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1 **RESEARCH NOTE**

2

3 **Putative sex-linked heteroplasmy in the tellinid bivalve *Limecola balthica* (Linnaeus, 1758)**

4

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9

10 Doubly uniparental inheritance (DUI) is a remarkable exception to the maternal inheritance of
11 mitochondria in metazoans. Found in bivalves, DUI is characterized by females transmitting ‘female’
12 (F) mitochondria that persist in oocytes and in somatic tissues of both sexes, whereas males pass on
13 ‘male’ (M) mitochondria that persist in the male germ line (reviewed by [Zouros, 2013](#)). The evolution
14 and maintenance of this feature remains unclear, especially as its prevalence among bivalve species
15 is still unknown. DUI can be detected by looking for distinct mitochondrial DNA sequences (or
16 mitotypes) in the sperm and somatic tissues of males. Although it has been found in about 40 species
17 ([Dégletagne et al., 2015](#)), authors have pointed out the difficulties of detecting DUI, as the genetic
18 distance between the F and M mitotypes can be very high, reaching 50% amino-acid divergence in
19 some unionoids ([Doucet-Beaupré et al., 2010](#)). Testing for the presence of DUI is therefore of prime
20 interest for understanding how this feature spread within the Bivalvia and how many times it
21 evolved.

22

23 We set out to test for the presence of DUI in *Limecola balthica* (Linnaeus, 1758) (previously known as
24 *Macoma balthica*; [Huber, 2015](#)). This marine bivalve is gonochoristic ([Lammens, 1967](#)) and belongs
25 to the family Tellinidae, for which DUI has not been reported to date. The closest relative of *L.*

26 *balthica* for which DUI has been detected is another tellinoidean, *Donax trunculus* (Theologidis *et al.*,
27 2008). A nuclear genome scan on *L. balthica* suggested that mito–nuclear genetic incompatibilities
28 may be involved in maintaining barriers to gene flow among European populations, as multiple genes
29 involved in ATP production and transport were detected as F_{ST} outliers (Pante *et al.*, 2012). If *L.*
30 *balthica* is characterized by DUI, mito–genetic incompatibilities could be expressed not only between
31 the F mitotype and nuclear genes (in somatic tissues and oocytes in females), but also between the
32 M mitotype and nuclear genes in sperm of interpopulational hybrids. This context makes the search
33 for DUI particularly relevant for this species.

34

35 Ten individuals of *L. balthica* were sampled on the sandy mudflat of Aytré, France (46°07'32.51" N,
36 1°7'42.94" W). This population belongs to the *rubra* lineage, based on mitochondrial and nuclear
37 data (Becquet *et al.*, 2012). Sampling was conducted on 20 April, 2016, when most individuals had
38 fully mature gametes (Saunier, 2015). Gonads were nicked with a clean scalpel and gametes were
39 pipetted out. Individuals were sexed by observing gametes under a light microscope; individuals that
40 could not be unambiguously sexed were left out of the study. Gametes and foot tissue were sampled
41 for one female and four males; gametes from a fifth male were sampled. Total DNA was extracted
42 from ≤ 15 mg of tissue (gametes and foot muscle) using the Nucleospin Tissue kit (Machery-Nagel,
43 Düren, Germany) following the manufacturer's protocol. DNA was quantified using a Nanodrop ND-
44 2000 spectrophotometer (Thermo Scientific, Waltham, USA) and gel electrophoresis (1% agarose).

45

46 To investigate the presence of sex-linked heteroplasmy, three mitochondrial genes that are evenly
47 spaced on the mitogenome of *L. balthica* were selected: *cox1*, *cox2* and *rnl* (Saunier *et al.*, 2014).
48 DNA amplifications were performed using the Perfect Taq PCR kit (5 Prime; Dominique Dutscher,
49 Brumath, France) with the following conditions: 0.75 unit of Taq, 1X reaction buffer, 200 μ M of each
50 dNTP, 0.2 μ M of each primer, 1.5 mM of $MgCl_2$, 15-60 ng of template DNA in a total volume of 25 μ l.
51 Reactions were performed on a SensoQuest (Göttingen, Germany) thermal cycler; thermal profiles

52 are provided in Table 1. We ran both an extraction blank and PCR negative controls. PCR purification
53 and Sanger sequencing (ABI 3730xl) were done by GATC (Konstanz, Germany).

54

55 A first attempt to amplify the mitochondrial *cox1* and *cox2* genes was made using primers specific to
56 *L. balthica* (Table 1). While sharp bands were obtained from ovocytes and foot muscle from both
57 sexes, sperm samples yielded very little to no amplification (example gel in Fig. 1). When sperm PCR
58 was concentrated enough to attempt sequencing, the same haplotype was systematically retrieved
59 from sperm and muscle, for each individual tested (this was true for both *cox1* and *cox2*). The
60 sequences retrieved were blasted (blastn; Zhang *et al.*, 2000) and showed matches of 99–100% with
61 known *cox1* and *cox2* haplotypes (Luttikhuisen *et al.*, 2003; Saunier *et al.*, 2014). We relaxed the PCR
62 conditions by (1) attempting a *cox1* amplification using primers that should amplify broadly among
63 molluscs (Folmer *et al.*, 1994; Table 1) and (2) running a gradient PCR on *cox2*, but the same result
64 was obtained (no sequencing was attempted).

65

66 We therefore targeted a more conserved mitochondrial gene, *rnL*, with broad-spectrum primers
67 (Palumbi *et al.*, 2002; Table 1); sharp PCR amplifications were obtained from sperm, ovocytes and
68 foot tissues from all individuals of both sexes. Sequences were initially 470 bp long and quality-
69 trimmed to 287 (sperm) and 284 bp (all other sequences). Trimmed sequences from ovocytes, male
70 and female muscle tissues were identical (GenBank acc. no. KX831970) and blasted to known *L.*
71 *balthica* haplotypes (blastn: 100% query coverage, 99% identity, e-value < 1e-140; best matches:
72 Saunier *et al.*, 2014). The two *rnL* haplotypes (0.7% divergence; GenBank acc. nos KX831969,
73 KX838910) recovered from trimmed sperm sequences blasted to other tellinid bivalves (74-84%
74 query coverage, 81-83% identity, e-value < 1e-40; *Pinguitellina* sp., AB751357; *Tellina staurella*,
75 AB751346; *Moerella rutila*, AB751334; *L. balthica*, KM373201; *Nitidotellina iridella*, AB751340;
76 *Loxoglypta* sp. AB751338, AB751339).

77

78 These results taken together (failed amplification for sperm at *cox1* and *cox2* and sex-linked
79 heteroplasmy at *rnL*) suggest that the presence of DUI should be further investigated in *L. balthica*. In
80 the case of *cox1* and *cox2*, we were able to get very faint PCR product for some sperm samples. In
81 the presence of DUI, these products might reflect contamination of sperm samples by somatic cells
82 containing the F mitotype (Venetis *et al.*, 2006), a pattern that was also reported for *D. trunculus*
83 (Theologidis *et al.*, 2008). At *rnL*, the Kimura 2-parameter distance between the ovocyte/muscle
84 haplotype and the sperm strict consensus sequence was 25.7% (alignment in Supplementary
85 Material). This is on par with the F/M mitotype distance observed for *rnL* in *D. trunculus* (Kimura 2-
86 parameter = 36.6%; 68% of our sequenced gene fragment overlaps with that of *D. trunculus*;
87 Theologidis *et al.*, 2008). The male sequences were 3 bp longer than the female ones, a pattern that
88 was also reported in e.g. *Meretrix lamarckii* (Bettinazzi *et al.*, 2016). We thus conclude that DUI is
89 probably present in the Tellinidae, a bivalve family for which DUI had not been detected before.

90

91 Future work should now focus on sequencing the full male mitochondrial genome for *L. balthica* in
92 order to measure the overall divergence between male and female mitotypes and evaluate its
93 functional significance.

94

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100

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145 **Table 1.** Primers used for PCR amplification and thermal profiles.

146

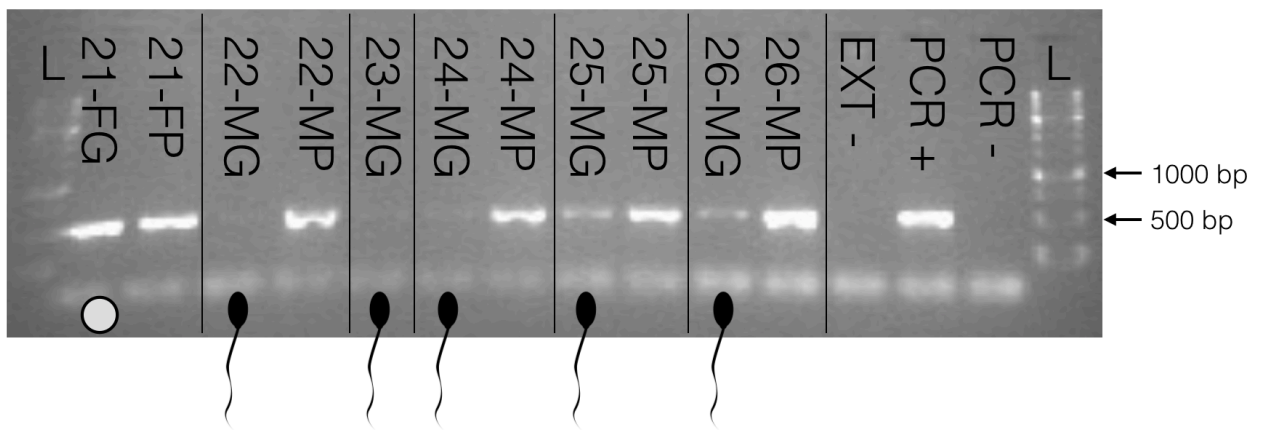
| Gene | Primer name | Sequence (5' - 3') | Tm (°C) | Cycle | Size (bp) | Reference |
|-------------|-------------|----------------------------|---------|---------------------------|-----------|-----------------------------------|
| <i>cox1</i> | 1 CO1F | TTAGTGACTTCACACGGTTTGC | 55.6 | 1-2: 93:30,57:30,72:40 | 437 | Luttikhuizen <i>et al.</i> (2003) |
| | 2 CO1R | AGTACAGGTATAGCAACCACCAG | 56.1 | | | |
| | 3 LCO1490-F | GGTCAACAAATCATAAAGATATTGC | 51.0 | 3-4: 93:30,45:30,72:30 | 580 | Folmer <i>et al.</i> (1994) |
| | 4 HCO2198-R | TAAACTTCAGGGTGACCAAAAAATCA | 55.3 | | | |
| <i>cox2</i> | 5 CO2F | GGTTGTGCTAGTGGTTTGGG | 59.4 | 5-6: 93:30,57:30,72:40 | 516 | This study |
| | 6 CO2R | AAACTGCCCATACCGGAAGC | 59.4 | | | |
| <i>rnL</i> | 7 16Sar-L | CGCCTGTTTATCAAAAACAT | 49.2 | 7-8: 93:30,43:30,72:30 | 515 | Palumbi <i>et al.</i> (2002) |
| | 8 16Sbr-H | CCGGTCTGAACTCAGATCACGT | 58.6 | | | |

147

148 Size: PCR fragment size calculated based on sequence from the full *cox1*, *cox2* and *rnL* genes
 149 (complete genome from individual A6, KM373201; Saunier *et al.*, 2014). Cycle: thermal profiles
 150 (temperature in °C: time in s; 30-32 cycles) following an initial denaturation step at 93 °C for 3 min
 151 and preceding a final elongation step at 72 °C for 5 min.

152 **Figure 1.** Example of PCR results on mitochondrial DNA amplification from gamete and foot tissue of
153 *Limecola balthica*. Gel electrophoresis (1% agarose) for the *cox2* gene. Specimens are labeled as
154 follows: individual number followed by a dash, followed by the sex of the individual (M, male; F,
155 female) and the tissue type (G, gamete; P, foot tissue); L, 1kb DNA ladder; EXT, the PCR amplification
156 of extraction negative control; PCR + and -, PCR positive and negative controls, respectively.

157



159 **Supplementary Material.**

160

161 Alignment of *rnL* sequences retrieved from sperm (24-MG) and from foot tissue (24-MP) from
162 a single individual of *Limecola balthica*, showing the extent of intra-individual sequence
163 divergence. Indels are symbolized with semi-colons. Sequence differences (point mutations
164 or indels) are symbolized with filled circles. The coordinates on the *rnL* gene precede the
165 sequences; this number corresponds to the position on the gene based on the reference
166 genome A6 from foot muscle (GenBank Acc. KM373201). The raw chromatograms are
167 presented for the 1104-1113 portion of the alignment (black box). Alignment was performed
168 in Sequencher v. 5.4.1.

169

24-MG #941 accccatcgagcttggttacttatactatTTTTTA:tatg
 24-MP accctgttgagcttggt:aaggctagt:tctcttagaatt
 • • • • • • • • • • • •

24-MG #981 tgag:gatgTTTTgtcggggctgataggga:gggaaagaa
 24-MP agagctatgTTTgttcggggctga:agggaagggagataa
 • • • • • • • • • • • •

24-MG #1021 tgcctcctttgTTaata:tgaagatccggcattaggccga
 24-MP tgccttctatgTTa:tagaaaagatcc::aatttgattga
 • • • • • • • • • • • •

24-MG #1061 ttggaagaagatagttaccgtggggataacagcgcaat:g
 24-MP ttggtggaagatagttaccacagggataacagcgcaatca
 • • • • • • • • • • • •

24-MG #1101 ttccattaagcacatatttgagggggcggttgcgacctc
 24-MP ttctgat:aaggccatatttaaggagtggTTTgcgacctc
 • • • • • • • • • • • •

24-MG #1141 gatgTTgaatcaggattatctattaggtgcagcagcttag
 24-MP gatgTTgaatcaaaatt:tctactaggtgtagcagcttag
 • • • • • • • • • • • •

24-MG #1181 taagtgggactgTtcgTcctttgaaatcctacgtgatctg
 24-MP taagtgggactgTtcgTcctttaaaattttacgtgatctg
 • • • • • • • • • • • •

24-MG #1221 agttcagaccgg
 24-MP agttcagaccgg

