and 1000 bootstrap replicates. Neutrality of sequence evolution was assessed through the McDonald and Kreitman (1991) test using DNAsp 5.10.01 (Librado and Rozas 2009) and comparing F and M sequences. Neutrality was also investigated through the codon based *Z* test of selection as implemented in MEGA 5.03 using five different methods, with pairwise deletion of gaps and 1000 bootstrap replicates; for the modified Nei Gojobori method, a fixed transition/transversion ratio of 2 was set (see Nei and Kumar 2000; and references therein).

A phylogenetic analysis was conducted using those markers available for venerid DUI species known to date, i.e. *cox1* and *rrnS*. *Hiatella arc tica* (Hiatellidae), *Acanthocardia tuberculata* (Cardiidae), and *Coelomac tra antiquata* (Mactridae) were used as outgroups. Sequences other than those obtained from *M. lamarckii* for the present study were retrieved from GenBank; the complete phylogenetic dataset is shown in Table 1. Sequences were managed through CLC Sequence Viewer 6.6.2 (CLC bio A/S, Aarhus, Denmark), MICROSOFT EXCEL® 2007, and MEGA 5.03. Genes were separately aligned with MAFFT 6 (Katoh et al. 2002); Q INS i algorithm (accounting for secondary structures; Katoh and Toh 2008) was chosen for *rrnS*, while G INS i algorithm (Katoh et al. 2005) was selected for *cox1*. Each alignment was masked to eliminate noisy positions, not suitable for phylogenetic analysis, with the software BMGE (Criscuolo and Gribaldo 2010), which computes the information entropy associated to each single site of the alignment and discards those with an entropy level which could hamper phylogenetic inference. Indels found in each alignment were coded following the simple indel method proposed by Simmons and Ochoterena (2000) as implemented in the software GAPCODER (Young and Healy 2003).

The two genes were concatenated in a single alignment and best fitting models of molecular evolution for either gene were selected with Kakusan4 (Tanabe 2007, 2011) using Treefinder (Jobb et al. 2004). Fol- lowing our previous experience with bivalve phylogeny (Plazzi and Pas- samonti 2010; Plazzi et al. 2011), data were partitioned into the two genes and were allowed to evolve under different molecular evolution models; moreover, we compared four different phylogenetic models for *cox1* partition: (1) a standard '4by4' nucleotide analysis; (2) a codon analysis (Goldman and Yang 1994; Muse and Gaut 1994) with equal level of selection for all amino acid sites ('codon equal'); (3) a codon analysis using the M3 model ('codon m3'); (4) a codon analysis using the Ny98 model ('codon ny98'; Nielsen and Yang 1998). The Akaike Information Criterion (AIC; Akaike 1973) and the Bayes Factor (BF; Kass and Raftery 1995) were used as described in Plazzi and Passamon ti (2010 and reference therein) to select the best *cox1* model for our dataset.

Bayesian analyses were carried out with MRBAYES 3.2.1 (Ronquist et al. 2011) hosted at the University of Oslo Bioportal (http://www.bioportal.uio.no). Each tree inference was carried out with two runs of 10 000 000 MC^3 generations with 4 chains each; the default analysis was chosen for restriction data, using the option coding variable and modelling substitution occurrence with four discrete, gamma distributed categories. Log likelihood value of trees, PSRF (Gelman and Rubin 1992), and standard deviation of average split frequencies sampled every 1000 generations were used as proxies for convergence. Trees were sampled every 100 generations, and the consensus was computed after burnin removal with the command sumt. MrBayes 3.2.1 was also used to calculate the probability of each codon site being in a positively selected class. All trees were graphically edited by PHYLOWIDGET (Jordan and Piel 2008) and DEND ROSCOPE (Huson et al. 2007) softwares.

Results

Genetic distances

A total of 160 sequences were obtained for this study: they are detailed in Table 2, along with the number of haplotypes for each gene/sex. Genetic data, along with voucher numbers, were uploaded to GenBank with accession numbers KF360089

Table 1. GenBank accession numbers of sequences used for phylogenetic reconstruction in this study. Bold entries were obtained for the present work, while others were downloaded from GenBank. Identical sequences from individuals sharing the same haplotype were deposited in GenBank only once; after the first reference, they are italicized. Labcodes were retrieved from GenBank (whenever available) or assigned to *Meretrix lamarckii* specimens by the authors

Species	Authority	Specimen voucher	Labcode	*cox1*	*rrnS*
Acanthocardia tuberculata	(Linnaeus, 1758)			NC 008452	NC 008452
Coelomactra antiquata	Spengler, 1802			NC 021375	NC 021375
Cyclina sinensis	(Gmelin, 1791)		F	AB040835	
			M1	AB040833	
			M2	AB040834	
Hiatella arctica	(Linnaeus, 1767)			NC 008451	NC 008451
Meretrix lamarckii	Deshayes, 1853			NC 016174	NC 016174
		BES:TKJ:004	F02	**KF360109**	**KF360174**
		BES:TKJ:005	F03	**KF360110**	**KF360175**
		BES:TKJ:007	F04	***KF360110***	
		BES:TKJ:008	F05	***KF360110***	**KF360176**
		BES:TKJ:010	F06		**KF360177**
		BES:TKJ:012	F08	***KF360110***	
		BES:TKJ:013	F09	***KF360110***	**KF360178**
		BES:TKJ:014	F10	**KF360111**	**KF360179**
		BES:TKJ:031	F11	**KF360112**	**KF360180**
		BES:TKJ:033	F12	**KF360113**	**KF360181**
		BES:TKJ:034	F13	***KF360110***	***KF360179***
		BES:TKJ:036	F15	**KF360114**	**KF360182**
		BES:TKJ:039	F18	***KF360110***	**KF360183**
		BES:TKJ:040	F19	***KF360110***	***KF360176***
		BES:TKJ:009	M02	**KF360115**	**KF360184**
		BES:TKJ:032	M03	**KF360116**	**KF360185**
		BES:TKJ:041	M04		**KF360186**
		BES:TKJ:042	M05	**KF360117**	***KF360186***
		BES:TKJ:043	M06	**KF360118**	
		BES:TKJ:044	M07		**KF360187**
		BES:TKJ:046	M08	**KF360119**	***KF360186***
		BES:TKJ:047	M09		**KF360188**
		BES:TKJ:048	M10	**KF360120**	***KF360188***
		BES:TKJ:025	M14	***KF360117***	
Meretrix lusoria	(Roding, 1798)			NC 014809	NC 014809
Meretrix meretrix	(Linnaeus, 1758)			NC 013188	NC 013188
Meretrix petechialis	(Lamarck, 1818)			NC 012767	NC 012767
Paphia amabilis	(Philippi, 1847)			NC 016889	NC 016889
Paphia euglypta	(Philippi, 1847)			NC 014579	NC 014579
Paphia textile	(Gmelin, 1791)			NC 016890	NC 016890
Paphia undulata	(Born, 1778)			NC 016891	NC 016891
Timoclea ovata	(Pennant, 1777)			JF496777	JF496752
Venus casina	Linnaeus, 1758			DQ458496	JF496753
Venerupis philippinarum	(A. Adams & Reeve, 1850)		F	NC 003354	NC 003354
			FA1	AF484332	AF484332
			FA2	AF484333	AF484333
			FA3	AF484334	AF484334
			FA4	AF484335	AF484335
			FA5	AF484336	AF484336
			M	AB065374	AB065374
			MA1	AF484337	AF484337
			MA2	AF484338	AF484338
			MA3	AF484339	AF484339
			MA4	AF484340	AF484340

KF360190. Specimens were deposited in the collection of one of the authors (M. P.) at the Museum of Zoology of the University of Bologna, hosted by the Department of Biological, Geological and Environmental Sciences (Bologna, Italy). For specimen vouchers, see Table 1. In all cases, different haplotypes were found for each gene, with the only exception of *C. chione*: the four female and the six male *cox1* sequences obtained from *C. chione* were all identical (Table 2). Table 3 shows *p* distance comparisons within groups (F and M) and between groups (F versus M). Generally, genetic variability in F mtDNA is some what lower than in M mtDNA, even if this difference in most cases is not significant given the standard errors. The *p* distance between F and M sequences is always intermediate to the values within the two groups.

The situation is different for *M. lamarckii*. The within group *p* distances are between 0.0028 and 0.0046, while, given that intervals computed using standard errors (±0.0009 and ±0.0015, respectively) do not overlap, between groups *p* distances are significantly higher, being 0.0980 ± 0.0095 for *cox1* and 0.1168 ± 0.0128 for *rrnS* (Table 3). In fact, most variable sites in the *cox1*/*rrnS* *M. lamarckii* alignments (Datasets S1 and S2) are diagnostic differences between F and M sequences (Fig. 2) in *M. lamarckii*. Most mutations in *cox1* appear to be synonymous: aminoacid *p* distance is 0.0026 ± 0.0018 among F

Table 2. Number of sequences obtained for each species for each marker. In most cases, it was possible to sequence two out of three genes, while for some species (*Callista chione*, *Mya arenaria*, and *Venus verrucosa*) only *cox1* could be amplified. F and M haplotypes are counted together. GenBank accession numbers are also shown

	Sequences						Haplotypes					
	cox1		*rrnL*		*rrnS*		*cox1*		*rrnL*		*rrnS*	
Species	Female	Male	Female	Male	Female	Male	Count	GenBank IDs	Count	GenBank IDs	Count	GenBank IDs
C. chione	4	6	0	0	0	0	1	KF360089	n/a		n/a	
Ensis siliqua minor	5	7	0	0	6	12	12	KF360147 158	n/a		8	KF360166 173
Mercenaria mercenaria	11	10	9	7	0	0	13	KF360121 133	7	KF360159 165	n/a	
Meretrix lamarckii	13	7	0	0	12	8	12	KF360109 120	n/a		15	KF360174 188
M. arenaria	10	10	0	0	0	0	13	KF360134 146	n/a		n/a	
Ruditapes decussatus	10	12	0	0	2	0	9	KF360090 098	n/a		2	KF360189 190
V. verrucosa	6	6	0	0	0	0	10	KF360099 108	n/a		n/a	

sequences and 0.0041 ± 0.0020 among M ones, while it is 0.0459 ± 0.0126 between F and M clusters, nearly half the value of the respective nucleotide *p* distances (Table 3). The probability of rejecting the null hypothesis of strict neutrality was always not significant, regardless of the test/method used (Table 4).

Phylogenetic analysis

The complete alignment, after BMGE masking, was 400 bp (+1 0/1 coded indel character) long for *cox1* and 354 bp (+31 0/1 coded indel characters) long for *rrnS*, for a total of 51 operational taxonomic units (OTUs) × 786 sites available for phylogenetic inference. GTR+G (Tavaré 1986) was selected as the best fitting molecular evolution model for *cox1*, while it was HKY85+G (Hasegawa et al. 1985) for *rrnS*. Both AIC and BF (Tables S4 and S5) tests ranked the four models in the same order, sharply favouring codon m3 over competitors: this is in good agreement with the methodological pipeline described in Plazzi and Passamonti (2010). As a consequence, we regard the tree obtained from the codon m3 analysis as our preferred phylogenetic tree computed for this study and it is shown in Fig. 3.

The tree has high node support values, with only two excep tions: (1) the family Veneridae is not recovered as monophyletic, due to the branch leading to the mactrid *Coelomactra antiquata* that makes it paraphyletic; (2) the subfamily Meretric inae has only weak posterior probability (PP) support (0.786), and therefore, its node has been collapsed. Conversely, subfam ilies Tapetinae and Cyclininae are supported in our tree with PP 1.000; all genera and species are monophyletic (0.996 < PP < 1.000); the species *V. philippinarum* exhibits its expected sex specific distribution of DUI related sequences, F and M clusters being separated with PP 1.000. Veneridae are subdivided in two large clades: on one side, the topology is (Chioninae + Venerinae) + Tapetinae; on the other side, Cyclin inae and Meretricinae cluster together. Within the species *M. lamarckii*, two main clades are splitted with PP 0.998: these correspond to F sequences and M sequences; the *M. la marckii* sequence downloaded from GenBank (accession num ber NC 016174) clusters with F sequences. Conversely, within the genus *Cyclina*, the F sequence clusters with the M2 sequence of this species, while *C. sinensis* M1 is basal to both.

Finally, posterior values of levels of selection are low (ω_1 0.0017; ω_2 0.0221; ω_3 0.0810), with a minimal vari

Table 3. *p* Distances for each species for each marker

	cox1			*rrnL*			*rrnS*		
Species	F	M	F/M	F	M	F/M	F	M	F/M
Callista chione	0.0000 ±0.0000	0.0000 ±0.0000	0.0000 ±0.0000						
Ensis siliqua minor	0.0127 ±0.0022	0.0225 ±0.0026	0.0176 ±0.0019				0.0000 ±0.0000	0.0026 ±0.0008	0.0014 ±0.0004
Mercenaria mercenaria	0.0049 ±0.0014	0.0075 ±0.0019	0.0063 ±0.0012	0.0067 ±0.0027	0.0043 ±0.0021	0.0056 ±0.0023			
Meretrix lamarckii	0.0028 ±0.0009	0.0044 ±0.0014	**0.0980 ±0.0095**				0.0046 ±0.0012	0.0033 ±0.0015	**0.1168 ±0.0128**
Mya arenaria	0.0028 ±0.0011	0.0039 ±0.0013	0.0038 ±0.0012						
Ruditapes decussatus	0.0055 ±0.0011	0.0111 ±0.0021	0.0080 ±0.0015						
Venus verrucosa	0.0060 ±0.0015	0.0036 ±0.0012	0.0045 ±0.0010						

F, intrafemale divergence; M, intramale divergence; F/M, divergence between females and males.
Standard errors are given under the distance value. *Meretrix lamarckii* F/M comparisons, a compelling evidence for the presence of DUI in this species, are shown in bold.

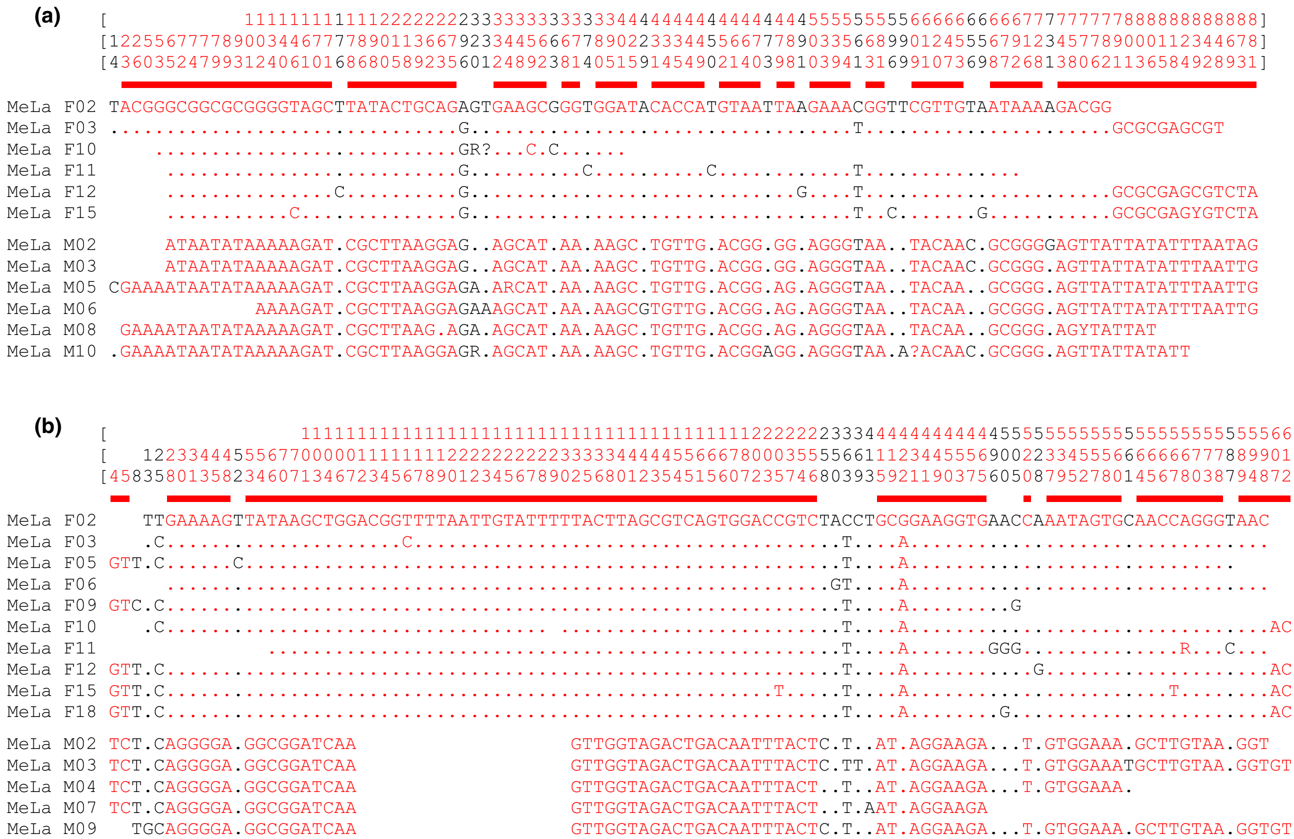

Fig. 2. Variable sites found in *cox1* (a) and *rrnS* (b) alignments of *Meretrix lamarckii* haplotypes. Red sites are diagnostic to distinguish among F and M sequences. Haplotypes are named by 'MeLa' (i.e. '*M. lamarckii*'), followed by either 'F' for females or 'M' for males and the specimen number. Site numbers are shown above the alignments and refer to the complete alignments available as Datasets S1 (*cox1*) and S2 (*rrnS*) in FASTA format. A dot indicates a character identical to the first line.

Table 4. Results of neutrality tests

Test	Statistic		p value
McDonald and Kreitman test	NI	0.099	0.0066**
Codon based Z test of selection	Nei Gojobori method	11.7615	0.0000***
	Modified Nei Gojobori method	11.7636	0.0000***
	Li Wu Luo method	9.0246	0.0000***
	Pamilo Bianchi Li method	9.1804	0.0000***
	Kumar method	9.3444	0.0000***

NI, neutrality index; p value, probability of rejecting the null hypothesis of strict neutrality.

Highly (p value <0.01) and very highly (p value <0.005) significant results are marked with two and three asterisks, respectively.

ance (<0.0002); in any case, the posterior probability of each codon site being positively selected is always zero.

Discussion

The quest for Doubly Uniparental Inheritance in heterodont bivalves

Neither marker evidenced a gender associated pattern in the mtDNA for the analysed species *C. chione*, *E. siliqua minor*, *M. mercenaria*, *M. arenaria*, *R. decussatus*, and *V. verrucosa*. For this reason, we may conclude that we do not have, at present, evidence of DUI in these species; similar results were indeed reported by Theologidis et al. (2008) for *C. chione*, *R. decussatus*, and *V. verrucosa*.

To the contrary, both *p* distance analyses and phylogenetic analyses strongly support a sex linked clustering of F and M mitochondrial sequences in *M. lamarckii* (Table 3; Fig. 3). These data are based on two partial mitochondrial genes and not on the complete mitochondrial genomes; yet, this approach already proved to be sound for DUI detection (see, e.g. Passamonti and Scali 2001; Passamonti 2007; Theologidis et al. 2008; Boyle and Etter 2013).

The presence of DUI will only be definitively ascertained by *in vivo* observation of mitochondria behaviour, with special reference to the localization of male mitochondria into the germline. In fact, such an aggregate pattern of male mitochondria during the early embryonic development is typical of other DUI species (Cao et al. 2004; Obata and Komaru 2005; Cogswell et al. 2006; Obata et al. 2008; Milani et al. 2012). Meanwhile, yet, the find ing of two distinct, sex linked mtDNAs has to be taken as a compelling evidence for the presence of DUI in this species.

First-glance characterization of DUI in *Meretrix lamarckii*

In fact, F and M mtDNA lineages of *M. lamarckii* show high levels of divergence, being almost 10% for *cox1* and 12% for *rrnS* (Table 3); these values are similar to those obtained from other DUI species, like *Musculista senhousia* (Benson in Cantor, 1842) (Passamonti et al. 2011), *Mytilus galloprovincialis* Lamarck, 1819 (Mizi et al. 2005), and *V. philippinarum* (Passa-

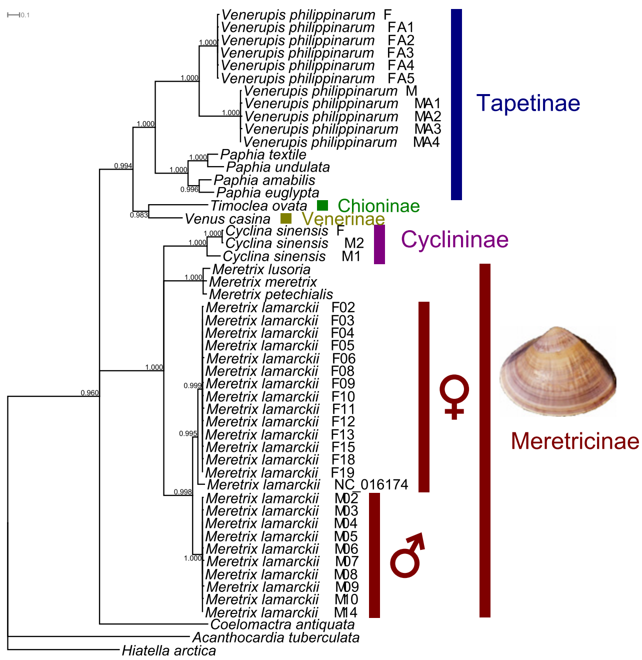

Fig. 3. Bayesian phylogenetic tree obtained from the concatenated *cox1 rrnS* dataset. For *cox1*, the M3 codon model was chosen (see text for details). Posterior probabilities (PP) are shown at each node and nodes with PP < 0.95 were collapsed. For venerids, traditional subfamilies are also shown.

monti et al. 2003), but do not reach those high values observed in *Donax trunculus* (Theologidis et al. 2008) and in Unionoidea (Doucet Beaupré et al. 2010).

The amount of detected polymorphism is comparable between F and M mtDNAs (Table 3), and it is slightly higher within either M (for *cox1*) or F (for *rrnS*) lineages. Thus, our preliminary survey cannot detect a higher amount of variation in M mito chondrial DNA in *M. lamarckii*, a commonly observed pattern in DUI species (Zouros 2013; and references therein).

Yet, the recent high throughput sequencing approach by Ghiselli et al. (2013) demonstrated that PCR based techniques may lead to an unavoidable underestimation of mtDNA variability, because they are not able to detect low frequency SNPs. Conse quently, the commonly established idea of a faster evolution of the M type mitochondrial genome (see, e.g. Zbawicka et al. 2010; Doucet Beaupré et al. 2010; and references therein), which has been commonly based on PCR data, has to be reconsidered. Since our data may represent a partial sampling of the real mtD NAs variability, it is not surprising that male branches may appear similar to, but not longer than, female branches in our phylogenetic tree (Fig. 3).

Our neutrality tests indicate purifying selection, which is generally assumed for mitochondrial genomes (Ballard and Kreitman 1995): given the importance of mitochondrial genes for the cell machinery, adaptive mutations are considered very rare, and del eterious changes should be quickly removed, leaving only the neutral quote of variation (Galtier et al. 2009). Yet, it has been repeatedly underlined that such an assumption does not hold completely (see, e.g. Ballard and Whitlock 2004; Ballard and Rand 2005; Galtier et al. 2009; Parmakelis et al. 2013; and refer ences therein). Many statistical tests are available to estimate the probability of rejection of the null hypothesis of neutrality (Bal lard and Kreitman 1994; Gerber et al. 2001), but, as noted by Ballard and Whitlock (2004), ‘a negative result cannot be taken as evidence of a lack of positive selection when the sample size is small’. Put in other words, our dataset of 51 OTUs cannot unveil such an adaptive selection through ‘selective sweeps’ that Bazin et al. (2006) unveiled across a mitochondrial dataset of >1600 animal species.

Ghiselli et al. (2013) found many different non neutral SNPs both in F and M mitochondrial populations of the DUI species *V. philippinarum*. It was also observed that their distribution was not random: the occurrence of SNPs with a potentially high impact on phenotype is higher within the F lineage than within the M lineage, even if they are rare; conversely, mid frequency alleles are much more common in M type (Ghiselli et al. 2013). All this considered, the impossibility of rejecting the null hypothesis of neutrality only indicates that no obvious selective pressures are working on our dataset, thus supporting the validity of our phylogenetic analysis.

Contrastingly, in some molluscan groups, positive traces of positive selection on mtDNA have been unveiled, as, for example, in pulmonate gastropods (Parmakelis et al. 2013). Whether or not DUI mitochondrial genomes are evolving under true neutral conditions is a question far beyond the present study and surely deserves further investigation, perhaps using high through put technologies as in the pioneering work of Ghiselli et al. (2013).

Phylogenetic pattern of sex-linked sequences and the origin of DUI

Meretrix lamarckii sex linked mtDNAs exhibit a phylogenetic pattern similar to that of *V. philippinarum* (Fig. 3): F and M mtDNA clusters are sister groups, and the species is retrieved as monophyletic. This is not the case for the family Unionidae and the genus *Mytilus*, where F sequences of different species cluster together, and so do M ones (see, e.g. Doucet Beaupré et al. 2010). The unclear situation of *Cyclina sinensis*, where the single F sequence clusters with only one of the two M sequences (Fig. 3), deserves further investigations.

We have to mention that the complete mtDNA of *M. lama rckii,* which was downloaded from GenBank (accession number NC 016174), clusters with our F sequences: this is also expected, as DNA extraction is generally carried out from somatic tissues in this case, the foot muscle (Wang et al. 2011) where mostly F mtDNA is present in both sexes. In fact, in DUI species, M mtDNA could be better recovered by extract ing DNA from sperm.

These data seem at first to suggest that DUI had multiple origins: specifically, at the base of Unionidae, of the genus *Mytilus*, of the species *V. philippinarum*, and of the species *M. lamarckii*. As mentioned, the possibility that DUI regulating ORFs have a viral origin (Milani et al. 2012) makes this hypothesis worthy to consider. On the other hand, DUI species are still being sig nalled: recently, for example, a species included in a superfamily where DUI was previously not known (Nuculanoidea) has been added to the list (Boyle and Etter 2013).

All that considered, it is still difficult to draw definitive conclusions, at least until the bivalve evolutionary tree is poorly sampled with respect to DUI. In this manuscript, we focused on Heterodonta and provide evidence for its presence in a venerid species, *M. lamarckii*, and we could not obtain any evidence for it in the other heterodont species we analysed. However, many more (large) families are currently waiting to be screened in the future, like Cardiidae, Mactridae, and Semelidae. The sharper definition of DUI distribution across the huge bivalve biodiver sity is by far one of the most compelling needs to advance our knowledge about this peculiar way of mitochondrial inheritance.

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Riassunto

La ricerca dell'Eredità Uniparentale Doppia nei bivalvi eterodonti ed il suo rilevamento in *Meretrix lamarckii* (Veneridae: Meretricinae)

L'Eredità Uniparentale Doppia (DUI) é probabilmente la più significativa eccezione della normale eredità materna dei mitocondri e da essa discendono una serie di interessanti domande riguardo, tra l'altro, la funzione di questi organelli, la determinazione del sesso, lo sviluppo embrionale e tutte le possibili conseguenze evolutive. Al momento la DUI è stata trovata in poche specie di molluschi bivalvi, ma è necessario un maggiore sforzo di ricerca per ottenere un quadro più chiaro della sua distribuzione in questo gruppo, quadro che è necessario per trarre ogni conclusione ben suffragata sulla sua origine e sul suo significato evolutivo. Se la DUI abbia avuto una singola origine o se sia comparsa più volte è ancora una questione aperta e dibattuta. In questo lavoro abbiamo analizzato sette specie di bivalvi eterodonti e forniamo i dati per sostenere la presenza della DUI nel veneride Meretrix lamarckii.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Individuals analysed for this study by species and sex. Format: PDF.

Table S2. Primers used in this study for routine PCR amplifi cations. Working condition column lists annealing conditions of each primer pair. Format: PDF.

Table S3. Lengths of amplified fragments by species and gene. l_{min}, minimum length; l_{max}, maximum length; align, align ment length. Format: PDF.

Table S4. Akaike Information Criterion. LnL, natural loga rithm of likelihood; K, free parameters; AIC, Akaike Information Criterion. The lower the AIC value, the better the model fits to the data. Format: PDF.

Table S5. Bayes Factor. For evolutionary model nomenclature, see text. A value >10 is a very strong evidence favouring the left model over the above model. Format: PDF.

Dataset S1. Alignment of *cox1 Meretrix lamarckii* haplotypes. FASTA built with MEGA 5.03 (Tamura et al. 2011).

Dataset S2. Alignment of *rrnS Meretrix lamarckii* haplotypes. FASTA built with MEGA 5.03 (Tamura et al. 2011).