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1	REVIEW
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4	DUI and beyond: the contribution of the Manila clam Ruditapes philippinarum
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6	Running title: DUI and beyond
7	
8	Marco Passamonti ^{1*} , Federico Plazzi ¹
9	
10	¹ Department of Biological, Geological, and Environmental Sciences – University of
11	Bologna.
12	
13	* Corresponding Author: marco.passamonti@unibo.it
14	
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16	inheritance; mitochondrial biology; genomic conflicts.
17	

18 Abstract

19 The Manila clam, *Ruditapes philippinarum*, is a widespread and commercially important

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

29

30 Introduction

31 Ruditapes philippinarum (Adams & Reeve, 1850), a clam species that is largely harvested 32 in the Adriatic Sea, shows an unusual mtDNA inheritance system, which has been observed, 33 so far, in some bivalves: the Doubly Uniparental Inheritance (DUI). DUI was first detected 34 and sketched out in Mytilus edulis (Skibinski, Gallagher, & Beynon, 1994a,b; Zouros, 35 Oberhauser Ball, Saavedra, & Freeman, 1994a,b). Since 1994, DUI has been found in other 36 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 37 bivalves have two mitochondrial (mt) lineages, one transmitted through eggs (F), the other 38 through sperm (M), whose mitochondrial genomes (mtDNAs) often show higher levels of 39 nucleotide divergence. After amphimixis, the DUI embryo is heteroplasmic for its mtDNA, a 40 41 status eventually maintained only in males (F being localized in somatic tissues, while M 42 being in both sperm and soma). Conversely, in females M is normally degraded (or diluted 43 below detection limits) and homoplasy is restored (Breton, Doucet-Beaupré, Stewart, Hoeh, & Blier, 2007; Passamonti & Ghiselli, 2009; Doucet-Beaupré et al., 2010; Zouros, 2013; 44 45 Zouros & Rodakis, 2019) (Fig. 1). The presence of DUI in a given species is generally detected using sex-linked 46 47 heteroplasmy as a proxy (see, f.i., Passamonti & Scali, 2001; Theologidis, Fodelianakis, Gaspar, & Zouros, 2008; Boyle & Etter, 2013; Plazzi, Cassano, & Passamonti, 2015; Plazzi, 48

49 2015; Vargas, Pérez, Toro, & Astorga, 2015; Dégletagne, Abele, & Held, 2016; Gusman et

50 al., 2016; Lucentini et al., submitted). In a nutshell, after dissection and microscopic

51 inspection in order to determine the sex, DNA is extracted from the gonadal content (i.e., eggs

52 or sperm) of sexually mature specimens; on the other side, DNA is extracted from somatic

53 tissues, typically foot or adductor muscles. The same mitochondrial markers are sequenced

54 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

56 haplotypes, the sex-linked heteroplasmy is demonstrated and this builds a strong argument for

57 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.,

64 the invasion of the M lineage by the female genome) may reset the divergence of the two

65 genomes, though they are actually inherited under DUI (Theologidis et al., 2008; Stewart,

66 Breton, Blier, & Hoeh, 2009; Zouros, 2013; Gusman et al., 2016). Masculinization of F

67 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;

69 Stewart et al., 2009; Walker et al., 2006) nor in Veneridae (Passamonti, 2007; Passamonti &

70 Scali, 2001; Stewart et al., 2009).

71 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 72 has been carried out among gastropods (Parakatselaki, Saavedra, & Ladoukakis, 2016; 73 74 Gusman, Azuelos, & Breton, 2017), yet DUI is probably widespread within bivalves, and a 75 large part of DUI species are still to be detected (Gusman et al., 2016). Moreover, in some 76 groups, such as the family Unionidae, DUI is common and widespread among gonochoric 77 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 78 79 (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 80 81 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 82 83 within the class is somewhat reduced to draw ultimate conclusions. Nonetheless, significant molecular differences among different DUI systems have been described (Zouros, 2013; 84 85 Plazzi, 2015; Plazzi & Passamonti, 2019).

86

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

88 The first evidence of Doubly Uniparental Inheritance (DUI) in the Manila clam *Ruditapes*

89 *philippinarum* was published in 2001 (Passamonti & Scali, 2001). Although it came later than

90 the one in *Mytilus* (Skibinski et al., 1994a,b; Zouros et al., 1994a,b) and unionid freshwater

91 mussels (Hoeh, Stewart, Sutherland, & Zouros, 1996b; Liu, Mitton, & Wu, 1996), R.

92 *philippinarum* was the first heterodont bivalve to show DUI, and this widened the taxonomic

93 occurrence of this unusual mitochondrial inheritance mechanism to all Autolamellibranchiata94 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

98 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 99 100 males (i.e. they are heteroplasmic for both mitotypes) allowed some interesting tests for some 101 of the more basic aspects of mitochondrial biology (f.i., mitochondrial inheritance). In 102 Passamonti, Boore, & Scali (2003) a case of possible recombination between M and F 103 mitotypes was recorded, thus confirming the coeval claims that there is no molecular reason 104 to consider mitochondrial DNA recombination absent from mitochondria (e.g., Thyagarajan, 105 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 106 107 2001a). However, in R. philippinarum, recombinant sequences were not detected in mtDNAs 108 obtained from gonads (Passamonti et al., 2003), and this was taken as an indication that clams 109 do not transmit recombinants to their progeny at a detectable level, even if recombination may 110 occur in their cells with characteristics similar to that of M. edulis (Laudokakis & Zouros 111 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 112 113 next generation might be the effect of the so-called mitochondrial bottleneck and, maybe, 114 some different selective constraints between somatic and germ-line mitochondria. However, 115 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

120 both issues, as we will see later on.

121 In a previous review (Passamonti & Ghiselli, 2009), we proposed that the function of DUI

122 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

124 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.

126 On the other hand, we can also imagine that this linkage is 'coincidental', i.e. the two sex-

127 linked mitochondrial genomes are just carried by the two gametes, with no apparent function.

128 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*.

130 *philippinarum* point out to the fact that the first has more chances to be true.

131

132 The segregation pattern and distribution of M and F types during early embryo development133 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).

141 The specific mechanisms of segregation of M- and F-type mitochondria during embryo 142 development and germline differentiation have been studied in depth in *R. philippinarum*, by 143 both molecular and cytogenetic approaches. A first assay using RealTime quantitative PCR (Ghiselli, Milani, & Passamonti, 2011) demonstrated that there is a strict segregation of 144 mitochondria in both clams' germlines (i.e., no mitochondrial DNA of the other sex is 145 detected in sperms or eggs). The absence of F-type mtDNAs in sperm is, according to 146 147 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 148 149 controversial for eggs: different levels of M-type mtDNAs have been detected in Mytilus 150 galloprovincialis eggs (Obata, Kamiya, Kawamura, & Komaru, 2006.; Obata, Kamiya, Kawamura, & Komaru, 2007; Sano, Obata, & Komaru, 2007), but there is no evidence of M-151 152 type mtDNAs in the eggs of *R. philippinarum*. An interesting observation came from some 153 female *R. philippinarum* showing somatic heteroplasmy, with variable levels of M-type 154 mtDNAs in their somatic tissues; this indicates that the elimination mechanism of sperm 155 mitochondria is not unfailing for somatic tissues. Quite remarkably, their eggs were 156 homplasmic for F-type, indicating that, at least in *R. philippinarum*, a strict homoplasmy is a 157 prerequisite for proper gamete formation in both sexes (Ghiselli et al., 2011). 158 In a few cases, DUI has been directly investigated by tracking mitochondria in the zygote 159 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 160 161 the developing embryo (Cao, Kenchington, & Zouros, 2004; Cogswell, Kenchington, & Zouros, 2006; Kenchington, Hamilton, Cogswell, & Zouros, 2009). On one side, sperm 162 163 mitochondria concentrate in a limited region of the embryo, close to the first cleavage furrow, 164 and will presumably end in populating the germline: this is called the 'aggregated' pattern and 165 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).

173 The very same patterns were observed in *M. galloprovincialis* (Obata & Komaru, 2005) 174 and R. philippinarum (Milani, Ghiselli, & Passamonti, 2012). Conversely, the same patterns 175 were not observed in Crassostrea gigas, where male mitochondria seem to distribute at 176 random (Obata, Shimizu, Sano, & Komaru, 2008): this observation, along with the complete 177 lack of evidence about sex-linked heteroplasmy, leads to the conclusion that DUI is not 178 present in the Pacific oyster. To date, C. gigas is thus to our knowledge the only species 179 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 180 181 heteroplasmy.

The area where paternal mitochondria concentrate in the aggregated pattern is the same 182 183 embryonic area in which also germ plasm is transferred, as is suggested by the observation 184 that it co-localizes with the protein VASA (Milani, Ghiselli, Maurizii, & Passamonti, 2011): 185 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; 186 187 Gustafson Wessel, 2010) and its inactivation suppresses the formation of primordial germ 188 cells (Lasko & Ashburner, 1988; Williamson & Lehmann, 1996; Knaut, Pelegri, Bohmann, 189 Schwarz, & Nuesslein-Volhard, 2000; Kuznicki et al., 2000). 190 In fluorescent analyses, a central role of microtubules is evident, as the M-type 191 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).

200 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: 201 202 they found that both mtDNAs do not detectably replicate during early embryogenesis, and 203 that the M line might be lost from females around 24 h of age. A rise in mtDNA copy number 204 was observed before the first reproductive season in both sexes, with the M mitochondrial 205 genome replicating more than the F in males, and we associate these boosts to the early phase 206 of gonad production. In parallel, the cytogenetic analyses evidenced that at each reproductive 207 season a proliferation of germline "primordial stem cells", originating from the simple 208 columnar epithelium of the gut and in the connective tissue nearby, contributes to the seasonal 209 gonad reconstitution (Milani et al., 2017a,b).

210 Finally, by an in-depth immunolocalization of F- and M-type variants of three 211 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 212 213 heteroplasmic, while gametes are invariably homoplasmic. Thus, the condition of 214 homoplasmy of germline is reached during gametogenesis, rather than earlier embryo 215 development, with a process that reminds a meiotic drive (Ghiselli et al, 2019). If this 216 scenario is true, then the homoplasmy condition is not causally linked to the 217 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 218 219 both sexes. If this is true, checkpoints 1 and 2 of our previously hypothesized model are just 220 ineffective, and the homoplasmic condition of gametes is restored at checkpoint (previously 221 numbered as) 3.

222

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

225 Mitochondrial transcriptomes provide further insights into DUI details and machinery. 226 Despite being (most likely) transcribed as a single polycistrone, different regions of the 227 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 228 229 regulations (Lynch, 2007; Scheffler, 2008). Moreover, in R. philippinarum, the two mitochondrial genomes globally show lineage-specific transcription levels for all protein 230 231 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 232

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.,

235 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*.

237 *philippinarum* and *R. decussatus*, notwithstanding the fact that the former shows the DUI

phenomenon, while the latter does not (Iannello et al., 2019).

239 Therefore, we conclude from these pieces of evidence that the onset of DUI entails a 240 remodulation of evolutionary-fixed levels of mitochondrial transcription aiming to optimize 241 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 242 243 OXPHOS genes does not seem to increase following the mitochondrial evolution (be it 244 connected or not with DUI). Although the overall evolutionary rate is quite high when 245 compared to other animal taxa, and although mitochondrial genes are much more expressed 246 than nuclear counterparts (from 12× to 100×), dN/dS ratios are comparable (Iannello et al., 247 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 248 249 of genes retained on the organellar genome may be directly regulated by the redox state of the respective products/complexes. 250

Similarly, selective pressure is somewhat different on the two lineages. It has been 251 252 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; 253 254 Breton, Burger, Stewart, & Blier, 2006; Zbawicka, Burzynski, Skibinski, & Wenne, 2010; 255 Doucet-Beaupré et al., 2010; Zouros, 2013; Zouros & Rodakis, 2019; but see Passamonti, 256 2007; Plazzi, Puccio, & Passamonti, 2016). However, in the first characterization of 257 mitochondrial transcriptome and polymorphism of a DUI species with high-throughput data, 258 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 259 260 amount of rare mutations, while the M lineage shows much more intermediate-frequency 261 alleles: interestingly, the overall number of SNPs is higher in the female genomes, as well as 262 is the number of "high-effect" (nonsynonymous) SNPs, being they polyallelic or monoallelic. 263 Therefore, the higher rates of molecular evolution of the M lineages (if confirmed) are not 264 connected to a higher polymorphism in male germ line mitochondria (Ghiselli et al., 2013). 265 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 266 267 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 ofnatural selection on deleterious SNPs.

270 Yet, mitochondria are not run by the just 13 protein which are typically encoded on animal 271 mitochondrial genomes. Wallace (2005) reports that approximately 1,500 genes that were 272 originally encoded by the mitochondrial genome are currently scattered throughout the 273 nuclear counterpart, and this is roughly the estimated size of the mitochondrial proteome, at 274 least in humans (Rabilloud et al., 1998; Lopez et al., 2000). The products of these genes 275 interact with the products of mitochondrial genes within respiratory complexes: what is the 276 nature of this interactions when DUI splits the sex-linked mitochondrial genomes the nuclear 277 genome has to interact with?

278 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.,

280 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*

282 peninsularis and Venustaconcha ellipsiformis the number of sex-biased genes is one order of

283 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*

290 margaritifera, yielded far less genes, since only 1,993 genes were detected

291 (Teaniniuraitemoana et al., 2014), a value comparable to that obtained for *R. philippinarum*.

292 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

295 mussels).

296 Within sex-biased genes, sex-specific genes were also detected, as well as sex-specific

297 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

299 gonochoric, DUI species U. peninsularis with the hermaphroditic U. imbecillis resulted in 567

300 genes specific to the hermaphroditic individuals, 59 genes specific to male individuals, and

301 100 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).
- 304 Most sex-biased genes are male-biased in R. philippinarum (Ghiselli et al., 2012) and U. 305 peninsularis (Capt et al., 2018), as has already been reported for other species (Meiklejohn, 306 Parsch, Ranz, & Hartl, 2003; Ranz, Castillo-Davis, Meiklejohn, & Hartl, 2003; Ellegren & 307 Parsch 2007). Most probably, this pattern is due to the fact that female-biased genes are very 308 commonly connected to essential functions in the context of gonad development, 309 gametogenesis, fertilization, and alike, and are therefore present and expressed in both sexes (Zhang, Hambuch, & Parsch, 2004; Proschel, Zhang, & Parsch, 2006; Clark, Findlay, Yi, 310 Maccoss, & Swanson, 2007; Ellegren & Parsch 2007; Larracuente et al., 2008; Ghiselli et al., 311 312 2012; Capt et al., 2018); nonetheless, 25,911 female-biased genes were found in H. schlegelii 313 against 19,511 male-biased genes (Shi et al., 2015). 314 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-315 biased ones, which leads to some difficulties in annotation. However, genes showing a 316 317 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 318 319 (and are actually much more; Ghiselli et al., 2018). 320 In fact, female- and male-biased genes appear to be somewhat conserved across different 321 species, and even across different mitochondrial inheritance systems (Capt et al., 2018; 322 Ghiselli et al., 2018; Capt et al., 2019). However, Ghiselli and colleagues (2018) 323 demonstrated that often sex biases are not maintained across the strictly related species R. 324 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
- 327 reversed. Albeit not always in top-enriched GO terms for sex-biased genes (Capt et al., 2018),
- 328 some functions are repeatedly associated to these orthologs. Some of these functions are
- 329 expected for genes connected to sex determination, like embryo/gonad development,
- 330 gametogenesis, fertilization (Ghiselli et al., 2012, 2018); other functions deserve a more
- 331 careful investigation.
- Indeed, many studies focused on the ubiquitination pathway: ubiquitin (Ub) is a universal
- 333 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,

336 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) 337 338 demonstrated that, as previously found in the Mytilus complex (Saavedra, Reyero, & Zouros 339 1997; Kenchington, MacDonald, Cao, Tsagkarakis, & Zouros, 2002; Cogswell et al., 2006; 340 Kenchington et al., 2009), families with different sex-biases are present in *R. philippinarum*, 341 leading to male-dominated or female-dominated offspring. Milani, Ghiselli, Nuzhdin, & 342 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 343 344 known to be involved in male sexual differentiation (Shimada, Kanematsu, Tanaka, Yokosawa, & Kawahara, 2006) and which was found to be highly differentially expressed 345 346 also in Mytilus edulis male-biased eggs (Diz et al., 2009). BIRC contains a domain termed 347 RING, which has been linked to degradation through the ubiquitin-proteasome system 348 (Milani et al., 2013b; Joazeiro & Weissman, 2000). ANUBL1 shows a ubiquitin-like domain 349 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 350 351 species (often with the same, male-oriented, bias), along with DNMT1, a DNA-352 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 353 354 with respect to female individuals of the latter species, along with fbx039, also a gene 355 connected with ubiquitination (Capt et al., 2019). Furthermore, ~380 ortholog transcripts 356 connected to ubiquitinating enzymes were detected in the R. decussatus and R. philippinarum 357 transcriptomes, be they Ub-activating enzymes, Ub-conjugating enzymes, Ub-ligases, 358 deubiquitinating enzymes, or proteasome subunits (Punzi, Milani, Ghiselli, & Passamonti, 359 2018). Currently, it is still unclear the actual mechanism which is involved in sperm 360 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. 361 362 philippinarum may lead to the idea that an evolutionary conserved signal on the outer 363 membrane (ubiquitinated prohibitins?) is masked by a DUI-specific factor in male zygotes; 364 alternatively, the presence of a transmembrane Ub-ligase with a strong male bias which is 365 present in R. decussatus, but not in R. philippinarum, may lead to the idea that in the latter, 366 DUI species, the labeling signal itself is modified upstream (Punzi et al., 2018). 367

368 The ORFans and their supposed role on DUI appearance

369 Mitochondrial ORFans are ORFs with no evident homology to the common animal 13

370 mitochondrial protein coding genes (Fischer & Eisenberg, 1999; Breton et al., 2014). ORFans

371 were often described in bivalve mitochondrial genomes (Breton et al., 2014; Plazzi et al.,

372 2016); albeit present in species where evidence of DUI is lacking as well, like the basal

373 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

376 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).

379 As said, mitochondrial ORFans are often found in bivalve mitochondrial genomes and they 380 have been claimed to be involved in DUI establishment/maintaining: ORFans, with few 381 exception, are lineage-specific and have been shown to be translated in functional protein, 382 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 383 384 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 385 386 infected some mitochondria of a hermaphroditic population conferring the ability to avoid 387 degradation in embryos if carried through sperm, and distorting segregation towards a 388 preferential transmission through generations? Actually, it has been argued that such an event 389 may well have triggered a shift to gonochorism and DUI in a given taxon (Milani et al., 2016) 390 as a way to resolve the conflict between nuclear and mitochondrial genome(s), a theme which 391 was originally put forward by Passamonti and Ghiselli (2009).

392

393 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

401 (Bettinazzi, Plazzi, & Passamonti, 2016). Similarly, the F-M divergence reaches 47.04 in *R*.

402 *philippinarum* amino acids (25.80 being the average value for venerid/mytilids) and 86.82 in

403 *Mytilus californianus* coding nucleotides (66.97 being the average; Bettinazzi et al., 2016).

404 Male and female lineages exhibit a K2P distance of 0.41 for *cytb* and 0.210 for *rrnL* in the

405 nuculanid *Ledella ultima*, which means a degree of divergence up to the 27% (Boyle & Etter,

406 2013); a similar value was observed for *Solen marginatus* (16% for *cox1*, 21% for *rrnL*;

407 Lucentini et al., submitted); an even higher value was recorded for *Donax trunculus* (28% for

408 *rrnL*, 36.5% for *cytb*; Theologidis et al., 2008).

409 To date, DUI seems to be scattered throughout the bivalve evolutionary tree, and the

410 patterns of its distribution are unclear (Theologidis et al., 2008; Plazzi, 2015; Gusman et al.,

411 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).

436 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 437 438 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 439 consistently retrieved by most, if not all, studies dealing with DUI distribution among 440 441 bivalves: in fact, the more DUI species added to the phylogenetic tree, the more evidence are 442 growing towards a polyphyletic origin of DUI. Remarkably, the idea of several DUI origins 443 within bivalves is consistent with other lines of evidence but the polyphyly with respect to a 444 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 445 of molecular synapomorphies were found (Plazzi & Passamonti, 2019), lending further 446 support to a multiple origin of DUI. It is tempting to connect this hypothesis to the previously 447 448 reported proposal of a viral origin for supernumerary ORFs in DUI mtDNAs (Milani et al., 449 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 450 and finally resulting in DUI would explain the evolutionary parallelism which hides behind a 451 452 polyphyletic distribution of DUI very well.

453

454 The smithRNAs and their role

455 Very recently, in *R. philippinarum*, we were able to identify several putative sncRNAs that 456 are transcribed from mtDNA at a very high level, yet they are predicted to act on nuclear 457 targets (Pozzi, Plazzi, Milani, Ghiselli, & Passamonti, 2017). Therefore, we proposed a new 458 category of interfering RNAs, named small mitochondrial highly transcribed RNAs 459 (smithRNAs). The invention of sncRNAs of mitochondrial origin is not completely new (Ro 460 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 461 462 affect nuclear gene expression: for example, we found clues that mt-derived small non-coding 463 RNAs might be involved in gonad formation (Pozzi et al., 2017). The possibility that mtDNA 464 may act on nuclear gene regulation has never been suggested before, and the relevance of this 465 mechanism for eukaryote gene regulation has to be assessed. Indeed, the smithRNAs may be 466 a new unprecedented form of retrograde signaling (i.e., the mitochondria-to-nucleus 467 signaling; Arnould, Michel, & Renard, 2015; Cagina & Enriqueza, 2015; Monaghan & 468 Whitmarsh, 2015).

469 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 470 471 formation, as originally suggested by Passamonti and Ghiselli (2009), could be in fact in 472 charge of these mitochondrial determinants. In this conception, while the ORF protein of viral 473 origin may have driven DUI appearance and evolution, acting as a selfish meiotic drive 474 distorter, the sex-linked mitochondria have soon evolved (or maybe reinforced) the capacity 475 of driving gamete formation trough RNA interference. This is not surprising because mtDNA 476 could be somehow prone to quickly evolve new functions through small interfering RNA, 477 because of hairpin structures in intergenic regions (needed for correct RNA cleavage), as well 478 as tRNAs, could be easily exapted to interfering RNAs. Moreover, mitochondria have been 479 implicated in the evolution of sex under several hypotheses, so it is at least conceivable that 480 mtDNA may act as a sex-determining factor or be involved in sexual differentiation. Finally, 481 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, & 482 Smith, 1997; Werren & O'Neill, 1997; Stouthamer, Breeuwer, & Hurst, 1999; Cheng et al., 483 484 2000; Weeks, Marec, & Breeuwer, 2001), although it is still unclear how this is achieved. 485

486 A final overview

487 Mitochondria are a fundamental component of eukaryotic life. Nevertheless, the current 488 knowledge about their biology and function is largely incomplete, and mostly biased toward a 489 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. 490 491 Mitochondrial genome is very compact, with few and short intergenic regions (except the 492 Control Region), so that one may consider that there is little space for other functions, other 493 than the usual content of 37 genes (Boore, 1999; Gissi, Iannelli, & Pesole, 2008; Breton et al. 494 2014). This, however, seems not to be the case, as evidenced above (see also Breton et al., 495 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.

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- 507 of making DUI (and especially *R. philippinarum*) a profitable model for mitochondrial
- 508 studies.
- 509

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900 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.

904 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 905 the zygote stage and early development all individuals are heteroplasmic; at a certain point 906 907 (how and when is still to be fully clarified) females become homoplasmic for the F-type, 908 hence transmitting the F-type with eggs, while males are, at various levels, heteroplasmic in 909 somatic tissues, and homoplasmic for M-type in gonad, hence transmitting the M-type with 910 spermatozoa. Phylogenetic distribution of DUI in bivalves (central part): clockwise from above, in blue, 911 912 Heterodonta (Mactridae, Veneridae, Arcticidae, Solenidae, Donacidae, Semelidae); in brown, Palaeoheterodonta (Unionida); in red, Pteryomorpha (Nuculanidae, Yoldiidae, Mytilidae). 913

914 The proposed phylogeny is based on mitochondrial genes.

915



918 Fig. 1