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)

1 **Additional taxonomic coverage of the DUI (Doubly Uniparental Inheritance) in bivalves:**
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5 **Running Title: Evidence of DUI in the razor clam *Solen marginatus* Pulteney, 1799**

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28

Abstract

In animals, Doubly Uniparental Inheritance (DUI) is a major exception to the common Strict

Maternal Inheritance of mitochondria. To date, DUI has only been found in many bivalve species,

but its distribution is still unclear. Given the great species richness of the class, much effort is

needed to further investigate the occurrence of DUI in unsampled species. A compelling evidence

of DUI is generally the presence of a sex-linked heteroplasmy, where two divergent mitochondrial

lineages are found: one is isolated from the male germline, the other one is isolated from the female

germline and, normally, from the soma of both sexes. In the present study, we investigated the sex-

linked heteroplasmy in the razor clam *Solen marginatus* Pulteney, 1799 and in the lagoon cockle

Cerastoderma glaucum (Bruguière, 1789) using two mitochondrial markers (*cox1* and *rrnL*). We

found evidence of DUI in the species *S. marginatus*, with a divergence up to 21% for the *rrnL* gene,

but not in *C. glaucum*. Moreover, our phylogenetic reconstruction includes all the available data for

heterodont species with sex-linked heteroplasmy and suggests multiple origins of DUI in this

subclass, as well as the presence of DUI in other species of the genus *Solen*.

43

2

1. Introduction

45

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

among different DUI systems (Zouros, 2013; Plazzi, 2015; Plazzi & Passamonti, 2019; and reference therein), are consistent with the idea that DUI evolved multiple times in different groups of bivalves (Milani, Ghiselli, Guerra, Breton, & Passamonti, 2013; Milani, Ghiselli, & Passamonti, 2016; Zouros, 2013; Plazzi & Passamonti, 2019). However, 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 still insufficient to draw conclusions. The DUI phenomenon is apparently restricted to bivalves, although only a limited research has been carried out among gastropods (Parakatselaki, Saavedra, & Ladoukakis, 2016; Gusman, Azuelos, & Breton, 2017); however, DUI is probably widespread within bivalves (Gusman et al., 2016).

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

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

4

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

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

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

2. Materials and methods

2.1 Sample collection and tissue preparation

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

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

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

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 GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-

156 TAAACTTCAGGGTGACCAAAAAATCA-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 MgCl$_2$), 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, & Jermiin, 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

8

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.

As repeatedly observed (e.g., Theologidis et al., 2008; Passamonti & Plazzi, submitted) a sex-linked heteroplasmy might be present, but standard PCR-based methods may fail to detect it. If the two mitochondrial genomes are significantly divergent, the selected primer pair might amplify only either, typically the female one: therefore, recall that a minimal amount of contaminating somatic cells are always present in gonadal extracts, this would result in the amplification of the female genome in all the considered tissues. Notably, most *cox1* sequences obtained from sperm in *S. marginatus* were the shortest in the alignment because of the low quality of the electropherograms, which in turn is most probably due to a lower efficiency of the universal primers on the male allele. Conversely, if the divergence between the two genomes is very low (e.g., due to a young origin of DUI in this species, or due to a recent masculinization event; Stewart, Breton, Blier, & Hoeh, 2009; Zouros, 2013; and reference therein), two markers might be not enough to detect diagnostic substitutions. Thus, additional types of data (e.g., massive sequencing of amplicons) are required in order to completely dismiss the hypothesis of *C. glaucum* to be a DUI species.

Conversely, the detection of sex-linked heteroplasmy in *S. marginatus* is a strong clue for the existence of DUI in this species; moreover, the phylogenetic reconstruction suggests that DUI arose before the separation of the three species included in our dataset. However, as aforementioned the only available putative M-type sequence from *S. grandis* is possibly contaminated, thus additional samples of the male germline from other species of the genus *Solen* are mandatory to confirm the present finding.

Moreover, this sex-specific pattern is neither the rule nor an exception in our phylogenetic tree. Given our relatively restricted dataset, the present phylogenetic reconstruction of Imparidentia mitochondrial lineages has definitely to be taken as preliminary: there is sure enough evidence of little saturation in our datasets (Supporting Information Figure S1, Supporting Information Table S2) and many UF-Boot support values ranged from 60 to 95. Nonetheless, the pattern of sex-linked heteroplasmy in the family Veneridae is completely different: for each species (*R. philippinarum*, *M. lamarckii*, and *C. sinensis*) the F- and the M- type cluster together. In this family, three independent origins of the DUI phenomenon can be claimed, recalling that masculinization, which is common among mytilids and would reset the divergence between the two lineages (Zouros, 2013), has never been directly observed for venerids (Plazzi & Passamonti, 2019; and reference therein).

Within the family Mactridae, the DUI species *P. sachalinensis* shows a species-specific pattern similar to that shown by Veneridae, but in this case a single species with sex-linked heteroplasmy is currently known. The finding of more mactrid species with a sex-linked heteroplasmy will allow to test for the consistency of this pattern. The situation is more difficult to disentangle for the

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

As a second conclusion, the first Imparidentia phylogenetic tree spanning over all the available sex-linked sequences, as well as over many complete mitochondrial sequences, is presented in this study. It supports the hypothesis of multiple DUI origins (Figure 2), which has become more than a speculation in recent years (Milani et al., 2013, 2016; Plazzi & Passamonti, 2019; Zouros, 2013). More information is needed to further clarify the distribution and the patterns of DUI evolution among Imparidentia, and the complete mitochondrial genomes of DUI species are mandatory in order to obtain robust phylogenetic results. Finally, we report strong evidence for the existence of a DUI system in the genus *Solen* (corroborating a previous claim by Gusman et al., 2016), and specifically for the European species *S. marginatus*, which deserves further characterization *per se*.

4. Conclusions

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

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

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

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592

Figure legends

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

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

List of Supporting Information

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

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

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

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

		cox1 (nt[‡])			cox1 (aa[§])			rrnL		
Solen marginatus	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
Cerastoderma glaucum	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.

cox1 (nt[‡])   cox1 (aa[§])   rrnL