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REVIEW

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

Running title: DUI and beyond

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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.

132 The segregation pattern and distribution of M and F types during early embryo development
133 and gonad differentiation.
134 The general, DUI-defining pattern of separated F and M lineages has different features in
135 different bivalve groups. As already mentioned, while male somatic tissues do mainly contain
136 the F lineage in the genus *Mytilus* (Garrido-Ramos et al., 1998; Dalziel & Stewart, 2002; but
137 see Kyriakou, Zouros, & Rodakis, 2010), male soma was found heteroplasmic at various
138 degrees (as in *R. philippinarum*): for instance, it appears to be dominated by M lineage in
139 *Perumytilus purpuratus* (Vargas et al., 2015). Moreover, heteroplasmic females may be found
140 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
144 (Ghiselli, Milani, & Passamonti, 2011) demonstrated that there is a strict segregation of
145 mitochondria in both clams' germlines (i.e., no mitochondrial DNA of the other sex is
146 detected in sperms or eggs). The absence of F-type mtDNAs in sperm is, according to
147 Venetis, Theologidis, Zouros, & Rodakis (2006), a basic requirement for the stability of DUI:
148 data on *R. philippinarum* confirmed this. On the other hand, the situation is more
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,
151 Kawamura, & Komaru, 2007; Sano, Obata, & Komaru, 2007), but there is no evidence of M-
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
160 patterns were detected in the DUI species *Mytilus edulis*, most likely in relation to the sex of
161 the developing embryo (Cao, Kenchington, & Zouros, 2004; Cogswell, Kenchington, &
162 Zouros, 2006; Kenchington, Hamilton, Cogswell, & Zouros, 2009). On one side, sperm
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

166 offspring. On the other side, sperm mitochondria are scattered throughout the embryo and
167 seem actually to be distributed at random: this is called the 'dispersed' pattern and is typical
168 of female-biased mothers, i.e. female individuals that give birth to almost only female
169 offspring. The presence of these two patterns of sperm mitochondria has been considered a
170 strong evidence of DUI, in that it links the differential inheritance of mitochondria in the two
171 sexes to a concrete mechanism for mitochondria to be preserved in the germline (for male
172 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
180 observation, rather than excluded as unlikely because of the absence of sex-linked
181 heteroplasmy.
182 The area where paternal mitochondria concentrate in the aggregated pattern is the same
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
186 in many other animals (Hay, Ackerman, Barbel, Jan, & Jan, 1988; Hay, Jan, & Jan, 1988;
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
192 after cytodieresis (Milani et al., 2011, 2012). Moreover, no replication boost of either M- and
193 F-type mtDNAs is evidenced in the earliest embryo development (Milani et al., 2012).
194 Based on the observations above, we proposed a three-step model for germ line
195 segregation under DUI, evidencing three checkpoints for proper segregation of sex-linked
196 mitochondrial lineages. The model includes an early segregation mechanism in embryos (the
197 aggregated/dispersed pattern we mentioned above), a second step in which M types are
198 actively eliminated/retained according to sex, and a final mechanism of sex-linked selection
199 during gamete formation (Ghiselli et al., 2011).

200 Subsequent researches focused on testing this three-step model. Guerra and colleagues
201 (2016) used a qPCR approach to follow both mtDNA lineages starting from early embryos:
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
212 stages, we evidenced that undifferentiated germ cells of both sexes, as well as male soma, are
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
218 population of germ cells, is reached at every reproductive term during gonad development of
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
223 The -omics breakthrough – Putative candidate genes for sex determination in bivalves and
224 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
228 levels of mature transcripts (Ghiselli et al., 2013) because of multiple post-transcriptional
229 regulations (Lynch, 2007; Scheffler, 2008). Moreover, in *R. philippinarum*, the two
230 mitochondrial genomes globally show lineage-specific transcription levels for all protein
231 coding genes, as well as for *rrnS* (Ghiselli et al., 2013; Iannello, Puccio, Piccinini,
232 Passamonti, & Ghiselli, 2019); conversely, all the nuclear components of the electron
233 transport chain but the subunits of complex III are transcribed from the nuclear genome to

234 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
236 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
238 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
242 clues of nuclear molecular compensation, which entails that dN/dS values observed in nuclear
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
248 hypothesis (Allen, 2003): in a nutshell, the idea that states that the expression of the handful
249 of genes retained on the organellar genome may be directly regulated by the redox state of the
250 respective products/complexes.
251 Similarly, selective pressure is somewhat different on the two lineages. It has been
252 repeatedly stated that the M lineage of DUI species evolves faster than the F counterpart
253 (Hoeh, Stewart, Sutherland, & Zouros, 1996a; Mizi, Zouros, Moschonas, & Rodakis, 2005;
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
259 polymorphism, in *R. philippinarum*. More in detail, the F lineage shows a significantly higher
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
266 lineage, recall that a single egg carries hundreds of mtDNAs. Conversely, *R. philippinarum*
267 spermatozoa contain only four mitochondria (Milani et al., 2011), which translates into a

268 handful of mtDNAs (Ghiselli et al., 2011), which increases the power and the effectiveness of
269 natural 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
279 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,
281 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,
284 Hong, Sheng, Peng, & Wang, 2015; Capt et al., 2018). Specifically, the number of sex-biased
285 genes in *V. ellipsiformis* was estimated between 11,408 and 52,257 with two different
286 approaches; recall that a draft genome of this species recovered roughly 43,000 annotated
287 open reading frames (Renaut et al., 2018), an important fraction of the protein coding genome
288 seems to be involved in sex-linked differential transcription in these species. Conversely, the
289 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
293 somewhat unexpected, given that these animals do show very limited sexual dimorphism (but
294 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*
298 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

302 *R. philippinarum*, 166 sex-specific SNPs were detected, mostly connected with sperm
303 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
310 (Zhang, Hambuch, & Parsch, 2004; Proschel, Zhang, & Parsch, 2006; Clark, Findlay, Yi,
311 Maccoss, & Swanson, 2007; Ellegren & Parsch 2007; Larracuente et al., 2008; Ghiselli et al.,
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
315 genes in *R. philipinarum*, and it seemed that male-biased genes evolve faster than female-
316 biased ones, which leads to some difficulties in annotation. However, genes showing a
317 conserved bias across *R. philippinarum* (a DUI species) and *R. decussatus* (a non-DUI
318 species) show higher dN/dS values when conserving a bias towards females in both species
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
325 be female-biased in the other one or vice versa: among 3,102 ortholog genes, 1,284 were
326 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.
332 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
334 directly involved in gametogenesis, with special reference to male gametogenesis (f.i.,
335 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
337 the mitochondrial membrane, the prohibitin (Sutovsky et al., 2000). Ghiselli et al. (2012)
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
343 male-biased families: *psa*, *birc*, and *anubl1*. PSA is a subunit of the proteasome, which is
344 known to be involved in male sexual differentiation (Shimada, Kanematsu, Tanaka,
345 Yokosawa, & Kawahara, 2006) and which was found to be highly differentially expressed
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).
350 Orthologs of the very same genes were found among sex-biased genes in unionid DUI
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
353 upregulated in the hermaphroditic *U. imbecillis* and in male individuals of *U. peninsularis*
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
361 midbody level (Milani et al., 2011). Ub-connected genes present in both *R. decussatus* and *R.*
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
374 hypothesized to be connected with DUI (Breton et al., 2009, 2011; Milani, Ghiselli, Guerra,
375 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,
377 Guerra, Stewart, & Breton, 2016; Plazzi et al., 2016) – and these supernumerary genes are
378 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
383 al., 2009, 2011; Milani et al., 2013a). Moreover, many clues were identified which would
384 relate lineage specific ORFans of DUI species to selfish viral elements (Milani et al., 2013a;
385 Milani, Ghiselli, & Passamonti, 2016). What would have happened if a viral element had
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
394 The divergence between the two mitochondrial M and F lineages can be very high under
395 DUI. The average amino acid Kimura distance among protein coding genes was reported to
396 be 83.30 among different species of the superfamily Unionoidea, yet in some DUI species of
397 this superfamily the F-M divergence is higher, up to 86.40 in *Utterbackia peninsularis*.
398 Moreover, the average nucleotide Jin-Nei Gamma distance computed on all coding regions of
399 the genome was reported to be 100.64 within Unionoidea, but it again is higher for some of
400 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).
412 Following Theologidis and colleagues (2008), it is possible to identify three phylogenetic
413 patterns of DUI-related sequences: a gender-joining pattern, a taxon-joining pattern, and a
414 mixed pattern. In a gender-joining pattern, F sequences of a given group of DUI species are
415 recovered as monophyletic; the same holds for the corresponding M sequences, and the two
416 clades are sister taxa. In a taxon-joining pattern, each F sequence is the sister taxon to the M
417 counterpart, and species are therefore monophyletic. In mixed patterns, F and M sequences
418 are interwoven.
419 The most remarkable example of gender-joining pattern is the superfamily Unionoidea: F
420 mitochondrial genomes of the different families comprising Unionoidea are consistently
421 retrieved as monophyletic (including non-DUI species), and M mitochondrial genomes are
422 monophyletic as well. The two clades are sister clades, and within each clade the same
423 topology is mirrored (e.g., Doucet-Beaupré et al., 2010; Gusman et al., 2016).
424 A typical taxon-joining pattern is found within Heterodonta. To date, DUI species
425 belonging to this subclass are quite unrelated: *Cyclina sinensis*, *M. lamarckii*, *Pseudocardium*
426 *sachalinense*, *Scrobicularia plana*, *S. marginatus*, and *R. philippinarum* are monophyletic,
427 resulting from the respective (F + M) cluster. The phylogenetic relationships among species
428 are basically the same that are obtained when DUI is not taken into account and only one
429 (typically female) sequence is included in the analysis (e.g., Plazzi, 2015; Gusman et al.,
430 2016; Plazzi & Passamonti, 2019).
431 While most mytilids are associated to taxon-joining patterns (Vargas et al., 2015; Gusman
432 et al., 2016), a mixed pattern is normally shown within the genus *Mytilus*: the M lineage of *M.*
433 *californianus* is typically recovered as the sister taxon to a clade comprised by the
434 corresponding F lineage and the DUI species *M. edulis*, *M. galloprovincialis*, and *M.*
435 *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
437 originated once and was subsequently lost in many bivalve lineages (Zouros, 2013). Given its
438 presence in *L. ultima*, the origin of DUI should be dated back to at least the early Cambrian
439 (Boyle & Etter, 2013). However, most of the aforementioned phylogenetic relationships are
440 consistently retrieved by most, if not all, studies dealing with DUI distribution among
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
445 detected for branches leading to a DUI assemblage in a phylogenetic tree, but only a handful
446 of molecular synapomorphies were found (Plazzi & Passamonti, 2019), lending further
447 support to a multiple origin of DUI. It is tempting to connect this hypothesis to the previously
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
450 selfish viral element acting on the same background mechanism of mitochondrial inheritance
451 and finally resulting in DUI would explain the evolutionary parallelism which hides behind a
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
461 Mazo. 2018; Riggs et al. 2018), but smithRNAs provide an unprecedented way for mtDNA to
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
470 speculate that the supposed capacity of DUI sex-linked mitochondrial DNAs to drive gamete
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
482 closest relatives of mitochondria, the α-proteobacteria (e.g., *Wolbachia*; Terry, Dunn, &
483 Smith, 1997; Werren & O'Neill, 1997; Stouthamer, Breeuwer, & Hurst, 1999; Cheng et al.,
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
490 entails the risk of losing a big part of the molecular and functional diversity of mitochondria.
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).
496 *R. philippinarum* has proven to be a successful model species for studies on mitochondrial
497 biology and Doubly Uniparental Inheritance (DUI). Thanks to its unusual features, DUI can
498 shed light on mitochondrial inheritance and biogenesis, and, above all, on the relationship
499 between mitochondria and germ line. Moreover, DUI is a unique experimental system for
500 studying mitochondrial heteroplasmy, and two processes that shape genome evolution:
501 genomic conflicts and mito-nuclear coevolution, which are at the very root of eukaryotic life.
502

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900 Figure legends

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

904 Inheritance routes (outer part): pink, F-type mitochondria; blue, M-type mitochondria.
905 Clockwise from above: eggs and sperms are homoplasmic for F- and M-type, respectively; at
906 the zygote stage and early development all individuals are heteroplasmic; at a certain point
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.

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

915

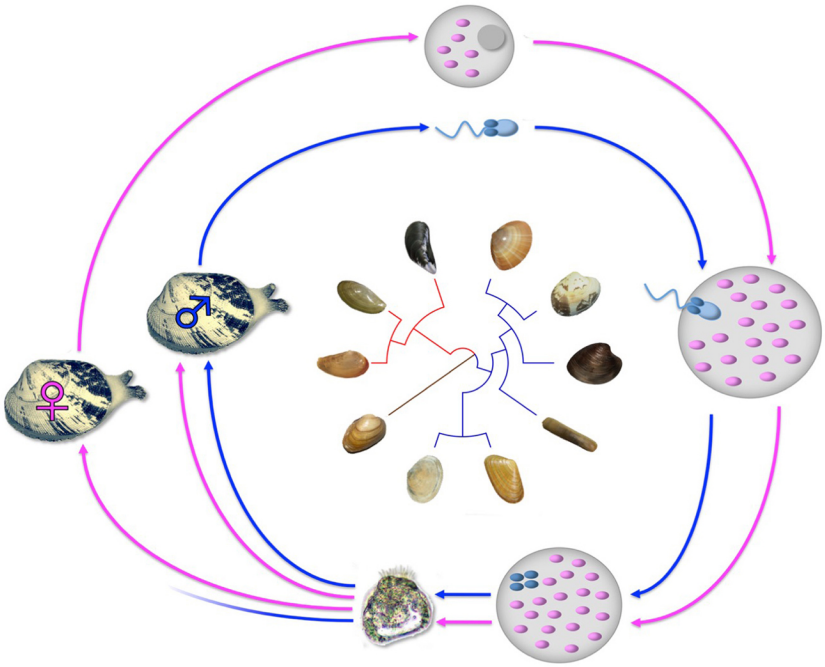

Fig. 1