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Additional taxonomic coverage of the doubly uniparental inheritance in bivalves: Evidence of sex-linked heteroplasmy in the razor clam *Solen marginatus* Pulteney, 1799, but not in the lagoon cockle *Cerastoderma glaucum* (Bruguière, 1789)

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1 **Additional taxonomic coverage of the DUI (Doubly Uniparental Inheritance) in bivalves:**
2 **evidence of sex-linked heteroplasmy in the razor clam *Solen marginatus* Pulteney, 1799, but**
3 **not in the lagoon cockle *Cerastoderma glaucum* (Bruguère, 1789)**

4

5 **Running Title: Evidence of DUI in the razor clam *Solen marginatus* Pulteney, 1799**

6

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27 ***marginatus*, *Cerastoderma glaucum***

28

29 **Abstract**

30 In animals, Doubly Uniparental Inheritance (DUI) is a major exception to the common Strict
31 Maternal Inheritance of mitochondria. To date, DUI has only been found in many bivalve species,
32 but its distribution is still unclear. Given the great species richness of the class, much effort is
33 needed to further investigate the occurrence of DUI in unsampled species. A compelling evidence
34 of DUI is generally the presence of a sex-linked heteroplasmy, where two divergent mitochondrial
35 lineages are found: one is isolated from the male germline, the other one is isolated from the female
36 germline and, normally, from the soma of both sexes. In the present study, we investigated the sex-
37 linked heteroplasmy in the razor clam *Solen marginatus* Pulteney, 1799 and in the lagoon cockle
38 *Cerastoderma glaucum* (Bruguière, 1789) using two mitochondrial markers (*cox1* and *rrnL*). We
39 found evidence of DUI in the species *S. marginatus*, with a divergence up to 21% for the *rrnL* gene,
40 but not in *C. glaucum*. Moreover, our phylogenetic reconstruction includes all the available data for
41 heterodont species with sex-linked heteroplasmy and suggests multiple origins of DUI in this
42 subclass, as well as the presence of DUI in other species of the genus *Solen*.

43

44

45 **1. Introduction**

46 Mitochondria, beside being the well-known cell compartments where the TCA cycle and
47 oxidative phosphorylation take place, play different, yet pivotal roles in many eukaryotic cellular
48 processes, spanning from apoptosis to aging, from cell differentiation to fertilization, from signaling
49 to nuclear gene regulation through ncRNAs (see as examples [Spikings, Alderson, & St. John, 2007](#);
50 [Scheffler, 2008](#); [Van Blerkom, 2011](#); [López-Otín, Blasco, Partridge, Serrano, & Kroemer, 2013](#);
51 [Chandel, 2014](#); [Babayev et al., 2016](#); [Bottje et al., 2017](#); [Pozzi, Plazzi, Milani, Ghiselli, &](#)
52 [Passamonti, 2017](#); [Prieto & Torres, 2017](#); [Riggs et al., 2018](#); [Hill, 2019](#)). As a keynote feature of
53 these multifaceted organelles, mitochondrial inheritance was also shown to involve different
54 mechanisms. While the Strict Maternal Inheritance (SMI) of mitochondria probably represents the
55 rule in animals ([Birky, 2001](#)), a mechanism alternative to SMI is the Doubly Uniparental
56 Inheritance (DUI), which has been reported in many species of bivalve molluscs ([Breton, Doucet-](#)
57 [Beaupré, Stewart, Hoeh, & Blier, 2007](#); [Passamonti & Ghiselli, 2009](#); [Zouros, 2013](#); [Gusman,](#)
58 [Lecomte, Stewart, Passamonti, & Breton, 2016](#); [Zouros & Rodakis, 2019](#)).

59 In DUI species, both parental mitochondrial lineages pass to the zygote: a paternal, male type
60 (M), which occurs in sperm, and a maternal, female type (F), occurring in oocytes. Thus, zygotes
61 are heteroplasmic: in embryos developing to females, M-type mitochondria are dispersed and
62 disrupted; in embryos developing to males, they are clustered together towards the primordial
63 germline ([Cao, Kenchington, & Zouros, 2004](#); [Milani, Ghiselli, & Passamonti, 2012](#)). Among adult
64 specimens, females are typically homoplasmic for the F lineage, whereas males maintain
65 heteroplasmy: the germline is dominated by M-type mitochondria and somatic cells show different
66 proportions of M-type and (often dominating) F-type mitochondria ([Garrido-Ramos, Stewart,](#)
67 [Sutherland, & Zouros, 1998](#); [Chakrabarti et al., 2007](#); [Kyriakou, Zouros, & Rodakis, 2010](#); [Batista,](#)
68 [Lallias, Taris, Guerdes-Pinto, & Beaumont, 2011](#); [Ghiselli, Milani, & Passamonti, 2011](#); [Obata,](#)
69 [Sano, & Komaru, 2011](#), [Brannock, Roberts, & Hilbish, 2013](#)).

70 DUI is generally detected using sex-linked heteroplasmy as a proxy (for example, [Passamonti &](#)
71 [Scali, 2001](#); [Theologidis, Fodelianakis, Gaspar, & Zouros, 2008](#); [Boyle & Etter, 2013](#); [Plazzi,](#)
72 [Cassano, & Passamonti, 2015](#); [Plazzi, 2015](#); [Vargas, Pérez, Toro, & Astorga, 2015](#); [Dégletagne,](#)
73 [Abele, & Held, 2016](#); [Gusman et al., 2016](#)). Over one hundred species have been currently reported
74 to show this peculiar phenomenon; however, it is possible that for many other bivalves, if not
75 molluscans, DUI species are still to be discovered ([Gusman et al., 2016](#)). Furthermore, evidence is
76 growing towards a multiple-origin scenario: the scattered distribution of DUI within bivalve species
77 ([Gusman et al., 2016](#); [Plazzi & Passamonti, 2019](#)), as well as significant molecular differences

78 among different DUI systems (Zouros, 2013; Plazzi, 2015; Plazzi & Passamonti, 2019; and
79 reference therein), are consistent with the idea that DUI evolved multiple times in different groups
80 of bivalves (Milani, Ghiselli, Guerra, Breton, & Passamonti, 2013; Milani, Ghiselli, & Passamonti,
81 2016; Zouros, 2013; Plazzi & Passamonti, 2019). However, with more than 3,500 extant and extinct
82 genera (Millard, 2001), the diversity of bivalves overwhelms the availability of empirical data on
83 sex-linked heteroplasmy, and the current knowledge of DUI distribution within the class is still
84 insufficient to draw conclusions. The DUI phenomenon is apparently restricted to bivalves,
85 although only a limited research has been carried out among gastropods (Parakatselaki, Saavedra, &
86 Ladoukakis, 2016; Gusman, Azuelos, & Breton, 2017); however, DUI is probably widespread
87 within bivalves (Gusman et al., 2016).

88 The razor clam *Solen marginatus* Pulteney, 1799, order Solenoidea, family Solenidae, has a broad
89 geographic distribution extending from Norway to the Mediterranean Sea, North Africa, the
90 Southeast and Western coasts of England (Darriba Couñago & Fernandez Tajés, 2011; Ayache et
91 al., 2016). The so-called “razor clam” is an infaunal bivalve (Semeraro et al., 2016) living in soft
92 sea beds and present in the deepest sediments, generally up to 20–50 cm below the surface
93 (Macedo, Macedo & Borges, 1999). Despite its economic interest, especially in the Southern
94 Mediterranean area (Ayache et al., 2016), limited data are available on its biology, ecology and
95 bioaccumulation profiles (see Sfriso et al., 2018 and references within). Moreover, few data are
96 available on molecular markers (Fernandez Tajés & Mendez, 2007; Francisco Candeira, Gonzalez
97 Tizon, Varela, & Martinez Lage, 2007), genetic diversity of its populations (Semeraro et al., 2016;
98 Hmida, Fassatoui, Ayed, Ayache, & Romdhane, 2012), gene structures and arrangements (Gonzalez
99 Romero, Ausio, Mendez, & Eirin-Lopez, 2009; Mesías Gansbiller, et al., 2012) and cytogenetics
100 (Fernandez Tajés, Gonzalez-Tizon, Martinez-Lage, & Mendez, 2003). To date, sex-linked
101 heteroplasmy has been suggested for the congeneric, Indo-Pacific species *Solen grandis* Dunker,
102 1862; however, only three sequences have been released in GenBank (Accession Numbers
103 AB064983, AB064984 and AB064985) and they are still unpublished.

104 The lagoon cockle *Cerastoderma glaucum* (Bruguière, 1789), order Veneroidea, family Cardiidae, is
105 also a benthic bivalve occurring in surface soft bottom sediments (Karray et al., 2015) or inside the
106 algal biomass. The species is distributed from the Atlantic coast of Norway to the Caspian Sea
107 (Brock, 1979) and in Mediterranean coastal lagoons. Compared to the closely related common
108 cockle *Cerastoderma edule* (Linnaeus, 1758), *C. glaucum* prefers semi-enclosed, shallow and
109 nontidal lagoons (Brock, 1979) or choked areas. *C. glaucum* has been used in different
110 environments as a bioindicator species of environmental contamination (see Karray et al., 2015;
111 Sfriso et al., 2018 and citations within). Moreover, since it represents an interesting model of a

112 benthic organism with a fragmented distribution, genetic diversity of its populations have been
113 extensively investigated, by traditional Sanger sequencing of ITS and mtDNA sequences (Nikula &
114 Vainola, 2003; Freire, Arias, Mendez, & Insua, 2010; Ladhar Chaabouni, Hamza Chaffai,
115 Hardivillier, Chenais, & Denis, 2010; Tarnowska, Chenuil, Nikula, Feral, & Wolowicz, 2010;
116 Tarnowska et al., 2012; Vergara Chen, Gonzalez Wanguemert, Marcos, Perez Ruzafa, 2013;
117 Sromek et al., 2016) by allozymic (Mariani, Ketmaier, & de Matthaëis, 2002; Sromek et al., 2016
118 and references within), and by microsatellite markers (Sromek et al., 2016). More recently,
119 population genomics has been investigated by NGS-based RAD markers (Sromek, Forcioli, Lasota,
120 Furla, & Wolowicz, 2019). Data have also been collected on its karyotype (Thiriot Quievreux &
121 Wolowicz, 1996).

122 Despite the occurrence of genetic and genomic data available for lagoon cockle, to our knowledge
123 there are no published papers regarding the possible occurrence of DUI phenomenon; up to now,
124 there is no evidence of DUI from cardiids (Gusman et al., 2016).

125 Therefore, in the present study *S. marginatus* and *C. glaucum* have been selected as target species
126 for a new study on the DUI phenomenon in bivalves.

127

128 **2. Materials and methods**

129 ***2.1 Sample collection and tissue preparation***

130 Mature specimens of *S. marginatus* (3 females and 6 males) and *C. glaucum* (5 females and 7
131 males) were collected in Summer 2017 in the Venice Lagoon (Northern Adriatic Sea) in two
132 stations facing the west side of the Malamocco-Marghera Canal. Razor clams were collected at
133 Verto Sud (sexagesimal coordinates: VS - 45.382987°/12.254941°); lagoon cockles were collected
134 at Torretta Bianca (TB - 45.393239°/12.264009°). The individuals were sampled by hand and
135 transported to the laboratory in an aerated basin with seawater.

136 Sample dissections were carried out within 24 hours following protocols already tested for previous
137 DUI analyses (Gusman et al., 2016). In detail, each individual was dissected, and the gonadal
138 content was analyzed under a light microscope (100×) to identify the occurrence of eggs or sperm.
139 Unambiguously sexed individuals were then selected for genetic analyses.

140 Somatic tissues (mantle and foot) and gonadal content were carefully separated for each specimen
141 and preserved in absolute ethanol at -20°C for DNA extractions (see next section).

142

143 ***2.2 DNA extraction and purification***

144 Total DNA was isolated individually from both the mantle/foot and the gonadal content, using the
145 DNeasy Blood & Tissue kit (Qiagen, Germantown, MD, USA), following the manufacturer's
146 instructions.

147 The quality and quantity of DNA were assessed by electrophoresis on 1% agarose gels and
148 spectrophotometric analysis.

149

150 **2.3 Mitochondrial marker amplification and sequencing**

151 Amplification of two different mitochondrial gene regions was carried out to investigate the
152 occurrence of intraspecific F and M haplotypes (Gusman et al., 2016): cytochrome *c* oxidase
153 subunit 1 (*cox1*) and 16S (*rrnL*).

154 Amplifications were performed by using universal primers LCO1490 (5'-

155 GGTCACAAATCATAAAGATATTGG-3') and HCO2198 (5'-

156 TAAACTTCAGGGTGACCAAAAATCA-3') for the *cox1* fragment (Folmer, Black, Hoeh, Lutz,

157 & Vrijenhoek 1994) and by more specific primers 16 Sar-ALT (5'-

158 CGCCTGTTTATCAAAAACATSG-3') and 16 Sbr-ALT (5'-CCGGTCTGAACTCAGATCACGT-

159 3') designed for bivalves for *rrnL* fragment (Mikkelsen, Bieler, Kappner, & Rawlings, 2006).

160 The amplification reactions were performed in a total volume of 25 µl, including 15.2 µl of

161 sterilized distilled water, 5 µl of 5× colorless GoTaq reaction buffer (7.5 mM MgCl₂), 1 µl of each

162 10 µM primer, 0.5 µl of dNTP mixture, 0.3 µl Go Taq G2 (Promega, Madison, WI, USA), and 2 µl

163 of DNA.

164 For the *cox1* gene fragment, PCR was carried out for 10 min at denaturation temperature of 95°C,

165 followed by thirty-five cycles of 30 sec at 95 °C, 40 sec at 47 °C and 60 sec at 72 °C, followed by a

166 final extension of 10 min at 72 °C.

167 For the *rrnL* gene fragment, PCR amplifications were performed by denaturing DNA for 2 min at

168 95 °C, followed by thirty-five cycles of 30 sec at 94 °C, 40 sec at 52 °C and 1 min at 72 °C, and a

169 final extension of 10 min at 72 °C.

170 The amplification products were checked by electrophoresis in TBE buffer and in 2% agarose gel

171 containing SafeView Nucleic Acid Stain (NBS Biologicals, Huntingdon, Cambridgeshire, UK) and

172 visualized under UV light: products were approximately 750 and 510 bp long for *cox1* and *rrnL*

173 amplicons, respectively.

174 Amplicons were then purified with EXOSAP-IT (Thermo Fisher Scientific, Affymetrix Inc., Santa

175 Clara, CA 95051, USA) following the standard protocol and Sanger sequencing was conducted by

176 Eurofins Genomics Germany GmbH.

177

178 **2.4 Phylogenetic analysis**

179 Electropherograms were handled and edited using MEGA X (Kumar, Stecher, Li, Knyaz, &
180 Tamura 2018). The taxonomic identity of the obtained sequences was evaluated using BLAST+
181 (Camacho et al., 2009). Uncorrected p-distances within and between female and male samples were
182 computed using MEGA X.

183 The complete sequences of *cox1* and *rrnL* genes were downloaded from complete mitochondrial
184 genomes available in GenBank from the bivalvian clade Imparidentia *sensu* Combosch et al.
185 (2017), using the anomalodesmatan *Lyonsia norwegica* (Gmelin, 1791) (GenBank Accession
186 Number NC_034302) as an outgroup. *Ruditapes philippinarum* (Adams & Reeve, 1850) and
187 *Meretrix lamarckii* Deshayes, 1853 are DUI species whose complete mtDNA is available in
188 GenBank and were therefore included in the analysis.

189 Moreover, all the (currently) known *cox1* and *rrnL* sequences related to a sex-linked heteroplasmy
190 among Imparidentia were added to the dataset, following the list compiled by Gusman et al. (2016):
191 *Cyclina sinensis* (Gmelin, 1791), *Donax trunculus* Linnaeus, 1758, *Donax cuneatus* Linnaeus,
192 1758, *Donax faba* Gmelin, 1791, *Pseudocardium sachalinensis* (Schrenck, 1862), and
193 *Scrobicularia plana* (da Costa, 1778). The putative M-type sequence of *Solen grandis* Dunker,
194 1862 was released in GenBank under the Accession Number AB064985 (Gusman et al., 2016);
195 however, it has never been published and it is consistently placed outside the family Solenidae in all
196 preliminary analyses. As a possible contamination, we decided to exclude this sequence from our
197 dataset, along with the putative, unpublished F-type sequence extracted from the female gonad
198 (GenBank Accession Number AB064983), retaining only somatic sequences of *S. grandis*. All
199 sequences obtained for this study and downloaded from GenBank are listed in Supporting
200 Information Table S1.

201 Sequences were aligned with the T-Coffee algorithm (Notredame, Higgins & Heringa, 2000), using
202 the packages PSI-BLAST (Altschul et al., 1997), Muscle (Edgar, 2004), ProbconsRNA (Do,
203 Mahabhashyam, Brudno & Batzoglou, 2005), RNAplfold (Lorenz et al., 2011), and MAFFT (Katoh
204 & Standley 2013); the option Psicoffee was set for *cox1* amino acids and the MR-Coffee mode was
205 set for *rrnL* nucleotides. Aligned amino acids were retro translated into nucleotides using a custom-
206 tailored R script; sites with low or noisy phylogenetic signal were masked using
207 `masking_package_v1.1` (Plazzi, Puccio, & Passamonti, 2016; available at
208 https://github.com/mozoo/masking_package), retaining sites selected as phylogenetically useful by
209 at least four of the five tool Aliscore 2.0 (Misof & Misof, 2009), BMGE 1.1 (Criscuolo & Gribaldo,
210 2010), Gblocks 0.91b (Castresana, 2000), Noisy (Dress et al., 2008), and Zorro (Wu, Chatterji, &

211 Eisen, 2012). The *cox1* alignment was further subdivided into the three codon positions using a
212 custom-tailored Python script, obtaining four datasets: *cox1_1*, *cox1_2*, *cox1_3*, and *rrnL*.
213 We estimated the degree of saturation in our datasets using the substitution saturation test developed
214 by Xia and colleagues (Xia & Lemey, 2009; Xia, Xie, Salemi, Chen, & Wang, 2003); moreover, we
215 used the distmat application of EMBOSS 6.6.0 (Rice, Longden, & Bleasby, 2000) to compute
216 pairwise (uncorrected) p-distances and plotted them over pairwise ML distances computed with
217 RAxML 8.2.12 (Stamatakis, 2014). Since the *cox1_3* partition was detected to be highly saturated
218 (Supporting Information Figure S1, Supporting Information Table S2), it was excluded from
219 subsequent analyses.

220 The three remaining datasets were concatenated into the final dataset; the phylogenetic inference
221 was carried out using IQ-TREE 1.7-beta7 (Nguyen, Schmidt, von Haeseler, & Minh, 2015) with
222 1000 ultrafast bootstrap replicates (Hoang, Chernomor, von Haeseler, Minh, & Vinh, 2018).
223 Substitution models were selected using ModelFinder (Kalyaanamoorthy, Minh, Wong, von
224 Haeseler, & Jermin, 2017) and the best partitioning scheme was selected with the greedy strategy
225 implemented in ModelFinder (Chernomor, von Haeseler, & Minh, 2016; Lanfear, Calcott, Ho, &
226 Guindon, 2012). Nodes with an ultrafast bootstrap support value lower than 85 were collapsed with
227 PhyloWidget (Jordan & Piel, 2008) and the phylogenetic tree was graphically edited with
228 Dendroscope 3.6.3 (Huson & Scornavacca, 2012).

229

230 3. Results and Discussion

231 We obtained 13 sequences of *S. marginatus cox1* gene (2 from female germline, 1 from female
232 soma, 5 from male germline, 5 from male soma), and 10 sequences of the *C. glaucum cox1* gene (4
233 from female soma, 3 from male germline, 3 from male soma). Most *cox1* sequences ranged from
234 592 to 644 bp in length; due to a poor electropherogram quality, the *S. marginatus* F8 and *C.*
235 *glaucum* M7 somatic sequences were trimmed to 366 and 406 bp, respectively, and the *S.*
236 *marginatus* sequences obtained from the male gonad were trimmed to 140-231 bp, with the
237 exception of M7 (594 bp). All sequences were deposited in GenBank under the Accession Numbers
238 MN630857-MN630869 for *S. marginatus* and MN613229-MN613238 for *C. glaucum* (see
239 Supporting Information Table S1).

240 Conversely, 18 sequences of *S. marginatus rrnL* gene (3 from female germline, 3 from female
241 soma, 6 from male germline, 6 from male soma) and 21 sequences of *C. glaucum rrnL* gene (2 from
242 female germline, 5 from female soma, 7 from male germline, 7 from male soma) were produced,
243 globally ranging from 419 to 469 bp. All sequences were deposited in GenBank under the

244 Accession Numbers MN603377-MN603394 for *S. marginatus* and MN602566-MN602586 for *C.*
245 *glaucum* (see Supporting Information Table S1).

246 Variable positions of *cox1* and *rrnL* alignments are shown in Figure 1. Within-group (i.e., within-
247 mitotype) uncorrected p-distances are generally low, ranging for nucleotides from 0.0004 for *S.*
248 *marginatus* F *rrnL* to 0.0871 for *S. marginatus* M *cox1* and for amino acids from 0 for *C. glaucum*
249 *cox1* to 0.0886 for *S. marginatus* M *cox1* (Table 1). However, while average uncorrected p-distance
250 between mitotypes is comparably low for *C. glaucum* (up to 0.0071 for *cox1* nucleotides), it is two
251 or three orders of magnitude higher for *S. marginatus* (up to 0.2122 for *rrnL*), which entails that
252 average sequence similarity between F-type and M-type lineages is not higher than ~85% for *cox1*
253 and ~80% for *rrnL* (Table 1). Therefore, there is no evidence for sex-linked heteroplasmy in *C.*
254 *glaucum* *cox1* and *rrnL* genes, while we provide strong evidence of sex-linked heteroplasmy for *S.*
255 *marginatus*. The only exception to this is the *cox1* sequence of the male specimen number 7 (see
256 Figure 1): it has been extracted from the gonad, but it turned out to be a F-type sequence, most
257 likely because of somatic tissue contaminating the germline.

258 The final dataset was comprised by 95 sequences and 614 sites: the phylogenetic tree is shown in
259 Figure 2 and supports the same conclusion about sex-linked heteroplasmy. The family Cardiidae
260 was retrieved as monophyletic with an ultrafast bootstrap (UF-Boot) support value of 100. The
261 cluster *Fulvia mutica* (Reeve, 1844) + *Vasticardium flavum* (Linnaeus, 1758) is the sister group of
262 remaining cardiids, which split into Tridacninae on one side, and *Acanthocardia* + *Cerastoderma*
263 (UF-Boot support value = 100) on the other side. However, *C. glaucum* sequences were uniformly
264 distributed and there were no strongly supported clusters with respect to sex or tissue.

265 Conversely, the family Solenidae was also recovered as monophyletic (UF-Boot support value =
266 100), but the cluster of *S. marginatus* M-type sequences (i.e., sequences extracted from male
267 germline) is strongly supported to be monophyletic (UF-Boot support value = 100) and the sister
268 group of the remaining F-type sequences (i.e., sequences extracted either from male soma or from
269 female tissues), which are also strongly supported (UF-Boot support value = 99). Notably, however,
270 the cluster of F-type sequences is comprised by all included F-type sequences from the genus *Solen*:
271 F-type sequences from *S. marginatus*, which were newly obtained for this study, are the sister group
272 of a cluster with the topology *S. strictus* + *S. grandis* (UF-Boot support value = 100).

273 All this considered, we suggest the presence of the DUI phenomenon in the species *S. marginatus*.
274 Contrastingly, there is no evidence supporting the same for *C. glaucum*. Actually, we did not find
275 sex-linked heteroplasmy in the latter species, which would have strongly suggested the presence of
276 DUI (as is the case for *S. marginatus*), but this cannot be taken as a definitive proof of the absence
277 of this phenomenon.

278 As repeatedly observed (e.g., Theologidis et al., 2008; Passamonti & Plazzi, submitted) a sex-linked
279 heteroplasmy might be present, but standard PCR-based methods may fail to detect it. If the two
280 mitochondrial genomes are significantly divergent, the selected primer pair might amplify only
281 either, typically the female one: therefore, recall that a minimal amount of contaminating somatic
282 cells are always present in gonadal extracts, this would result in the amplification of the female
283 genome in all the considered tissues. Notably, most *cox1* sequences obtained from sperm in *S.*
284 *marginatus* were the shortest in the alignment because of the low quality of the electropherograms,
285 which in turn is most probably due to a lower efficiency of the universal primers on the male allele.
286 Conversely, if the divergence between the two genomes is very low (e.g., due to a young origin of
287 DUI in this species, or due to a recent masculinization event; Stewart, Breton, Blier, & Hoeh, 2009;
288 Zouros, 2013; and reference therein), two markers might be not enough to detect diagnostic
289 substitutions. Thus, additional types of data (e.g., massive sequencing of amplicons) are required in
290 order to completely dismiss the hypothesis of *C. glaucum* to be a DUI species.
291 Conversely, the detection of sex-linked heteroplasmy in *S. marginatus* is a strong clue for the
292 existence of DUI in this species; moreover, the phylogenetic reconstruction suggests that DUI arose
293 before the separation of the three species included in our dataset. However, as aforementioned the
294 only available putative M-type sequence from *S. grandis* is possibly contaminated, thus additional
295 samples of the male germline from other species of the genus *Solen* are mandatory to confirm the
296 present finding.
297 Moreover, this sex-specific pattern is neither the rule nor an exception in our phylogenetic tree.
298 Given our relatively restricted dataset, the present phylogenetic reconstruction of Imparidentia
299 mitochondrial lineages has definitely to be taken as preliminary: there is sure enough evidence of
300 little saturation in our datasets (Supporting Information Figure S1, Supporting Information Table
301 S2) and many UF-Boot support values ranged from 60 to 95. Nonetheless, the pattern of sex-linked
302 heteroplasmy in the family Veneridae is completely different: for each species (*R. philippinarum*,
303 *M. lamarckii*, and *C. sinensis*) the F- and the M- type cluster together. In this family, three
304 independent origins of the DUI phenomenon can be claimed, recalling that masculinization, which
305 is common among mytilids and would reset the divergence between the two lineages (Zouros,
306 2013), has never been directly observed for venerids (Plazzi & Passamonti, 2019; and reference
307 therein).
308 Within the family Mactridae, the DUI species *P. sachalinensis* shows a species-specific pattern
309 similar to that shown by Veneridae, but in this case a single species with sex-linked heteroplasmy is
310 currently known. The finding of more mactrid species with a sex-linked heteroplasmy will allow to
311 test for the consistency of this pattern. The situation is more difficult to disentangle for the

312 superfamily Tellinoidea. The relationships between the different species are scarcely supported and
313 the present reconstruction would be compatible with both a sex-specific and a species-specific
314 pattern. Very long branches, like those leading to *D. faba* or *S. plana* male sequences, may hamper
315 the phylogenetic inference.

316 As a second conclusion, the first Imparidentia phylogenetic tree spanning over all the available sex-
317 linked sequences, as well as over many complete mitochondrial sequences, is presented in this
318 study. It supports the hypothesis of multiple DUI origins (Figure 2), which has become more than a
319 speculation in recent years (Milani et al., 2013, 2016; Plazzi & Passamonti, 2019; Zouros, 2013).

320 More information is needed to further clarify the distribution and the patterns of DUI evolution
321 among Imparidentia, and the complete mitochondrial genomes of DUI species are mandatory in
322 order to obtain robust phylogenetic results. Finally, we report strong evidence for the existence of a
323 DUI system in the genus *Solen* (corroborating a previous claim by Gusman et al., 2016), and
324 specifically for the European species *S. marginatus*, which deserves further characterization *per se*.

325

326 **4. Conclusions**

327 The present study focused on the taxonomic coverage of the DUI (Doubly Uniparental Inheritance)
328 in bivalves. In particular, the occurrence of DUI has been investigated in two species, namely razor
329 clam *S. marginatus* and lagoon cockle *C. glaucum*.

330 Cytochrome *c* oxidase subunit 1 (*cox1*) and 16S (*rrnL*) mitochondrial regions were selected to test
331 the presence of intraspecific F and M haplotypes in these two species.

332 Results herein collected suggested the occurrence of DUI phenomenon in the razor clam *S.*
333 *marginatus*, with a divergence up to the 21% for the *rrnL* gene, but not in the lagoon cockle *C.*
334 *glaucum*. Moreover, our phylogenetic reconstruction suggests multiple origins of DUI in the
335 heterodont subclass, as well as the presence of DUI in other species of the genus *Solen*, which
336 should be furtherly investigated.

337

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341

342 **5. References**

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592

593 **Figure legends**

594 **Figure 1.** Variable sites of *cox1* and *rrnL* alignments of newly obtained sequences. “SoMa” (“*Solen*
595 *marginatus*”) is followed by the sex of the specimen (either “F” or “M”), a specimen ID and the
596 source tissue (“G” for “gonad” and “S” for “soma”; but see text for specimen SoMaM7G). Site
597 numbers referring to the complete matrix are printed above each alignment. Pink color indicates F-
598 type sequences, blue color indicates M-type sequences.

599

600 **Figure 2.** Maximum Likelihood phylogenetic reconstruction of *Imparidentia sensu* [Combosch et al.](#)
601 [\(2017\)](#) using partial sequences of the mitochondrial markers *cox1* and *rrnL*. Node support is shown
602 as ultrafast bootstrap support value as computed by IQ-TREE. Newly obtained sequences are
603 indicated with the picture of relative species, and the species names are followed by either “F” for
604 “F-type” or “M” for “M-type” and a specimen ID. The entangled blue and pink rings pinpoint
605 systems with sex-linked heteroplasmy: again, species names are followed by either “F” or “M” in
606 that case. For newly obtained sequences and whenever available, the source tissue is also shown
607 (“G” for “gonad” and “S” for “soma”; but see text for specimen SoMaM7G).

608

609 **List of Supporting Information**

610 **Supporting Information Figure S1.** Pairwise uncorrected p-distances plotted over pairwise
611 Maximum Likelihood distances for the four available datasets.

612

613 **Supporting Information Table S1.** Sequences downloaded from GenBank for the present study. If
614 the complete mitochondrial genome was available and used to extract *cox1* and *rrnL* sequences, the
615 corresponding GenBank Accession Number is given; otherwise, separated Accession Numbers for
616 *cox1* and/or *rrnL* are shown. When applicable, sex (“F” for “female”, “M” for “male”), sequence
617 progressive number and source tissue (“G” for “gonad”, “S” for “soma”) are also provided after the
618 species name. The first entry is the outgroup. Taxonomy follows the World Register of Marine
619 Species available at <http://www.marinespecies.org>.

620

621 **Supporting Information Table S2.** Test of substitution saturation for the three *cox1* codon
622 positions and for *rrnL*. The analysis was performed on fully resolved sites only, assuming an
623 asymmetrical topology and removing duplicate sequences. For the sake of clarity, given the sample
624 size only results for 32 OTUs are shown.

625 **Table 1.** Uncorrected p-distances[†] within and between F-type and M-type sequences.

		<i>coxI</i> (nt [‡])		<i>coxI</i> (aa [§])		<i>rrnL</i>				
<i>Solen marginatus</i>	F	0.0010	±	0.0007	0.0031	±	0.0023	0.0004	±	0.0004
	M	0.0871	±	0.0112	0.0886	±	0.0217	0.0006	±	0.0006
	F vs M	0.1597	±	0.0205	0.1477	±	0.0347	0.2122	±	0.0178
<i>Cerastoderma glaucum</i>	F	0.0080	±	0.0020	0.0000	±	0.0000	0.0040	±	0.0019
	M	0.0094	±	0.0029	0.0000	±	0.0000	0.0040	±	0.0021
	F vs M	0.0071	±	0.0018	0.0000	±	0.0000	0.0037	±	0.0017

626 † Mean within- and between-groups uncorrected p-distance with pairwise deletion of gaps ± standard deviation (1,000
627 bootstrap replicates).

628 ‡ nt, nucleotides.

629 § aa, amino acids.