Doubly Uniparental Inheritance and beyond: The contribution of the Manila clam Ruditapes philippinarum.

REVIEW

DUI and beyond: the contribution of the Manila clam *Ruditapes philippinarum*

Running title: DUI and beyond

Marco Passamonti[1]*, Federico Plazzi[1]

[1] Department of Biological, Geological, and Environmental Sciences – University of Bologna.

* Corresponding Author: marco.passamonti@unibo.it

Keywords: Doubly Uniparental Inheritance (DUI); *Ruditapes philippinarum*; mitochondrial inheritance; mitochondrial biology; genomic conflicts.

Abstract

The Manila clam, *Ruditapes philippinarum*, is a widespread and commercially important bivalve species showing a peculiar way of mitochondrial inheritance known as Doubly Uniparental Inheritance (DUI), which is different from the strict maternal inheritance found in the broad majority of metazoans. Although DUI in *R. philippinarum* was characterized afterwards mytilids and unionids, nevertheless its ongoing in-depth analysis gave and is giving important contribution and insights to the characterization of this unusual mitochondrial inheritance mechanism. In this review, we recap the experimental results that were obtained mainly on *R. philippinarum* in the context of the available knowledge on DUI and discuss it in terms of significance for DUI characterization and, more broadly, for metazoan mitochondrial biology.

Introduction

Ruditapes philippinarum (Adams & Reeve, 1850), a clam species that is largely harvested in the Adriatic Sea, shows an unusual mtDNA inheritance system, which has been observed, so far, in some bivalves: the Doubly Uniparental Inheritance (DUI). DUI was first detected and sketched out in *Mytilus edulis* (Skibinski, Gallagher, & Beynon, 1994a,b; Zouros, Oberhauser Ball, Saavedra, & Freeman, 1994a,b). Since 1994, DUI has been found in other bivalve species, hitting triple digits in recent years (Gusman, Lecomte, Stewart, Passamonti, & Breton, 2016); nonetheless, the broad figure is somewhat consistent. Long story short, DUI bivalves have two mitochondrial (mt) lineages, one transmitted through eggs (F), the other through sperm (M), whose mitochondrial genomes (mtDNAs) often show higher levels of nucleotide divergence. After amphimixis, the DUI embryo is heteroplasmic for its mtDNA, a status eventually maintained only in males (F being localized in somatic tissues, while M being in both sperm and soma). Conversely, in females M is normally degraded (or diluted below detection limits) and homoplasy is restored (Breton, Doucet-Beaupré, Stewart, Hoeh, & Blier, 2007; Passamonti & Ghiselli, 2009; Doucet-Beaupré et al., 2010; Zouros, 2013; Zouros & Rodakis, 2019) (Fig. 1).

The presence of DUI in a given species is generally detected using sex-linked heteroplasmy as a proxy (see, f.i., Passamonti & Scali, 2001; Theologidis, Fodelianakis, Gaspar, & Zouros, 2008; Boyle & Etter, 2013; Plazzi, Cassano, & Passamonti, 2015; Plazzi, 2015; Vargas, Pérez, Toro, & Astorga, 2015; Dégletagne, Abele, & Held, 2016; Gusman et al., 2016; Lucentini et al., submitted). In a nutshell, after dissection and microscopic inspection in order to determine the sex, DNA is extracted from the gonadal content (i.e., eggs or sperm) of sexually mature specimens; on the other side, DNA is extracted from somatic tissues, typically foot or adductor muscles. The same mitochondrial markers are sequenced from all samples: if the female germline (i.e., eggs) shows the same haplotypes of somatic tissues of both sexes, while the male germline (i.e., sperm) consistently shows different haplotypes, the sex-linked heteroplasmy is demonstrated and this builds a strong argument for DUI to be present in the focal species.

The use of sex-linked heteroplasmy as a proxy for DUI has proved to be very effective, but it is important to reckon some drawbacks: (i) in case of highly divergent genomes (which is often claimed to be the case for the M lineage; f.i., Gusman et al., 2016; Zouros, 2013; but see Passamonti, 2007; Ghiselli et al., 2013; Plazzi, Puccio, & Passamonti, 2016; Plazzi and Passamonti, 2019), PCR primers may fail to amplify both haplotypes (Theologidis et al., 2008; Zouros, 2013; Gusman et al., 2016); and (ii) the masculinization of the F lineage (i.e.,

the invasion of the M lineage by the female genome) may reset the divergence of the two genomes, though they are actually inherited under DUI (Theologidis et al., 2008; Stewart, Breton, Blier, & Hoeh, 2009; Zouros, 2013; Gusman et al., 2016). Masculinization of F lineage was repeatedly observed in *Mytilus* species (Stewart et al., 2009; Zouros, 2013; and reference therein), but was never detected in Unionidae (Curole & Kocher, 2002, 2005; Stewart et al., 2009; Walker et al., 2006) nor in Veneridae (Passamonti, 2007; Passamonti & Scali, 2001; Stewart et al., 2009).

Following these lines of evidence, more than one hundred species of bivalves have been currently reported to show the peculiar phenomenon of DUI. Although only limited research has been carried out among gastropods (Parakatselaki, Saavedra, & Ladoukakis, 2016; Gusman, Azuelos, & Breton, 2017), yet DUI is probably widespread within bivalves, and a large part of DUI species are still to be detected (Gusman et al., 2016). Moreover, in some groups, such as the family Unionidae, DUI is common and widespread among gonochoric taxa and strictly absent in hermaphroditic species (Breton et al., 2011; Guerra et al., 2017); in other cases, only one or two species were reported to show DUI evidence in a given family (Gusman et al., 2016), and many gonochoric species do not show evidence of the phenomenon (Plazzi et al., 2015; Lucentini et al., submitted). With more than 3,500 extant and extinct genera (Millard, 2001), the diversity of bivalves overwhelms the availability of empirical data on sex-linked heteroplasmy, and the current knowledge of DUI distribution within the class is somewhat reduced to draw ultimate conclusions. Nonetheless, significant molecular differences among different DUI systems have been described (Zouros, 2013; Plazzi, 2015; Plazzi & Passamonti, 2019).

The discovery of DUI in *Ruditapes philippinarum* and its characterization

The first evidence of Doubly Uniparental Inheritance (DUI) in the Manila clam *Ruditapes philippinarum* was published in 2001 (Passamonti & Scali, 2001). Although it came later than the one in *Mytilus* (Skibinski et al., 1994a,b; Zouros et al., 1994a,b) and unionid freshwater mussels (Hoeh, Stewart, Sutherland, & Zouros, 1996b; Liu, Mitton, & Wu, 1996), *R. philippinarum* was the first heterodont bivalve to show DUI, and this widened the taxonomic occurrence of this unusual mitochondrial inheritance mechanism to all Autolamellibranchiata bivalves.

Soon the DUI system of this species showed both peculiarities and analogies with these already known ones. Many features of *R. philippinarum* DUI resemble the ones already described in *Mytilus*, but some differences were discovered, with especial reference to tissue

distribution of M and F mitotypes, with somatic tissues being richer in M-type mtDNAs than *Mytilus* counterparts. The peculiar distribution of M and F mitotypes in somatic tissues of males (i.e. they are heteroplasmic for both mitotypes) allowed some interesting tests for some of the more basic aspects of mitochondrial biology (f.i., mitochondrial inheritance). In Passamonti, Boore, & Scali (2003) a case of possible recombination between M and F mitotypes was recorded, thus confirming the coeval claims that there is no molecular reason to consider mitochondrial DNA recombination absent from mitochondria (e.g., Thyagarajan, Padua, & Campbell, 1996; Laudokakis & Zouros 2001a,b) and that the ratio of recombinants is directly proportional to the level of heteroplasmy of the cells (Laudokakis & Zouros 2001a). However, in *R. philippinarum*, recombinant sequences were not detected in mtDNAs obtained from gonads (Passamonti et al., 2003), and this was taken as an indication that clams do not transmit recombinants to their progeny at a detectable level, even if recombination may occur in their cells with characteristics similar to that of *M. edulis* (Laudokakis & Zouros 2001). Although the available data were just a handful of sequences (we were still not in the 'omics' era), this fact allowed to speculate that the lack of recombinant DNAs passing to the next generation might be the effect of the so-called mitochondrial bottleneck and, maybe, some different selective constraints between somatic and germ-line mitochondria. However, the details of both mechanisms are still largely unknown.

Since the beginning of DUI studies, two main questions have been repeatedly addressed: (i) which would be the functional significance (if any) of DUI (f.i., Everett, Williams, Gibson, & Stewart, 2004); and (ii) how (and how many times) DUI could have been originated (f.i., Hoeh et al., 1996b). The in-depth analysis of *R. philippinarum* gave relevant contributions on both issues, as we will see later on.

In a previous review (Passamonti & Ghiselli, 2009), we proposed that the function of DUI could be somehow related to sex determination, in that the mitochondrial genomes (M-type and F-type) may play a role in shifting gonad differentiation toward testes or ovaries. In this conception, the presence of M-type mitochondria in sperms, and F-type mitochondria in eggs, should be considered 'causal', and those mitochondria may regulate gamete differentiation. On the other hand, we can also imagine that this linkage is 'coincidental', i.e. the two sex-linked mitochondrial genomes are just carried by the two gametes, with no apparent function. If this second hypothesis is true, both mitotypes act as selfish elements. Both hypotheses have been proposed for the other DUI systems as well, but as we will see our data on *R. philippinarum* point out to the fact that the first has more chances to be true.

The segregation pattern and distribution of M and F types during early embryo development and gonad differentiation.

The general, DUI-defining pattern of separated F and M lineages has different features in different bivalve groups. As already mentioned, while male somatic tissues do mainly contain the F lineage in the genus *Mytilus* (Garrido-Ramos et al., 1998; Dalziel & Stewart, 2002; but see Kyriakou, Zouros, & Rodakis, 2010), male soma was found heteroplasmic at various degrees (as in *R. philippinarum*): for instance, it appears to be dominated by M lineage in *Perumytilus purpuratus* (Vargas et al., 2015). Moreover, heteroplasmic females may be found in *Mytilus* (Brannock, Roberts, & Hilbish, 2013).

The specific mechanisms of segregation of M- and F-type mitochondria during embryo development and germline differentiation have been studied in depth in *R. philippinarum*, by both molecular and cytogenetic approaches. A first assay using RealTime quantitative PCR (Ghiselli, Milani, & Passamonti, 2011) demonstrated that there is a strict segregation of mitochondria in both clams' germlines (i.e., no mitochondrial DNA of the other sex is detected in sperms or eggs). The absence of F-type mtDNAs in sperm is, according to Venetis, Theologidis, Zouros, & Rodakis (2006), a basic requirement for the stability of DUI: data on *R. philippinarum* confirmed this. On the other hand, the situation is more controversial for eggs: different levels of M-type mtDNAs have been detected in *Mytilus galloprovincialis* eggs (Obata, Kamiya, Kawamura, & Komaru, 2006.; Obata, Kamiya, Kawamura, & Komaru, 2007; Sano, Obata, & Komaru, 2007), but there is no evidence of M-type mtDNAs in the eggs of *R. philippinarum*. An interesting observation came from some female *R. philippinarum* showing somatic heteroplasmy, with variable levels of M-type mtDNAs in their somatic tissues; this indicates that the elimination mechanism of sperm mitochondria is not unfailing for somatic tissues. Quite remarkably, their eggs were homplasmic for F-type, indicating that, at least in *R. philippinarum*, a strict homoplasmy is a prerequisite for proper gamete formation in both sexes (Ghiselli et al., 2011).

In a few cases, DUI has been directly investigated by tracking mitochondria in the zygote and in the early segmentation (up to the trochophore larva in *M. edulis*). Two different patterns were detected in the DUI species *Mytilus edulis*, most likely in relation to the sex of the developing embryo (Cao, Kenchington, & Zouros, 2004; Cogswell, Kenchington, & Zouros, 2006; Kenchington, Hamilton, Cogswell, & Zouros, 2009). On one side, sperm mitochondria concentrate in a limited region of the embryo, close to the first cleavage furrow, and will presumably end in populating the germline: this is called the 'aggregated' pattern and is typical of male-biased mothers, i.e. female individuals that give birth to almost only male

offspring. On the other side, sperm mitochondria are scattered throughout the embryo and seem actually to be distributed at random: this is called the 'dispersed' pattern and is typical of female-biased mothers, i.e. female individuals that give birth to almost only female offspring. The presence of these two patterns of sperm mitochondria has been considered a strong evidence of DUI, in that it links the differential inheritance of mitochondria in the two sexes to a concrete mechanism for mitochondria to be preserved in the germline (for male offspring) or dispersed and degraded (for female offspring).

The very same patterns were observed in *M. galloprovincialis* (Obata & Komaru, 2005) and *R. philippinarum* (Milani, Ghiselli, & Passamonti, 2012). Conversely, the same patterns were not observed in *Crassostrea gigas*, where male mitochondria seem to distribute at random (Obata, Shimizu, Sano, & Komaru, 2008): this observation, along with the complete lack of evidence about sex-linked heteroplasmy, leads to the conclusion that DUI is not present in the Pacific oyster. To date, *C. gigas* is thus to our knowledge the only species where the presence of DUI was discarded on the basis of direct mitochondrial behavior observation, rather than excluded as unlikely because of the absence of sex-linked heteroplasmy.

The area where paternal mitochondria concentrate in the aggregated pattern is the same embryonic area in which also germ plasm is transferred, as is suggested by the observation that it co-localizes with the protein VASA (Milani, Ghiselli, Maurizii, & Passamonti, 2011): VASA is a DEAD-box RNA helicase described at first in *Drosophila melanogaster* and then in many other animals (Hay, Ackerman, Barbel, Jan, & Jan, 1988; Hay, Jan, & Jan, 1988; Gustafson& Wessel, 2010) and its inactivation suppresses the formation of primordial germ cells (Lasko & Ashburner, 1988; Williamson & Lehmann, 1996; Knaut, Pelegri, Bohmann, Schwarz, & Nuesslein-Volhard, 2000; Kuznicki et al., 2000).

In fluorescent analyses, a central role of microtubules is evident, as the M-type mitochondrial aggregate co-localize with the midbody, i.e. the residual of the mitotic spindle after cytodieresis (Milani et al., 2011, 2012). Moreover, no replication boost of either M- and F-type mtDNAs is evidenced in the earliest embryo development (Milani et al., 2012).

Based on the observations above, we proposed a three-step model for germ line segregation under DUI, evidencing three checkpoints for proper segregation of sex-linked mitochondrial lineages. The model includes an early segregation mechanism in embryos (the aggregated/dispersed pattern we mentioned above), a second step in which M types are actively eliminated/retained according to sex, and a final mechanism of sex-linked selection during gamete formation (Ghiselli et al., 2011).

Subsequent researches focused on testing this three-step model. Guerra and colleagues (2016) used a qPCR approach to follow both mtDNA lineages starting from early embryos: they found that both mtDNAs do not detectably replicate during early embryogenesis, and that the M line might be lost from females around 24 h of age. A rise in mtDNA copy number was observed before the first reproductive season in both sexes, with the M mitochondrial genome replicating more than the F in males, and we associate these boosts to the early phase of gonad production. In parallel, the cytogenetic analyses evidenced that at each reproductive season a proliferation of germline "primordial stem cells", originating from the simple columnar epithelium of the gut and in the connective tissue nearby, contributes to the seasonal gonad reconstitution (Milani et al., 2017a,b).

Finally, by an in-depth immunolocalization of F- and M-type variants of three mitochondrially-encoded proteins, in germline and somatic tissues at different developmental stages, we evidenced that undifferentiated germ cells of both sexes, as well as male soma, are heteroplasmic, while gametes are invariably homoplasmic. Thus, the condition of homoplasmy of germline is reached during gametogenesis, rather than earlier embryo development, with a process that reminds a meiotic drive (Ghiselli et al, 2019). If this scenario is true, then the homoplasmy condition is not causally linked to the aggregated/dispersed patters, as previously hypothesized, but starting from a heteroplasmic population of germ cells, is reached at every reproductive term during gonad development of both sexes. If this is true, checkpoints 1 and 2 of our previously hypothesized model are just ineffective, and the homoplasmic condition of gametes is restored at checkpoint (previously numbered as) 3.

The -omics breakthrough – Putative candidate genes for sex determination in bivalves and mitochondrial inheritance

Mitochondrial transcriptomes provide further insights into DUI details and machinery. Despite being (most likely) transcribed as a single polycistrone, different regions of the mitochondrial genome, as well as different regions within the same gene, yield different levels of mature transcripts (Ghiselli et al., 2013) because of multiple post-transcriptional regulations (Lynch, 2007; Scheffler, 2008). Moreover, in *R. philippinarum*, the two mitochondrial genomes globally show lineage-specific transcription levels for all protein coding genes, as well as for *rrnS* (Ghiselli et al., 2013; Iannello, Puccio, Piccinini, Passamonti, & Ghiselli, 2019); conversely, all the nuclear components of the electron transport chain but the subunits of complex III are transcribed from the nuclear genome to

approximately the same level in the two sexes (Ghiselli et al., 2013; see also Iannello et al., 2019). Moreover, a strong correlation was observed between levels of OXPHOS gene expression (either nuclear or mitochondrial ones) in the two strictly related species *R. philippinarum* and *R. decussatus*, notwithstanding the fact that the former shows the DUI phenomenon, while the latter does not (Iannello et al., 2019).

Therefore, we conclude from these pieces of evidence that the onset of DUI entails a remodulation of evolutionary-fixed levels of mitochondrial transcription aiming to optimize the functionality of both genomes. However, Iannello and collegues (2019) did not find any clues of nuclear molecular compensation, which entails that dN/dS values observed in nuclear OXPHOS genes does not seem to increase following the mitochondrial evolution (be it connected or not with DUI). Although the overall evolutionary rate is quite high when compared to other animal taxa, and although mitochondrial genes are much more expressed than nuclear counterparts (from 12× to 100×), dN/dS ratios are comparable (Iannello et al., 2019). Eventually, the different regulation of F and M genomes is consistent with the CORR hypothesis (Allen, 2003): in a nutshell, the idea that states that the expression of the handful of genes retained on the organellar genome may be directly regulated by the redox state of the respective products/complexes.

Similarly, selective pressure is somewhat different on the two lineages. It has been repeatedly stated that the M lineage of DUI species evolves faster than the F counterpart (Hoeh, Stewart, Sutherland, & Zouros, 1996a; Mizi, Zouros, Moschonas, & Rodakis, 2005; Breton, Burger, Stewart, & Blier, 2006; Zbawicka, Burzynski, Skibinski, & Wenne, 2010; Doucet-Beaupré et al., 2010; Zouros, 2013; Zouros & Rodakis, 2019; but see Passamonti, 2007; Plazzi, Puccio, & Passamonti, 2016). However, in the first characterization of mitochondrial transcriptome and polymorphism of a DUI species with high-throughput data, Ghiselli and colleagues (2013) found similar amount of polymorphism, but different kinds of polymorphism, in *R. philippinarum*. More in detail, the F lineage shows a significantly higher amount of rare mutations, while the M lineage shows much more intermediate-frequency alleles: interestingly, the overall number of SNPs is higher in the female genomes, as well as is the number of "high-effect" (nonsynonymous) SNPs, being they polyallelic or monoallelic. Therefore, the higher rates of molecular evolution of the M lineages (if confirmed) are not connected to a higher polymorphism in male germ line mitochondria (Ghiselli et al., 2013).

A buffering effect may allow higher degrees of (possibly deleterious) mutations in the F lineage, recall that a single egg carries hundreds of mtDNAs. Conversely, *R. philippinarum* spermatozoa contain only four mitochondria (Milani et al., 2011), which translates into a

handful of mtDNAs (Ghiselli et al., 2011), which increases the power and the effectiveness of natural selection on deleterious SNPs.

Yet, mitochondria are not run by the just 13 protein which are typically encoded on animal mitochondrial genomes. Wallace (2005) reports that approximately 1,500 genes that were originally encoded by the mitochondrial genome are currently scattered throughout the nuclear counterpart, and this is roughly the estimated size of the mitochondrial proteome, at least in humans (Rabilloud et al., 1998; Lopez et al., 2000). The products of these genes interact with the products of mitochondrial genes within respiratory complexes: what is the nature of this interactions when DUI splits the sex-linked mitochondrial genomes the nuclear genome has to interact with?

The *de novo* assembly of the complete transcriptome of *R. philippinarum* spotted out about 1,500 genes with a differential expression in male and female individuals (Ghiselli et al., 2012), which are therefore expected to be connected to sex determination and, in DUI species, with DUI itself. In the palaeoheterodont DUI species *Hyriopsis schlegelii*, *Utterbackia peninsularis* and *Venustaconcha ellipsiformis* the number of sex-biased genes is one order of magnitude higher, ranging from 7,281 to 52,257 (depending on the detection method; Shi, Hong, Sheng, Peng, & Wang, 2015; Capt et al., 2018). Specifically, the number of sex-biased genes in *V. ellipsiformis* was estimated between 11,408 and 52,257 with two different approaches; recall that a draft genome of this species recovered roughly 43,000 annotated open reading frames (Renaut et al., 2018), an important fraction of the protein coding genome seems to be involved in sex-linked differential transcription in these species. Conversely, the characterization of sex-biased transcripts in a (as far as we know) non-DUI species, *Pinctada margaritifera*, yielded far less genes, since only 1,993 genes were detected (Teaniniuraitemoana et al., 2014), a value comparable to that obtained for *R. philippinarum*. Generally speaking, such high numbers of genes connected with sex determination are somewhat unexpected, given that these animals do show very limited sexual dimorphism (but see Capt et al., 2018; and reference therein for the peculiar case of unionoid freshwater mussels).

Within sex-biased genes, sex-specific genes were also detected, as well as sex-specific SNPs. Six male-specific and three female-specific genes were detected among *U. peninsularis* and *V. ellipsiformis* ortholog, sex-biased genes (Capt et al. 2018); the comparison of the gonochoric, DUI species *U. peninsularis* with the hermaphroditic *U. imbecillis* resulted in 567 genes specific to the hermaphroditic individuals, 59 genes specific to male individuals, and 100 genes specific to female individuals (Capt, Renaut, Stewart, Johnson, & Breton, 2019). In

R. philippinarum, 166 sex-specific SNPs were detected, mostly connected with sperm motility and ubiquitination (Ghiselli et al., 2011).

Most sex-biased genes are male-biased in *R. philippinarum* (Ghiselli et al., 2012) and *U. peninsularis* (Capt et al., 2018), as has already been reported for other species (Meiklejohn, Parsch, Ranz, & Hartl, 2003; Ranz, Castillo-Davis, Meiklejohn, & Hartl, 2003; Ellegren & Parsch 2007). Most probably, this pattern is due to the fact that female-biased genes are very commonly connected to essential functions in the context of gonad development, gametogenesis, fertilization, and alike, and are therefore present and expressed in both sexes (Zhang, Hambuch, & Parsch, 2004; Proschel, Zhang, & Parsch, 2006; Clark, Findlay, Yi, Maccoss, & Swanson, 2007; Ellegren & Parsch 2007; Larracuente et al., 2008; Ghiselli et al., 2012; Capt et al., 2018); nonetheless, 25,911 female-biased genes were found in *H. schlegelii* against 19,511 male-biased genes (Shi et al., 2015).

Ghiselli et al. (2012) found sex-biased genes to be more variable compared to unbiased genes in *R. philipinarum*, and it seemed that male-biased genes evolve faster than female-biased ones, which leads to some difficulties in annotation. However, genes showing a conserved bias across *R. philippinarum* (a DUI species) and *R. decussatus* (a non-DUI species) show higher dN/dS values when conserving a bias towards females in both species (and are actually much more; Ghiselli et al., 2018).

In fact, female- and male-biased genes appear to be somewhat conserved across different species, and even across different mitochondrial inheritance systems (Capt et al., 2018; Ghiselli et al., 2018; Capt et al., 2019). However, Ghiselli and colleagues (2018) demonstrated that often sex biases are not maintained across the strictly related species *R. decussatus* and *R. philippinarum*, i.e. the ortholog of a male-biased gene in one species may be female-biased in the other one or vice versa: among 3,102 ortholog genes, 1,284 were found to be sex-biased, but the sex bias was maintained only in 430 cases, and in 17 it was reversed. Albeit not always in top-enriched GO terms for sex-biased genes (Capt et al., 2018), some functions are repeatedly associated to these orthologs. Some of these functions are expected for genes connected to sex determination, like embryo/gonad development, gametogenesis, fertilization (Ghiselli et al., 2012, 2018); other functions deserve a more careful investigation.

Indeed, many studies focused on the ubiquitination pathway: ubiquitin (Ub) is a universal protein connected to protein quality control and turnover in cells, and ubiquitination is directly involved in gametogenesis, with special reference to male gametogenesis (f.i., Richburg, Myers, & Bratton, 2014; Suresh, Lee, Kim, & Ramakrishna, 2016). In mammals,

Ub provide sperm mitochondria signals through the di-ubiquitination of a protein exposed on the mitochondrial membrane, the prohibitin (Sutovsky et al., 2000). Ghiselli et al. (2012) demonstrated that, as previously found in the *Mytilus* complex (Saavedra, Reyero, & Zouros 1997; Kenchington, MacDonald, Cao, Tsagkarakis, & Zouros, 2002; Cogswell et al., 2006; Kenchington et al., 2009), families with different sex-biases are present in *R. philippinarum*, leading to male-dominated or female-dominated offspring. Milani, Ghiselli, Nuzhdin, & Passamonti (2013) focused on three genes which are males-biased, or more expressed in male-biased families: *psa*, *birc*, and *anubl1*. PSA is a subunit of the proteasome, which is known to be involved in male sexual differentiation (Shimada, Kanematsu, Tanaka, Yokosawa, & Kawahara, 2006) and which was found to be highly differentially expressed also in *Mytilus edulis* male-biased eggs (Diz et al., 2009). BIRC contains a domain termed RING, which has been linked to degradation through the ubiquitin-proteasome system (Milani et al., 2013b; Joazeiro & Weissman, 2000). ANUBL1 shows a ubiquitin-like domain at the N-terminus and may tag in some way sperm mitochondria (Milani et al., 2013b).

Orthologs of the very same genes were found among sex-biased genes in unionid DUI species (often with the same, male-oriented, bias), along with DNMT1, a DNA-methyltransferase associated to mitochondria (Capt et al., 2018). These genes are also upregulated in the hermaphroditic *U. imbecillis* and in male individuals of *U. peninsularis* with respect to female individuals of the latter species, along with *fbx039*, also a gene connected with ubiquitination (Capt et al., 2019). Furthermore, ~380 ortholog transcripts connected to ubiquitinating enzymes were detected in the *R. decussatus* and *R. philippinarum* transcriptomes, be they Ub-activating enzymes, Ub-conjugating enzymes, Ub-ligases, deubiquitinating enzymes, or proteasome subunits (Punzi, Milani, Ghiselli, & Passamonti, 2018). Currently, it is still unclear the actual mechanism which is involved in sperm mitochondria tagging for degradation or aggregation in the first cleavage furrow, at the midbody level (Milani et al., 2011). Ub-connected genes present in both *R. decussatus* and *R. philippinarum* may lead to the idea that an evolutionary conserved signal on the outer membrane (ubiquitinated prohibitins?) is masked by a DUI-specific factor in male zygotes; alternatively, the presence of a transmembrane Ub-ligase with a strong male bias which is present in *R. decussatus*, but not in *R. philippinarum*, may lead to the idea that in the latter, DUI species, the labeling signal itself is modified upstream (Punzi et al., 2018).

The ORFans and their supposed role on DUI appearance

Mitochondrial ORFans are ORFs with no evident homology to the common animal 13 mitochondrial protein coding genes (Fischer & Eisenberg, 1999; Breton et al., 2014). ORFans were often described in bivalve mitochondrial genomes (Breton et al., 2014; Plazzi et al., 2016); albeit present in species where evidence of DUI is lacking as well, like the basal protobranch *Solemya velum* (Plazzi, Ribani, & Passamonti, 2013), they have been hypothesized to be connected with DUI (Breton et al., 2009, 2011; Milani, Ghiselli, Guerra, Breton, & Passamonti, 2013). However, it is challenging to state a sequence or structure similarity between ORFans of even related bivalve species (Milani et al., 2013a; Mitchell, Guerra, Stewart, & Breton, 2016; Plazzi et al., 2016) – and these supernumerary genes are possibly not homologous at all (Plazzi et al., 2016).

As said, mitochondrial ORFans are often found in bivalve mitochondrial genomes and they have been claimed to be involved in DUI establishment/maintaining: ORFans, with few exception, are lineage-specific and have been shown to be translated in functional protein, that are active in different cellular compartment, and can thus act as DUI regulators (Breton et al., 2009, 2011; Milani et al., 2013a). Moreover, many clues were identified which would relate lineage specific ORFans of DUI species to selfish viral elements (Milani et al., 2013a; Milani, Ghiselli, & Passamonti, 2016). What would have happened if a viral element had infected some mitochondria of a hermaphroditic population conferring the ability to avoid degradation in embryos if carried through sperm, and distorting segregation towards a preferential transmission through generations? Actually, it has been argued that such an event may well have triggered a shift to gonochorism and DUI in a given taxon (Milani et al., 2016) as a way to resolve the conflict between nuclear and mitochondrial genome(s), a theme which was originally put forward by Passamonti and Ghiselli (2009).

Chasing DUI in bivalves' phylogeny

The divergence between the two mitochondrial M and F lineages can be very high under DUI. The average amino acid Kimura distance among protein coding genes was reported to be 83.30 among different species of the superfamily Unionoidea, yet in some DUI species of this superfamily the F-M divergence is higher, up to 86.40 in *Utterbackia peninsularis*. Moreover, the average nucleotide Jin-Nei Gamma distance computed on all coding regions of the genome was reported to be 100.64 within Unionoidea, but it again is higher for some of the internal DUI comparisons, up to 113.61 between *Quadrula quadrula* F and M lineage (Bettinazzi, Plazzi, & Passamonti, 2016). Similarly, the F-M divergence reaches 47.04 in *R. philippinarum* amino acids (25.80 being the average value for venerid/mytilids) and 86.82 in

Mytilus californianus coding nucleotides (66.97 being the average; Bettinazzi et al., 2016). Male and female lineages exhibit a K2P distance of 0.41 for *cytb* and 0.210 for *rrnL* in the nuculanid *Ledella ultima*, which means a degree of divergence up to the 27% (Boyle & Etter, 2013); a similar value was observed for *Solen marginatus* (16% for *cox1*, 21% for *rrnL*; Lucentini et al., submitted); an even higher value was recorded for *Donax trunculus* (28% for *rrnL*, 36.5% for *cytb*; Theologidis et al., 2008).

To date, DUI seems to be scattered throughout the bivalve evolutionary tree, and the patterns of its distribution are unclear (Theologidis et al., 2008; Plazzi, 2015; Gusman et al., 2016; Plazzi & Passamonti, 2019; Lucentini et al., submitted).

Following Theologidis and colleagues (2008), it is possible to identify three phylogenetic patterns of DUI-related sequences: a gender-joining pattern, a taxon-joining pattern, and a mixed pattern. In a gender-joining pattern, F sequences of a given group of DUI species are recovered as monophyletic; the same holds for the corresponding M sequences, and the two clades are sister taxa. In a taxon-joining pattern, each F sequence is the sister taxon to the M counterpart, and species are therefore monophyletic. In mixed patterns, F and M sequences are interwoven.

The most remarkable example of gender-joining pattern is the superfamily Unionoidea: F mitochondrial genomes of the different families comprising Unionoidea are consistently retrieved as monophyletic (including non-DUI species), and M mitochondrial genomes are monophyletic as well. The two clades are sister clades, and within each clade the same topology is mirrored (e.g., Doucet-Beaupré et al., 2010; Gusman et al., 2016).

A typical taxon-joining pattern is found within Heterodonta. To date, DUI species belonging to this subclass are quite unrelated: *Cyclina sinensis*, *M. lamarckii*, *Pseudocardium sachalinense*, *Scrobicularia plana*, *S. marginatus*, and *R. philippinarum* are monophyletic, resulting from the respective (F + M) cluster. The phylogenetic relationships among species are basically the same that are obtained when DUI is not taken into account and only one (typically female) sequence is included in the analysis (e.g., Plazzi, 2015; Gusman et al., 2016; Plazzi & Passamonti, 2019).

While most mytilids are associated to taxon-joining patterns (Vargas et al., 2015; Gusman et al., 2016), a mixed pattern is normally shown within the genus *Mytilus*: the M lineage of *M. californianus* is typically recovered as the sister taxon to a clade comprised by the corresponding F lineage and the DUI species *M. edulis*, *M. galloprovincialis*, and *M. trossulus*. (Zouros, 2013; Plazzi & Passamonti, 2019; but see Vargas et al., 2015).

Since DUI ought to entail a complex molecular machinery, a possibility is that DUI originated once and was subsequently lost in many bivalve lineages (Zouros, 2013). Given its presence in *L. ultima*, the origin of DUI should be dated back to at least the early Cambrian (Boyle & Etter, 2013). However, most of the aforementioned phylogenetic relationships are consistently retrieved by most, if not all, studies dealing with DUI distribution among bivalves: in fact, the more DUI species added to the phylogenetic tree, the more evidence are growing towards a polyphyletic origin of DUI. Remarkably, the idea of several DUI origins within bivalves is consistent with other lines of evidence but the polyphyly with respect to a phylogenetic tree. Interestingly, selective footprints on mitochondrial genes have been clearly detected for branches leading to a DUI assemblage in a phylogenetic tree, but only a handful of molecular synapomorphies were found (Plazzi & Passamonti, 2019), lending further support to a multiple origin of DUI. It is tempting to connect this hypothesis to the previously reported proposal of a viral origin for supernumerary ORFs in DUI mtDNAs (Milani et al., 2016), if their involvement in DUI regulation and maintenance is confirmed. The idea of a selfish viral element acting on the same background mechanism of mitochondrial inheritance and finally resulting in DUI would explain the evolutionary parallelism which hides behind a polyphyletic distribution of DUI very well.

The smithRNAs and their role

Very recently, in *R. philippinarum*, we were able to identify several putative sncRNAs that are transcribed from mtDNA at a very high level, yet they are predicted to act on nuclear targets (Pozzi, Plazzi, Milani, Ghiselli, & Passamonti, 2017). Therefore, we proposed a new category of interfering RNAs, named small mitochondrial highly transcribed RNAs (smithRNAs). The invention of sncRNAs of mitochondrial origin is not completely new (Ro et al., 2013; Wu, Stone, Štorchová, & Sloan, 2015; Mercer et al. 2011; Larriba, Rial, & del Mazo. 2018; Riggs et al. 2018), but smithRNAs provide an unprecedented way for mtDNA to affect nuclear gene expression: for example, we found clues that mt-derived small non-coding RNAs might be involved in gonad formation (Pozzi et al., 2017). The possibility that mtDNA may act on nuclear gene regulation has never been suggested before, and the relevance of this mechanism for eukaryote gene regulation has to be assessed. Indeed, the smithRNAs may be a new unprecedented form of retrograde signaling (i.e., the mitochondria-to-nucleus signaling; Arnould, Michel, & Renard, 2015; Cagina & Enriqueza, 2015; Monaghan & Whitmarsh, 2015).

The role of smithRNAs in DUI has not been assessed yet. However, it is intriguing to speculate that the supposed capacity of DUI sex-linked mitochondrial DNAs to drive gamete formation, as originally suggested by Passamonti and Ghiselli (2009), could be in fact in charge of these mitochondrial determinants. In this conception, while the ORF protein of viral origin may have driven DUI appearance and evolution, acting as a selfish meiotic drive distorter, the sex-linked mitochondria have soon evolved (or maybe reinforced) the capacity of driving gamete formation trough RNA interference. This is not surprising because mtDNA could be somehow prone to quickly evolve new functions through small interfering RNA, because of hairpin structures in intergenic regions (needed for correct RNA cleavage), as well as tRNAs, could be easily exapted to interfering RNAs. Moreover, mitochondria have been implicated in the evolution of sex under several hypotheses, so it is at least conceivable that mtDNA may act as a sex-determining factor or be involved in sexual differentiation. Finally, similar capabilities of affecting sex determination are known to be widespread among the closest relatives of mitochondria, the α-proteobacteria (e.g., *Wolbachia*; Terry, Dunn, & Smith, 1997; Werren & O'Neill, 1997; Stouthamer, Breeuwer, & Hurst, 1999; Cheng et al., 2000; Weeks, Marec, & Breeuwer, 2001), although it is still unclear how this is achieved.

A final overview

Mitochondria are a fundamental component of eukaryotic life. Nevertheless, the current knowledge about their biology and function is largely incomplete, and mostly biased toward a few model species. In fact, focusing the research on a small and uneven subset of organisms entails the risk of losing a big part of the molecular and functional diversity of mitochondria. Mitochondrial genome is very compact, with few and short intergenic regions (except the Control Region), so that one may consider that there is little space for other functions, other than the usual content of 37 genes (Boore, 1999; Gissi, Iannelli, & Pesole, 2008; Breton et al. 2014). This, however, seems not to be the case, as evidenced above (see also Breton et al., 2014; Plazzi et al., 2016).

R. philippinarum has proven to be a successful model species for studies on mitochondrial biology and Doubly Uniparental Inheritance (DUI). Thanks to its unusual features, DUI can shed light on mitochondrial inheritance and biogenesis, and, above all, on the relationship between mitochondria and germ line. Moreover, DUI is a unique experimental system for studying mitochondrial heteroplasmy, and two processes that shape genome evolution: genomic conflicts and mito-nuclear coevolution, which are at the very root of eukaryotic life.

503 Acknowledgements

504 We wish to thank Elisabeth Haring for having conceived and proposed this special issue on
505 Doubly Uniparental Inheritance. Moreover, we wish to thank all colleagues and scholars that,
506 at various levels, have contributed during the last 20 years to the exciting scientific challenge
507 of making DUI (and especially *R. philippinarum*) a profitable model for mitochondrial
508 studies.

509

References

Allen, J. F. (2003). The function of genomes in bioenergetic organelles. *Philosophical Transactions of the Royal Society B-Biological Sciences*. 358, 19–37.

Arnould, T., Michel, S., & Renard, P. (2015). Mitochondria Retrograde Signaling and the UPRmt: Where Are We in Mammals? *International Journal of Molecular Sciences*, 16, 18224–18251.

Bettinazzi, S., Plazzi, F., & Passamonti, M. (2016). The Complete Female- and Male-Transmitted Mitochondrial Genome of *Meretrix lamarckii*. *PLoS ONE*, 11, e0153631.

Boore, J. L. (1999). Animal mitochondrial genomes. *Nucleic Acids Research*, 27:1767–1780.

Boyle, E. E., & Etter, R. J. (2013). Heteroplasmy in a deep-sea protobranch bivalve suggests an ancient origin of doubly uniparental inheritance of mitochondria in Bivalvia. *Marine Biology*, 160, 413–422.

Brannock, P. M., Roberts, M. A., & Hilbish, T. J. (2013). Ubiquitous heteroplasmy in *Mytilus* spp. resulting from disruption in doubly uniparental inheritance regulation. *Marine Ecology Progress Series*, 480, 131–143.

Breton, S., Burger, G., Stewart, D. T., & Blier, P. U. (2006). Comparative analysis of gender-associated complete mitochondrial genomes in marine mussels (*Mytilus* spp.). *Genetics*, 172, 1107–1119.

Breton, S., Doucet-Beaupré, H., Stewart, D. T., Hoeh, W. R., & Blier, P. U. (2007). The unusual system of doubly uniparental inheritance of mtDNA: isn't one enough? *Trends in Genetics*, 23, 465–474.

Breton, S., Doucet-Beaupré, H., Stewart, D. T., Piontkivska, H., Karmakar, M., Bogan, A. E., Blier, P. U., & Hoeh, W. R. (2009). Comparative mitochondrial genomics of freshwater mussels (Bivalvia: Unionoida) with doubly uniparental inheritance of mtDNA: Gender-specific open reading frames and putative origins of replication. *Genetics*, 183, 1575–1589.

Breton, S., Milani, L., Ghiselli, F., Guerra, D., Stewart, D. T., & Passamonti, M. (2014). A resourceful genome: updating the functional repertoire and evolutionary role of animal mitochondrial DNAs. *Trends in Genetics*, 30, 555–564.

Breton, S., Stewart, D. T., Shepardson, S., Trdan, R. J., Bogan, A. E., Chapman, E. G., Ruminas, A. J., Piontkivska, H., & Hoeh, W. R. (2011). Novel protein genes in animal mtDNA: A new sex determination system in freshwater mussels (Bivalvia: Unionoida)? *Molecular Biology and Evolution*, 28, 1645–1659.

Cagina, U., & Enriqueza, J. A. (2015). The complex crosstalk between mitochondria and the nucleus: What goes in between? *International Journal of Biochemistry & Cell Biology*, 63, 10–15.

Cao, L., Kenchington, E. L. R., & Zouros, E. (2004). Differential Segregation Patterns of Sperm Mitochondria in Embryos of the Blue Mussel (*Mytilus edulis*). *Genetics*, 166, 883–894.

Capt, C., Renaut, S., Ghiselli, F., Milani, L., Johnson, N. A., Sietman, B. E., Stewart, D. T., & Breton, S. (2018). Deciphering the Link between Doubly Uniparental Inheritance of mtDNA and Sex Determination in Bivalves: Clues from Comparative Transcriptomics *Genome Biology and Evolution*, 10, 577–590.

Capt, C., Renaut, S., Stewart, D. T., Johnson, N. A., & Breton, S. (2019). Putative Mitochondrial Sex Determination in the Bivalvia: Insights From a Hybrid Transcriptome Assembly in Freshwater Mussels. *Frontiers in Genetics*, 10, 840.

Chakrabarti, R., Walker, J. M., Chapman, E. G., Shepardson, S. P., Trdan, R. J., Curole, J. P., Watters, G. T., Stewart, D. T., Vijayaraghavan, S., & Hoeh, W. R. (2007). Reproductive function for a C-terminus extended, male-transmitted cytochrome c-oxidase subunit II protein expressed in both spermatozoa and eggs. *FEBS Letters*, 581, 5213–5219.

Chakrabarti, R., Walker, J. M., Stewart, D. T., Trdan, R. J., Vijayaraghavan, S., Curole, J. P., & Hoeh, W. R. (2006). Presence of a unique male-specific extension of C-terminus to the cytochrome c oxidase subunit II protein coded by the male-transmitted mitochondrial genome of *Venustaconcha ellipsiformis* (Bivalvia: Unionoidea). *FEBS Letters*, 580, 862–866.

Cheng, Q., Ruel, T. D., Zhou, W., Moloo, S. K., Majiwa, P., O'Neill, S. L. & Aksoy, S. (2000). Tissue distribution and prevalence of *Wolbachia* infections in tsetse flies, *Glossina* spp. *Medical and Veterinary Entomology*, 14, 44–50.

Clark, N. L., Findlay, G. D., Yi, X., Maccoss, M. J., & Swanson, W. J. (2007). Duplication and selection on abalone sperm lysin in an allopatric population. *Molecular Biology and Evolution*, 24, 2081–2090.

Cogswell, A. T., Kenchington, E. L. R., & Zouros, E. (2006). Segregation of sperm mitochondria in two- and four-cell embryos of the blue mussel *Mytilus edulis*: implications for the mechanism of doubly uniparental inheritance of mitochondrial DNA. *Genome*, 49, 799–807.

Curole, J. P., & Kocher, T. D. (2002). Ancient sex-specific extension of the cytochrome c oxidase II gene in bivalves and the fidelity of doubly-uniparental inheritance. *Molecular Biology and Evolution*, 19, 1323–1328.

577 Curole, J. P., & Kocher, T. D. (2005). Evolution of a unique mitotype-specific protein-
578 coding extension of the cytochrome c oxidase II gene in freshwater mussels (Bivalvia:
579 Unionoida). *Journal of Molecular Evolution*, 61, 381–389.
580 Dalziel, A. C., & Stewart, D. T. (2002). Tissue-specific expression of male-transmitted
581 mitochondrial DNA and its implications for rates of molecular evolution in *Mytilus* mussels
582 (Bivalvia: Mytilidae). *Genome*, 45, 348–355.
583 Dégletagne, C., Abele, D., & Held, C. (2016). A distinct mitochondrial genome with DUI-
584 like inheritance in the ocean quahog *Arctica islandica*. *Molecular Biology and Evolution*, 33,
585 375–383.
586 Diz, A. P., Dudley, E., MacDonald, B. W., Piña, B., Kenchington, E. L. R., Zouros,, E., &
587 Skibinski, D. O. F. (2009). Genetic variation underlying protein expression in eggs of the
588 marine mussel *Mytilus edulis*. *Molecular & Cellular Proteomics*, 8, 132–144.
589 Doucet-Beaupré, H., Breton, S., Chapman, E. G., Blier, P. U., Bogan, A. E., Stewart, D. T.,
590 & Hoeh, W. R. (2010). Mitochondrial phylogenomics of the Bivalvia (Mollusca): searching
591 for the origin and mitogenomic correlates of doubly uniparental inheritance of mtDNA. *BMC*
592 *Evolutionary Biology*, 10, 50.
593 Ellegren, H., & Parsch, J. (2007). The evolution of sex-biased genes and sex-biased gene
594 expression. *Nature Reviews Genetics*, 8, 689–698.
595 Everett, E. M., Williams, P. J., Gibson, G., & Stewart, D. T. (2004). Mitochondrial DNA
596 polymorphisms and sperm motility in *Mytilus edulis* (Bivalvia: Mytilidae). *Journal of*
597 *Experimental Zoology Part A-Comparative Experimental Biology*, 301, 906–910.
598 Fischer, D., & Eisenberg, D. (1999). Finding families for genomic ORFans.
599 *Bioinformatics*, 15, 759–762.
600 Garrido-Ramos, M. A., Stewart, D. T., Sutherland, B. W., & Zouros, E. (1998). The
601 distribution of male-transmitted and female-transmitted mitochondrial DNA types in somatic
602 tissues of blue mussels: Implications for the operation of doubly uniparental inheritance of
603 mitochondrial DNA. *Genome*, 41, 818–824.
604 Ghiselli, F., Iannello, M., Puccio, G., Chang, P. L., Plazzi, F., Nuzhdin, S. V., &
605 Passamonti, M. (2018). Comparative Transcriptomics in Two Bivalve Species Offers
606 Different Perspectives on the Evolution of Sex-Biased Genes. *Genome Biology and*
607 *Evolution*, 10, 1389–1402.
608 Ghiselli, F., Maurizii, M. G., Reunov, A., Ariño-Bassols, H., Cifaldi, C., Pecci, A.,
609 Alexandrova, Y., Bettini, S., Passamonti, M., Franceschini, V., & Milani, L. (2019). Natural

610 Heteroplasmy and Mitochondrial Inheritance in Bivalve Molluscs. *Integrative and*
611 *Comparative Biology*, 59, 1016–1032.
612 Ghiselli, F., Milani, L., & Passamonti, M. (2011). Strict sex-specific mtDNA segregation
613 in the germline of the DUI species *Venerupis philippinarum* (Bivalvia Veneridae). *molecular*
614 *biology and evolution*, 28, 949–961.
615 Ghiselli, F., Milani, L., Chang, P. L., Hedgecock, D., Davis, J. P., Nuzhdin, S. V., &
616 Passamonti, M. (2012). De Novo Assembly of the Manila Clam *Ruditapes philippinarum*
617 Transcriptome Provides New Insights into Expression Bias, Mitochondrial Doubly
618 Uniparental Inheritance and Sex Determination *Molecular Biology and Evolution*, 29, 771–
619 786.
620 Ghiselli, F., Milani, L., Guerra, D., Chang, P. L., Breton, S., Nuzhdin, S. V., &
621 Passamonti, M. (2013). Structure, transcription, and variability of metazoan mitochondrial
622 genome: Perspectives from an unusual mitochondrial inheritance system. *Genome Biology*
623 *and Evolution*, 5, 1535–1554.
624 Ghiselli, F., Milani. L., & Passamonti, M. (2011). Strict sex-specific mtDNA segregation
625 in the germ line of the DUI species *Venerupis philippinarum* (Bivalvia: Veneridae).
626 *Molecular Biology and Evolution*, 28, 949–961.
627 Gissi, C., Iannelli, F., & Pesole, G. (2008). Evolution of the mitochondrial genome of
628 Metazoa as exemplified by comparison of congeneric species. *Heredity*, 101, 301–320.
629 Guerra, D., Ghiselli, F., Milani, L., Breton, S., & Passamonti, M. (2016). Early replication
630 dynamics of sex-linked mitochondrial DNAs in the doubly uniparental inheritance species
631 *Ruditapes philippinarum* (Bivalvia Veneridae). *Heredity*, 116, 324–332.
632 Guerra, D., Plazzi, F., Stewart, D. T., Bogan, A. E., Hoeh, W. R., & Breton, S. (2017).
633 Evolution of sex-dependent mtDNA transmission in freshwater mussels (Bivalvia: Unionida).
634 *Scientific Reports*, 7, 1551.
635 Gusman, A., Azuelos, C., & Breton, S. (2017). No evidence of sex-linked heteroplasmy or
636 doubly-uniparental inheritance of mtDNA in five gastropod species. *Journal of Molluscan*
637 *Studies*, 83, 119–122.
638 Gusman, A., Lecomte, S., Stewart, D. T., Passamonti, M., & Breton, S. (2016). Pursuing
639 the quest for better understanding the taxonomic distribution of the system of doubly
640 uniparental inheritance of mtDNA. *PeerJ*, 4, e2760.
641 Gustafson, E. A., & Wessel, G. M. (2010). Vasa genes: Emerging roles in the germ line
642 and in multipotent cells. *Bioessays*, 32, 626–637.

643 Hay, B., Ackerman, L., Barbel, S., Jan, L. Y., & Jan, Y. N. (1988). Identification of a
644 component of *Drosophila* polar granules. *Development*, 103, 625–640.
645 Hay, B., Jan, L. Y, & Jan, Y. N. (1988). A protein component of *Drosophila* polar granules
646 is encoded by *vasa* and has extensive sequence similarity to ATP-dependent helicases. *Cell*,
647 55, 577–587.
648 Hoeh, W. R., Stewart, D. T., Sutherland, B. W., & Zouros, E. (1996a). Cytochrome *c*
649 oxidase sequence comparisons suggest an unusually high rate of mitochondrial DNA
650 evolution in *Mytilus* (Mollusca: Bivalvia). *Molecular Biology and Evolution*, 13, 418–421.
651 Hoeh, W. R., Stewart, D. T., Sutherland, B. W., & Zouros, E. (1996b). Multiple origins of
652 gender-associated mitochondrial DNA lineages in bivalves (Mollusca: Bivalvia). *Evolution*,
653 50, 2276–2286.
654 Iannello, M., Puccio, G., Piccinini, G., Passamonti, M. & Ghiselli, F. (2019). The
655 dynamics of mito-nuclear coevolution: A perspective from bivalve species with two different
656 mechanisms of mitochondrial inheritance. *Journal of Zoological Systematics and*
657 *Evolutionary Research*, 57, 534–547.
658 Joazeiro, C. A., & Weissman, A. M. (2000). RING finger proteins: mediators of ubiquitin
659 ligase activity. *Cell*, 102, 549–552.
660 Kenchington, E. L. R., Hamilton, L., Cogswell, A. T., & Zouros, E. (2009). Paternal
661 mtDNA and maleness are co-inherited but not causally linked in mytilid mussels. *PLoS ONE*,
662 4, e6976.
663 Kenchington, E. L. R., MacDonald, B., Cao, L., Tsagkarakis, D., & Zouros, E. (2002).
664 Genetics of mother-dependent sex ratio in blue mussels (*Mytilus* spp.) and implications for
665 doubly uniparental inheritance of mitochondrial DNA. *Genetics*, 161, 1579–1588.
666 Knaut, H., Pelegri, F., Bohmann, K., Schwarz, H., & Nuesslein-Volhard, C. (2000).
667 Zebrafish *vasa* RNA but not its protein is a component of the germ plasm and segregates
668 asymmetrically before germline specification. *Journal of Cell Biology*, 149, 875–888.
669 Kuznicki, K. A., Smith, P. A., Leung-Chiu, W. M., Estevez, A. O., Scott, H. C., & Bennett,
670 K. L. (2000). Combinatorial RNA interference indicates GLH-4 can compensate for GLH-1;
671 these two P granule components are critical for fertility in *C. elegans*. *Development*, 127,
672 2907–2916.
673 Kyriakou, E., Zouros, E., & Rodakis, G. C. (2010). The atypical presence of the paternal
674 mitochondrial DNA in somatic tissues of male and female individuals of the blue mussel
675 species *Mytilus galloprovincialis*. *BMC Research Notes*, 3, 222.

Ladoukakis, E. D., & Zouros, E. (2001a). Direct evidence for homologous recombination in mussel (*Mytilus galloprovincialis*) mitochondrial DNA. *Molecular Biology and Evolution*, 18, 1168–1175.

Ladoukakis, E. D., & Zouros, E. (2001b). Recombination in animal mitochondrial DNA: evidence from published sequences. *Molecular Biology and Evolution*, 18, 2127–2131.

Larracuente, A. M., Sackton, T. B., Greenberg, A. J., Wong, A., Singh, N. D., Sturgill, D., Zhang, Y., Oliver, B., & Clark, A. G. (2008). Evolution of protein coding genes in *Drosophila*. *Trends in Genetics*, 24, 114–123.

Larriba, E., Rial, E., & del Mazo, J. (2018). The landscape of mitochondrial small non-coding RNAs in the PGCs of male mice, spermatogonia, gametes and in zygotes. *BMC Genomics*, 19, 634.

Lasko, F., & Ashburner, M. (1988). The product of the *Drosophila* gene *vasa* is very similar to eukaryotic initiation factor-4A. *Nature*, 335, 611–617.

Liu, H. P., Mitton, J. B., & Wu, S.-K. (1996). Paternal mitochondrial DNA differentiation far exceeds maternal mitochondrial DNA and allozyme differentiation in the freshwater mussel, *Anodonta grandis grandis*. *Evolution*, 50, 952–957.

Lopez, M. F., Kristal, B. S., Chernokalskaya, E., Lazarev, A., Shestopalov, A. I., Bogdanova, A., & Robinson, M. (2000). High-throughput profiling of the mitochondrial proteome using affinity fractionation and automation. *Electrophoresis*, 21:3427–3440.

Lucentini, L., Plazzi, F., Sfriso A. A., Pizzirani, C., Sfriso, A., & Chiesa, S. (submitted). Additional taxonomic coverage of the DUI (Doubly Uniparental Inheritance) in bivalves: evidence of sex-linked heteroplasmy in the razor clam *Solen marginatus* Pulteney, 1799. *Journal of Zoological Systematics and Evolutionary Research*.

Lynch, M. (2007). *The origins of genome architecture*. Sunderland, MA: Sinauer Associates.

Meiklejohn, C. D., Parsch, J., Ranz, J. M., & Hartl, D. L. (2003). Rapid evolution of male-biased gene expression in *Drosophila*. *Proceedings of the National Academy of Science of the United States of America*, 100, 9894–9899.

Mercer, T. R., Neph, S., Dinger, M. E., Crawford, J., Smith, M. A., Shearwood, A. M., Haugen, E., Bracken, C. P., Rackham, O., Stamatoyannopoulos, J. A., Filipovska, A., & Mattick, J. S. (2011). The Human Mitochondrial Transcriptome. *Cell*, 146, 645–658.

Milani, L., Ghiselli, F., & Passamonti, M. (2012). Sex-Linked Mitochondrial Behavior During Early Embryo Development in *Ruditapes philippinarum* (Bivalvia Veneridae) a Species With the Doubly Uniparental Inheritance (DUI) Of Mitochondria. *Journal of*

Experimental Zoology Part B-Molecular and Developmental Evolution, 318, 182–189.

Milani, L., Ghiselli, F., & Passamonti, M. (2016). Mitochondrial selfish elements and the evolution of biological novelties. *Current Zoology*, 62, 687–697.

Milani, L., Ghiselli, F., Guerra, D., Breton, S., & Passamonti, M. (2013a). A Comparative Analysis of Mitochondrial ORFans: New Clues on Their Origin and Role in Species with Doubly Uniparental Inheritance of Mitochondria. *Genome Biology and Evolution*, 5, 1408–1434.

Milani, L., Ghiselli, F., Maurizii, M. G., & Passamonti, M. (2011). Doubly uniparental inheritance of mitochondria as a model system for studying germ line formation. *PLoS One*, 6, e28194.

Milani, L., Ghiselli, F., Maurizii, M. G., Nuzhdin, S. V., & Passamonti M. (2014). Paternally transmitted mitochondria express a new gene of potential viral origin. *Genome Biology and Evolution,* 6, 391–405.

Milani, L., Ghiselli, F., Nuzhdin, S. V., & Passamonti, M. (2013b). Nuclear Genes With Sex Bias in *Ruditapes philippinarum* (Bivalvia, Veneridae): Mitochondrial Inheritance and Sex Determination in DUI Species. *Journal of Experimental Zoology Part B-Molecular and Developmental Evolution*, 320, 442–454.

Milani, L., Ghiselli, F., Pecci, A., Maurizii, M. G., & Passamonti, M. (2015). The expression of a novel mitochondrially-encoded gene in gonadic precursors may drive paternal inheritance of mitochondria. *PLoS ONE*, 10, e0137468.

Milani, L., Pecci, A., Ghiselli, F., Passamonti, M., Franceschini, V., & Maurizii M. G. (2017a). Vasa expression suggests shared germ line dynamics in bivalve molluscs. *Histochemistry and Cell Biology*, 148, 157–171.

Milani, L., Pecci, A., Ghiselli, F., Passamonti, M., Lazzari, M., Franceschini, V., & Maurizii, M. G. (2017b). Germ cell line during the seasonal sexual rest of clams: finding niches of cells for gonad renewal. *Histochemistry and Cell Biology*, 149, 105–110.

Millard, V. (2001). *Classification of Mollusca: A classification of world wide Mollusca. 2nd edition*. South Africa.

Mitchell, A., Guerra, D., Stewart, D. T., & Breton, S. (2016). *In silico* analyses of mitochondrial ORFans in freshwater mussels (Bivalvia: Unionoida) provide a framework for future studies of their origin and function. *BMC Genomics*, *17*, 597. https://doi.org/10.1186/s12864-016-2986-6

Mizi, A., Zouros, E., Moschonas, N., & Rodakis, G. C. (2005). The complete maternal and paternal mitochondrial genomes of the Mediterranean mussel *Mytilus galloprovincialis*:

implications for the doubly uniparental inheritance mode of mtDNA. *Molecular Biology and Evolution*, 22, 952–967.

Monaghan, R. M., & Whitmarsh, A. J. (2015). Mitochondrial Proteins Moonlighting in the Nucleus. Trends in Biochemical Sciences, 40, 728–735.

Obata, M., & Komaru, A. (2005). Specific location of sperm mitochondria in mussel *Mytilus galloprovincialis* zygotes stained by MitoTracker. *Development Growth & Differentiation*, 47, 255–263.

Obata, M., Kamiya, C., Kawamura, K., & Komaru, A. (2006). Sperm mitochondrial DNA transmission to both male and female offspring in the blue mussel *Mytilus galloprovincialis*. *Development Growth & Differentiation*, 48, 253–261.

Obata, M., Sano, N., Kawamura, K., & Komaru, A. (2007). Inheritance of two M type mitochondrial DNA from sperm and unfertilized eggs to offspring in *Mytilus galloprovincialis*. *Development Growth & Differentiation*, 49, 335–344.

Obata, M., Shimizu, M., Sano, N., & Komaru, A. (2008). Maternal inheritance of mitochondrial DNA (mtDNA) in the Pacific oyster (*Crassostrea gigas*): a preliminary study using mtDNA sequence analysis with evidence of random distribution of MitoTracker-stained sperm mitochondria in fertilized eggs. *Zoological Science*, 25, 248–254.

Parakatselaki, M. E., Saavedra, C., & Ladoukakis, E. D. (2016). Searching for doubly uniparental inheritance of mtDNA in the apple snail *Pomacea diffusa*. *Mitochondrial DNA Part A*, 27, 4000–4002.

Passamonti, M. (2007). An unusual case of gender-associated mitochondrial DNA heteroplasmy: The mytilid *Musculista senhousia* (Mollusca Bivalvia). *BMC Evolutionary Biology*, 7(Suppl 2), S7.

Passamonti, M., & Ghiselli, F. (2009). Doubly Uniparental Inheritance: two mitochondrial genomes, one precious model for organelle DNA inheritance and evolution. *DNA and Cell Biology*, 28, 79–89.

Passamonti, M., & Scali, V. (2001). Gender-associated mitochondrial DNA heteroplasmy in the venerid clam *Tapes philippinarum* (Mollusca Bivalvia). *Current Genetics*, 39, 117–124.

Passamonti, M., Boore, J. L., & Scali, V. (2003). Molecular evolution and recombination in gender-associated mitochondrial DNAs of the Manila clam *Tapes philippinarum*. *Genetics*, 164, 603–611.

Passamonti, M., Ricci, A., Milani, L., & Ghiselli, F. (2011). Mitochondrial genomes and doubly uniparental inheritance: new insights from *Musculista senhousia* sex-linked mitochondrial DNAs (Bivalvia Mytilidae). *BMC Genomics*, 12, 442.

Plazzi, F. (2015). The detection of sex-linked heteroplasmy in *Pseudocardium sachalinense* (Bivalvia: Mactridae) and its implications for the distribution of doubly uniparental inheritance of mitochondrial DNA. *Journal of Zoological Systematics and Evolutionary Research*, 53, 205–210.

Plazzi, F., & Passamonti, M. (2019). Footprints of unconventional mitochondrial inheritance in bivalve phylogeny: Signatures of positive selection on clades with doubly uniparental inheritance. *Journal of Zoological Systematics and Evolutionary Research*, 57, 258–271.

Plazzi, F., Cassano, A., & Passamonti, M. (2015). The quest for doubly uniparental inheritance in heterodont bivalves and its detection in *Meretrix lamarckii* (Veneridae: meretricinae). *Journal of Zoological Systematics and Evolutionary Research*, 53, 87–94.

Plazzi, F., Puccio, G., & Passamonti, M. (2016). Comparative large-scale mitogenomics evidences clade-specific evolutionary trends in mitochondrial DNAs of Bivalvia. *Genome Biology and Evolution*, 8, 2544–2564.

Plazzi, F., Ribani, A., & Passamonti, M. (2013). The complete mitochondrial genome of Solemya velum (Mollusca: Bivalvia) and its relationships with Conchifera. *BMC Genomics*, 14, 409.

Pozzi, A., Plazzi, F., Milani, L., Ghiselli, F., & Passamonti, M. (2017). SmithRNAs: could mitochondria "bend" nuclear regulation? *Molecular Biology and Evolution*, 34, 1960–1973.

Proschel, M., Zhang, Z., & Parsch, J. (2006). Widespread adaptive evolution of *Drosophila* genes with sex-biased expression. *Genetics*, 174, 893–900.

Punzi, E., Milani, L., Ghiselli, F., & Passamonti, M. (2018). Lose it or keep it: (how bivalves can provide) insights into mitochondrial inheritance mechanisms. *Journal of Experimental Zoology Part B-Molecular and Developmental Evolution*, 330, 41–51.

Rabilloud, T., Kieffer, S., Procaccio, V., Louwagie, M., Courchesne, P. L., Patterson, S. D., Martinez, P., Garin, J., & Lunardi, J. (1998). Two-dimensional electrophoresis of human placental mitochondria and protein identification by mass spectrometry: Toward a human mitochondrial proteome. *Electrophoresis*, 19, 1006–1014.

Ranz, J. M., Castillo-Davis, C. I., Meiklejohn, C. D., & Hartl, D. L. (2003). Sex-dependent gene expression and evolution of the *Drosophila* transcriptome. *Science*, 300, 1742–1745.

Renaut, S., Guerra, D., Hoeh, W. R., Stewart, D. T., BoganA. E., Ghiselli, F., Milani, L., Passamonti, M., & Breton, S. (2018). Genome Survey of the Freshwater Mussel *Venustaconcha ellipsiformis* (Bivalvia: Unionida) Using a Hybrid De Novo Assembly Approach. *Genome Biology and Evolution*, 10, 1637–1646.

812 Richburg, J. H., Myers, J. L., & Bratton, S. B. (2014). The role of E3 ligases in the
813 ubiquitin-dependent regulation of spermatogenesis. *Seminars in Cell Development Biology*,
814 30, 27–35.
815 Riggs, C. L., Summers, A., Warren, D. E., Nilsson, G. E., Lefevre, S., Dowd, W. W.,
816 Milton, S., & Podrabsky, J. E. (2018). Small Non-coding RNA Expression and Vertebrate
817 Anoxia Tolerance. *Frontiers in Genetics*, 9, 230.
818 Ro, S., Ma, H. Y., Park, C., Ortogero, N., Song, R., Hennig, G. W., Zheng, H., Lin, Y. M.,
819 Moro, L., Hsieh, J. T, & Yan, W. (2013). The mitochondrial genome encodes abundant small
820 noncoding RNAs. *Cell Research*, 23, 759–774.
821 Saavedra, C., Reyero, M. I., & Zouros, E. (1997). Male-dependent doubly uniparental
822 inheritance of mitochondrial DNA and female-dependent sex-ratio in the mussel *Mytilus*
823 *galloprovincialis*. *Genetics*, 145, 1073–1082.
824 Sano, N., Obata, M., & Komaru A. (2007). Quantitation of the male and female types of
825 mitochondrial DNA in a blue mussel, *Mytilus galloprovincialis*, using real-time polymerase
826 chain reaction assay. Development Growth & Differentiation, 49, 67–72.
827 Scheffler, I. E. (2008). *Mitochondria*. 2nd ed. Hoboken, NJ: Wiley-Liss.
828 Shi, J., Hong, Y., Sheng, J., Peng, K., & Wang, J. (2015). *De novo* transcriptome
829 sequencing to identify the sex-determination genes in *Hyriopsis schlegelii*. *Bioscience*
830 *Biotechnology and Biochemistry*, 79, 1257–1265.
831 Shimada, M., Kanematsu, K., Tanaka, K., Yokosawa, H., & Kawahara, H. (2006).
832 Proteasomal ubiquitin receptor RPN-10 controls sex determination in *Caenorhabditis*
833 *elegans*. *Molecular Biology of the Cell*, 17, 5356–5371.
834 Skibinski, D. O. F., Gallagher, C., & Beynon, C. M. (1994a). Mitochondrial DNA
835 inheritance. Nature, 368, 817–818.
836 Skibinski, D. O. F., Gallagher, C., & Beynon, C. M. (1994b). Sex-limited mitochondrial
837 DNA transmission in the marine mussel *Mytilus edulis*. *Genetics*, 138, 801–809.
838 Stewart, D. T., Breton, S., Blier, P. U., & Hoeh, W. R. (2009). Masculinization events and
839 doubly uniparental inheritance of mitochondrial DNA: a model for understanding the
840 evolutionary dynamics of gender-asssociated mtDNA in mussels. In P. Pontarotti (Ed.),
841 *Evolutionary biology from concept to application II* (pp. 163–173). Berlin: Springer-Verlag.
842 Stouthamer, R., Breeuwer, J. A. & Hurst, G. D. (1999). *Wolbachia pipientis*: microbial
843 manipulator of arthropod reproduction. *Annual Review of Microbiology*, 53, 71–102.

Suresh, B., Lee, J., Kim, K-S., & Ramakrishna, S. (2016). The importance of ubiquitination and deubiquitination in cellular reprogramming. *Stem Cells International*, 2016, 6705927.

Sutovsky, P., Moreno, R. D., Ramalho-Santos, J., Dominko, T., Simerly, C., & Schatten, G. (2000). Ubiquitinated sperm mitochondria, selective proteolysis, and the regulation of mitochondrial inheritance in mammalian embryos. *Biology of Reproduction*, 63, 582–590.

Teaniniuraitemoana, V., Huvet, A., Levy, P., Klopp, C., Lhuillier, E., Gaertner-Mazouni, N., Gueguen, Y., & Le Moullac, G. (2014). Gonad transcriptome analysis of pearl oyster *Pinctada margaritifera*: identification of potential sex differentiation and sex determining genes. *BMC Genomics*, 15, 491.

Terry, R. S., Dunn, A. M., & Smith, J. E. (1997). Cellular distribution of a feminizing microsporidian parasite: a strategy for transovarial transmission. *Parasitology*, 115, 157–163.

Theologidis, I., Fodelianakis, S., Gaspar, M. B., & Zouros, E. (2008). Doubly uniparental inheritance (DUI) of mitochondrial DNA in *Donax trunculus* (Bivalvia: Donacidae) and the problem of its sporadic detection in Bivalvia. *Evolution*, 62, 959–970.

Thyagarajan, R. A., Padua, R. A., & Campbell, C. (1996). Mammalian Mitochondria Possess Homologous DNA Recombination Activity. *Journal of Biological Chemistry*, 271, 27536–27543.

Vargas, J., Pérez, M., Toro, J., & Astorga, M. P. (2015). Presence of two mitochondrial genomes in the mytilid *Perumytilus purpuratus*: phylogenetic evidence for doubly uniparental inheritance. *Genetics and Molecular Biology*, 38, 173–181.

Venetis, C., Theologidis, I., Zouros, E., & Rodakis, G. C. (2006). No evidence for presence of maternal mitochondrial DNA in the sperm of *Mytilus galloprovincialis* males. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 273, 2483–2489.

Walker, J. M., Curole, J. P., Wade, D. E., Chapman, E. G., Bogan, A. E., Watters, G. T., & Hoeh, W. R. (2006). Taxonomic distribution and phylogenetic utility of gender-associated mitochondrial genomes in the Unionoida (Bivalvia). *Malacologia*, 48, 265–282.

Wallace, D. C. (2005). A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: A dawn for evolutionary medicine. *Annual Review of Genetics*, *39*, 359–407.

Weeks, A. R., Marec, F., & Breeuwer, J. A. J. (2001). A mite species that consists entirely of haploid females. *Science*, 292, 2479–2482.

Werren, J. H., & O'Neill, S. L. (1997). The evolution of heritable symbionts. In S. L. O'Neill, A. Hoffman & J. H. Werren (Eds.), *Influential passengers: inherited microorganisms and arthropod reproduction* (pp. 1–41). Oxford: Oxford University Press.

Williamson, A., & Lehmann, R. (1996). Germ Cell Development in *Drosophila*. *Annual Review of Cell and Developmental Biology*, 12, 365–391.

Wu, Z., Stone, J. D., Štorchová, H., & Sloan, D. B. (2015). High Transcript Abundance, RNA Editing, and Small RNAs in Intergenic Regions within the Massive Mitochondrial Genome of the Angiosperm *Silene noctiflora*. *BMC Genomics*, 16, 938.

Zbawicka, M., Burzynski, A., Skibinski, D., & Wenne, R. (2010). Scottish *Mytilus trossulus* mussels retain ancestral mitochondrial DNA: complete sequences of male and female mtDNA genomes. *Gene*, 456, 45–53.

Zhang, Z., Hambuch, T. M., & Parsch, J. (2004). Molecular evolution of sex-biased genes in *Drosophila*. *Molecular Biology and Evolution*, 21, 2130–2139.

Zouros, E. (2013). Biparental inheritance through uniparental transmission: the doubly uniparental inheritance (DUI) of mitochondrial DNA. *Evolutionary Biology*, 40, 1–31.

Zouros, E., & Rodakis, G. C. (2019). Doubly Uniparental Inheritance of mtDNA: An Unappreciated Defiance of a General Rule. *Advances in anatomy embryology and cell biology*, 231, 25–49.

Zouros, E., Oberhauser Ball, A., Saavedra, C., & Freeman, K. R. (1994a). An unusual type of mitochondrial DNA inheritance in the blue mussel *Mytilus*. *Proceedings of the National Academy of Science of the United States of America*, 91, 7463–7467.

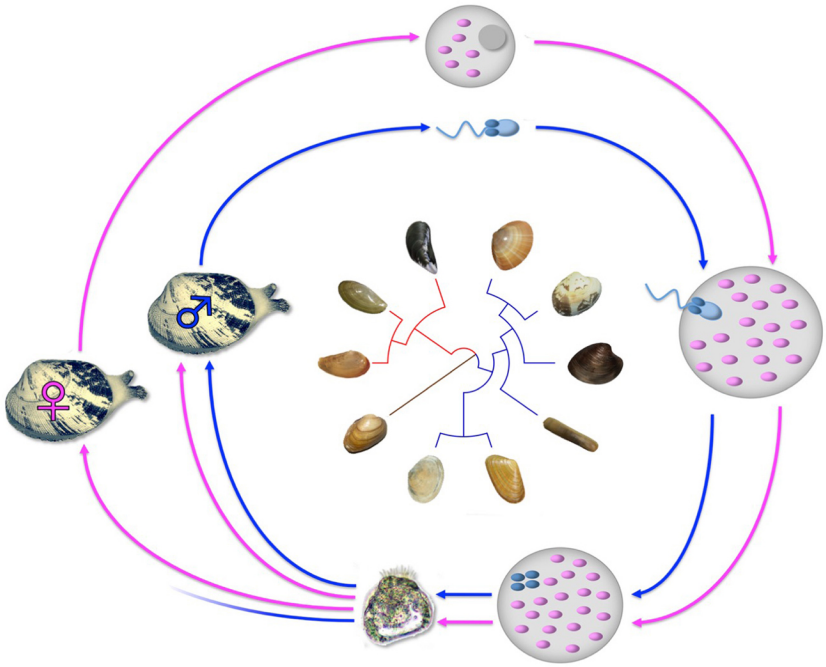
Zouros, E., Oberhauser Ball, A., Saavedra, C., & Freeman, K. R. (1994b). Mitochondrial DNA inheritance. *Nature*, 368, 818.

Figure legends

Fig. 1. Schematic drawing depicting the inheritance patterns of sex-linked mitochondrial lineages (and their DNA) under Doubly Uniparental Inheritance, and its phylogenetic distribution.

Inheritance routes (outer part): pink, F-type mitochondria; blue, M-type mitochondria. Clockwise from above: eggs and sperms are homoplasmic for F- and M-type, respectively; at the zygote stage and early development all individuals are heteroplasmic; at a certain point (how and when is still to be fully clarified) females become homoplasmic for the F-type, hence transmitting the F-type with eggs, while males are, at various levels, heteroplasmic in somatic tissues, and homoplasmic for M-type in gonad, hence transmitting the M-type with spermatozoa.

Phylogenetic distribution of DUI in bivalves (central part): clockwise from above, in blue, Heterodonta (Mactridae, Veneridae, Arcticidae, Solenidae, Donacidae, Semelidae); in brown, Palaeoheterodonta (Unionida); in red, Pteryomorpha (Nuculanidae, Yoldiidae, Mytilidae). The proposed phylogeny is based on mitochondrial genes.



916

917

918 Fig. 1