Discordant population structure inferred from male- and female-type mtDNAs from *Macoma balthica*, a bivalve species characterized by doubly uniparental inheritance of mitochondria

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Abstract

Doubly Uniparental Inheritance (DUI) of mitochondria is a remarkable exception to the Strictly Maternal Inheritance (SMI) in metazoans. In species characterized by DUI --almost exclusively gonochoric bivalve mollusks--, females (F) transmit mitochondria to offspring of both sexes, while males (M) pass on their mitochondria exclusively to their sons. Under DUI, males are heteroplasmic, somatic tissues containing F-transmitted mtDNA and gametic cells containing M-transmitted mtDNAs. The aforementioned transmission routes make M- and F-transmitted mtDNA interesting as sex-specific markers which can differ in their effective population sizes, mutation rates, and selective constraints. For these reasons, looking at both markers can provide significant insights into the genetic structure of populations and investigate its determinants. In this study, we document differences in genetic diversity, divergence, inter-populational genetic differentiation and biogeographic structure between M- and F-type *cox1* mt genes in the Baltic tellin (*Macoma balthica*) to test whether *cox1m* and *cox1f* genes bear the marks of similar phylogeographic histories. Both markers were sequenced for 302 male individuals sampled from the North Sea to the Gironde Estuary (Southern France). Nucleotide diversity and net divergence were over twice higher in *cox1m*

compared to *cox1f*. A strong southward decrease in nucleotide diversity was observed only at *cox1m*. Genetic differentiation between northern and southern populations was nearly 3 times higher at *cox1m* compared to *cox1*f (global ΦST = 0.345 and 0.126 respectively) and the geographic localization of the strongest genetic break significantly differed between the markers (Finistère Peninsula at *cox1f*; Cotentin Peninsula at *cox1m*). A higher mutation rate, relaxed negative selection and differences in effective population sizes (depending on locations) at *cox1m* could explain differences in population genetic structure. As both F- and M-type mtDNAs interact with nuclear genes for oxidative phosphorylation and ATP production, geographical discordances in genetic clines could be linked to mito-nuclear genetic incompatibilities in this system.

Keywords

Doubly Uniparental Inheritance, disruption, heteroplasmy, comparative biogeography, phylogeography, discordance, hybrid zone, genetic cline, mitochondria

Introduction

Some species show a remarkable exception to the maternal inheritance of mitochondria in metazoans: the doubly uniparental mode of inheritance (DUI). In this system, both males and females can transmit their mitochondria. The former transmits “female-inherited” (F-type) mitochondria to all their progeny and the latter pass on “male-inherited” (M-type) mitochondria to their male offspring, where the male mitogenomes (mt) are quartered in male germ line and gametes (reviewed in Zouros, 2013). To date, DUI species have only been discovered in the class Bivalvia, with over 100 DUI species (Gusman et al 2016) among the about 11,000 contained in this taxon (Huber 2010, 2015). They are all gonochoric, except for the hermaphroditic mussel *Semimytilus algosus* (Lubosny et al 2020). More than a simple peculiarity, DUI is suspected to play a role in sex-determination and gonad differentiation (Zouros 2000, Breton et al 2011, Guerra et al 2017, Capt et al 2018, 2019), and could well be involved in population structure through intrinsic (*e.g.* genetic incompatibilities; Saavedra et al 1996) and extrinsic (*e.g.* selection and demography, Stewart et al 1996) factors.

In DUI species, the divergence between F-type and M-type mitogenomes is variable but generally high, ranging from 6 to over 50% (reviewed in Breton et al 2007 and Gusman et al 2016), which questions the maintenance of mito-nuclear genetic coadaptation. Indeed, both F- and M-type mitochondria can be found in males and females but in majority, females are

homoplasmic for the F-type mtDNA whereas males are heteroplasmic, accommodating two highly divergent mitogenomes (F-type in somatic tissues and M-type in sperm). The presence of the M-type mtDNA in somatic tissues is considered as a paternal leak due to elimination or segregation failure of sperm mitochondria in female or male embryos, respectively (Milani et al 2012). Both F- and M-type mt lineages show rapid molecular evolution compared to other animals, the M-type mtDNA usually evolving faster than the F-type mtDNA (Zouros et al 2013). Coevolution and coadaptation of mitochondrial and nuclear genes are required for efficient cellular energy production (*i.e.* oxidative phosphorylation OXPHOS) and mito-nuclear genetic incompatibilities (MNIs) can lead to a desynchronization of this machinery (Burton & Baretto 2012,2013). DUI could, therefore, bear on the maintenance of genetic structure among populations of highly dispersive bivalve species at small spatial scales, and provide key insight into the establishment and maintenance of local adaptation.

Indeed, barriers to gene flow can arise and be maintained by a multitude of environmental and/or intrinsic factors (Barberousse et al 2010), from ecological isolation to genetic incompatibilities. Hybrid zones, which correspond to transition regions between spatially separated genetic stocks, are “natural laboratories” to study the interactions between intrinsic barriers and the environment, and the processes of adaptation and speciation.

Macoma balthica, a species in which DUI has recently been detected (Pante et al 2017), is a noteworthy model species to study hybrid zones in marine ecosystems (Strelkov et al 2007, Riginos & Cunningham 2007). It has a wide distribution range spanning from the west pacific coasts, in Japan and from Alaska to Oregon (USA, Luttikhuizen, 2003) to the North Atlantic, where the species is found in the west from Arctic to Virginia (USA; Meehan, 1985) and in the east from the north of Russia (Hummel et al, 1997) to the Arcachon Basin (Hily 2013 and this publication). The succession of glaciation and inter-glaciation periods has resulted in colonization events of the Atlantic marked by repeated isolation, invasion, and re-colonization events (Nikula et al, 2007). These episodic colonization events have created multiple opportunities for secondary contacts between different genetic stocks and the establishment of several hybrid zones in the Atlantic. Two subspecies of *M. balthica* co-occur in North Atlantic: a Pacific lineage (*M. b. balthica*) present in the Baltic Sea and the White Sea, and an Atlantic lineage (*M. b. rubra*) present in the Norwegian Sea, the North Sea and along the British coasts, down to the southern range limit of the species (Väinölä 2003, Luttikhuisen et al 2003, Nikula et al 2008). In Europe, genetic breaks were described in the Kattegat Detroit between Sweden and Denmark (Nikula et al. 2007, 2008) and in the North Finistère between the Channel and the Atlantic Ocean. Southern populations of *M. b. rubra*

exhibit F-type mtDNA signatures consistent with long-term isolation in the glacial refugium of the Bay of Biscay: high genetic diversity relative to previously glaciated areas, high prevalence of private alleles, and a sharp genetic break separating them from northern populations (Becquet et al 2012). Multiple genes involved in the oxidative phosphorylation (OXPHOS) system (including genes coding for ATP synthase subunits and an ADP/ATP transporter were detected as significantly differentiated among southern and northern populations by an F_{ST} scan (Pante et al 2012, 2019). These results suggested that incompatibilities between mitochondrial and nuclear genes encoding OXPHOS functions could be involved in endogenous barriers to gene flow in *M. b. rubra*.

Here, we present sharp differences in genetic diversity, divergence and population genetic structure between F- and M-type mtDNAs of *M. b. rubra* male individuals, in particular along the northeast Atlantic hybrid zone first described in Becquet et al (2012). Given the typically higher evolution rate and the potentially relaxed selection pressures acting on the M-type mitogenome, these comparative data allow us to start testing whether mitotypes are sufficiently different to cause genetic incompatibilities impeding gene flow in *M. b. rubra*.

Materials and Methods

Sampling

Individuals were collected from a total of 14 sampling sites ranging from Arcachon (southern range limit of the species, France) to Le Crotoy (Somme Bay, northern France) and from Kruiningen (the Netherlands) to Sylt (Germany) (Table S1). Individuals from sampling sites on the French coasts were treated as follows: 70 to 100 adults from 11 mm to 23 mm were randomly collected live at sexual maturity between 4th and 23rd of April 2018 at 9 locations ranging from the Bay of Biscay in southern France to Somme Bay (Table S1). Individuals were then held in aquaria until dissection at the LIENSs laboratory (LIttoral ENvironnement et Sociétés) in La Rochelle, France, with water temperature maintained at 10°C. They were fed with a multispecific microalgal mixture every other day and dead or dying individuals were removed daily. For each individual, the adductor muscle was carefully severed to separate the two valves, without damaging the gonad, and a sample of the mantle was taken. Sex and gonadal maturation stage were then determined with a dissecting microscope (x100 to x400). Two types of tissue samples were collected: a gonadic sample and a somatic sample (mantle). All tissue samples were flash-frozen in liquid nitrogen, and then stored at -80°C

until DNA extraction. At each step of this protocol, dissecting tools and bench surfaces were thoroughly cleaned in successive baths of 10 % bleach, demineralized water, and 95 % EtOH as to avoid DNA cross-contamination. Using this dataset, we checked that male mitochondria were limited to male gonads (*i.e.* absent from other male tissues and from females); thus making molecular sexing permissible (Le Cam et al in prep).

For the other sampling sites, whole individuals had been collected for a previous study (Becquet et al. 2012 and the BIOCOMBE project) and were preserved in 95% EtOH. A 3 mm^3 piece of tissue was sampled from the gonad, gDNA was purified and quantified as detailed below, and PCR amplifications were attempted with both the *cox1m* and *cox1f* primers. Individuals for which both gene regions could be amplified were considered as males and included in subsequent analyses.

Molecular Analyses

Total DNA was extracted with the Nucleospin Tissue Kit (Macherey Nagel), following the manufacturer's instructions. In Saint-Vaast-la-Hougue (VAA) and Mont-Saint-Michel (MSM), most specimens were sexually undifferentiated at the time of sampling. Therefore, for these samples and for the samples from all the other "non-French" localities, sex was determined by checking for the amplification of both male and female *cox1* markers in gonad DNA. DNA purity and potential contaminants were checked using a NanoDrop2000.

Specific primers were designed to independently amplify the 5' portion of the *cox1* gene of the M-type and F-type mitogenomes (*cox1m* and *cox1f* respectively). We used female and male mitogenomes from Saunier et al. (2014) and Capt et al. (2020), as well as transcriptomic sequences from Pante *et al.* (2012) to design these primers. Polymerase chain reaction (PCR) amplifications of *cox1f* and *cox1m* mitochondrial DNA regions were performed using the following primers (*cox1m*: cox1m14641F ATAGCTGGCCTGGTGTTTAGG, cox1m15560R TTGGACCCTTTCGAGCCAAG; *cox1f*: cox1f5434F TTAGTGACTTCACACGGTTTGC, cox1f6032R TGGGAAATAATCCCAAACCCG). Amplifications were realized in a total volume of 25 µL, with 0.1 µL of Taq Polymerase 5 $U.\mu L^{-1}$ (TaKaRa Ex Taq® Kit $MgCl_2$ Free Buffer) (TaKaRa Ex Taq, TaKaRa Bio, Shiga, Japan), 2.5 µL PCR Buffer 10X, 1.5 µL $MgCl_2$ 25 mM, 1 µL dNTP 2.5 mM each, 0.6 µL each primer 10 µM and 17.7 Milli-Q water, and 1 to 20 ng of template DNA. SensoQuest thermal cyclers (Göttingen, Germany) were used to perform the following PCR cycling profiles: (i) for the *cox1f*, 2 min of initial denaturation at 94°C, then 30 cycles consisting of 45 sec at 94°C followed by 30 sec of annealing at 57°C

and 40 sec of elongation at 72°C, and a 5 min final step of elongation at 72°C, and (ii) for the *cox1m*, 2 min at 94°C, then 30 cycles of 30 sec at 94°C, 30 sec at 60°C and 55 sec at 72°C, and a final elongation for 5 min at 72°C. PCR success (*i.e.* amplicon concentration, specificity and absence of amplicons for PCR and extraction negative controls), was tested on 1% agarose gels. PCR products were then sent to Eurofins GATC Biotech GmbH (Konstanz, Germany) to be purified and Sanger sequencing was performed on both directions.

Sequence Analyses

All sequences of *cox1f* sequences and *cox1m* fragments were quality-controlled and aligned in Geneious Prime 10.2.6 (Kearse et al 2012). Consensus sequences were trimmed to 479 bp and 676 bp for *cox1f* and *cox1m*, respectively. *cox1m* sequences, trimmed at the same length as *cox1f* (479bp), were used to compute further comparative analyses. Haplotype sequences and their frequencies were computed in R v3.3.3 (R Core Team, 2017) using the *pegas* package v0.11. (Paradis, 2010). In order to model intraspecific relationships among haplotypes, for both *cox1f* and *cox1m*, median-joining networks (Bandelt et al., 1999) were constructed with PopART v1.7 software (Leigh & Bryant, 2015). For each marker, we constructed a maximum-likelihood phylogenetic tree using IQ-TREE software (http://www.iqtree.org/) with 1,000 bootstrap replicates, and nucleotide acid-substitution models were inferred using ModelFinder with Bayesian information criteria (Kalyaanamoorthy et al. 2017; Nguyen et al. 2015). For *cox1f*, data from Nikula et al (2003) were used for phylogenetic cross-interpretation. From these genealogical relationships, mitochondrial lineages were defined (haplogroups) and their population relative frequency estimated.

Genetic diversity indices and population differentiation analyses at *cox1f* and *cox1m* were estimated only for sampling sites with a minimum sample size of 10 individuals. Genetic diversity indices were calculated using Arlequin v3.5.2.2 (Excoffier et al., 1992): haplotype number (H), haplotype diversity (Hd) and nucleotide diversity (π Tajima, 1983). Index distributions were statistically compared using paired non-parametric Mann-Whitney-Wilcoxon tests. Genetic differentiation was estimated in Arlequin using the Φ_{ST} fixation index and its statistical significance was tested by performing 10,000 permutations (Excoffier et al., 1992).

An analysis of molecular variance (AMOVA) was carried out to evaluate the genetic structure among geographic regions and overall sampling sites using 10,000 permutations with Arlequin v3.5.2.2 (Excoffier et al., 1992). We used jModeltest2 (Darriba et al., 2012;

Guindon & Gascuel, 2003) to choose the Tamura-Nei model of nucleotide substitution (with gamma shape parameters of 0.1150 and 0.1450 for *cox1f* and *cox1m*, respectively) as a measure of haplotype distances in Arlequin. Based on discordances in the distribution of the genetic diversity at *cox1f* and *cox1m*, several hierarchical groupings of subpopulations were tested for each marker to determine the best geographical delineation of genetic structure. Different measures of populations differentiation (pairwise and AMOVA) were also carried out using conventional F- statistics.

To test for differences in selective pressure on the female and male mitochondrial sequence sets, the Tajima's D (Tajima, 1989) test for deviations from the neutral theory model for a population of constant size was calculated in Arlequin v3.5.2.2 (Excoffier et al., 1992) for each marker and sampling sites. Its statistical significance was tested with 10,000 permutations. The McDonald and Kreitman test (McDonald and Kreitman 1991) was used to test for adaptive evolution of the male mitogenome in comparison with the female's (Chacon et al., 2020). The test was conducted in DNAsp (Librado and Rozas 2009) overall data and within genetically homogeneous groups at the north and south of the hybrid zone.

Because the difference in effective population size (Ne) between males and females is often invoked in the literature as a factor influencing the population genetics of M- and F-mitogenomes, we estimated this parameter based on the method used in Ladoukakis et al (2002), the ratio of the male and female genome effective size N_e was estimated based on:

(i) the expected effective number of alleles under neutrality formulated by Crow and Kimura (1970, equation 7.2.5)

$$n_e = C N_e \mu + 1$$ (not $n_e = C n_e \mu + 1$ as written Ladoukakis et al 2002)

C being variable according to the mutation model and the genome (mitochondrial *vs* nuclear). Conversely to Ladoukakis et al 2002, because we estimated a male mitogenome mutation rate (μ_M) twice as high as μ_F, the mutation rate term was μ was kept in the equation:

$$\frac{N_{eF}}{N_{eM}} = \frac{n_{eF}-1}{n_{eM}-1} x \frac{\mu_M}{\mu_F}$$

(ii) an estimate of n_e given by Zouros (1979, equation 15)

$$n_e = \frac{1}{1 - Hd}$$

From equations (i) and (ii):

$$\frac{N_{eF}}{N_{eM}} = \frac{\frac{1}{1 - Hd_F} - 1}{\frac{1}{1 - Hd_M} - 1} x \frac{\mu_M}{\mu_F}$$

This method solely relies on the estimate of haplotype diversity H_d and the mutation rate μ. The ratio μ_M / μ_F is independent of the time calibration point and can be formulated as $\frac{\mu_M}{\mu_F} = \frac{substitutions_per_site_M}{substitutions_per_site_F}$. The mean genetic distance (Kimura 2-parameters distance, Kimura 1980) among sequences of *cox1m* and *cox1f* haplotypes from Crildumersiel (CRI) and Arcachon (ARC) as extremes on the haplotype networks (Fig 1) were used.

Results

A total of 655 individuals from 14 sampling sites of the French Atlantic coast were dissected, and optical microscopy sexing reported 248 males, 185 females and 222 undifferentiated individuals. Undifferentiated specimens might have released their gametes or resorbed them, as lipid droplets were observed in some of the undifferentiated gonads. Molecular sexing was used to detect males in undifferentiated individuals.

Both *cox1m* and *cox1f* were successfully sequenced for 302 male individuals from 14 sampling sites (Table 1). Sampling size per site ranged from 3 to 33 overall sampling sites and from 28 to 33 when only considering sampling sites in France, apart from Arcachon (N=10).

Among the 302 studied individuals, 34 and 61 haplotypes were found for *cox1f* and *cox1m*, respectively. Among these, 59% were singletons at cox1f and 80% at *cox1m*. Median joining networks showed a similar geographical pattern with three main haplogroups encompassing mainly the northern/central and southern samples (Figure 1 A and C). Nonetheless, the networks revealed important differences between markers. First, *cox1m* presented a higher diversity, but a similar divergence level: a maximum of 25 and 26 mutation steps separated the most divergent haplotypes for *cox1f* and *cox1m*, respectively. We estimated maximum divergence rates of 0.027 s/s and 0.028 s/s for the m and f datasets, respectively. This difference is not significant based on the relative-rates test (No significant comparisons out of 2074 https://github.com/lyy005/Relative-Rate-Test).

Phylogenetic inferences from the *cox1f* dataset combined with Nikula et al (2007) data revealed that our dataset is only composed of the *M. balthica rubra* lineage (Supplementary material figure S1). The phylogenetic tree and the haplotype network were congruent: the "northern" haplogroup corresponded to the b2_b3 *M. b. rubra* lineage and our data revealed 2 sub-lineages within b1 lineage, b1a and b1b. The phylogeny of *cox1m* revealed a similar pattern, with three clades m1, m2, m3 (Supplementary material figure S2).

The haplogroup relative frequencies showed different patterns (Figure 2). The *cox1m* haplogroup m3 was present only in the North Sea and east Channel samples while the *cox1f* haplogroup b2/b3 encompassed individuals from the North Sea to the west English Channel (hybrid zone). Also, the haplogroup b1a of *cox1f* was shared among the North Sea, the English Channel and Atlantic samples, whereas the central haplogroup m2 of *cox1m* was found only in the North Sea and the Channel Sea. Interestingly, clear genetic breaks were revealed for each marker, and they were not located at the same geographical location. For the *cox1m*, the Cotentin peninsula represented a sharp transition zone between the haplogroups m1/m2 and m3, which was confirmed by pairwise Φ_{ST} values (Table S2). Two homogenous populations were observed north of the Cotentin peninsula and the south of the Finistère (within group pairwise Φ_{ST} ranged from -0.05 to 0.04) and these populations were highly differentiated (between group pairwise Φ_{ST} ranged from 0.38 to 0.61). In between, the BRI and MSM sampling sites were very differentiated (between group pairwise Φ_{ST} ranged from 0.50 to 0.61) from the North of the Cotentin but they were also weakly yet significantly different from some sites at the South of the Finistère (between group pairwise Φ_{ST} ranged from 0.02 to 0.04). For *cox1f*, the pattern was more complex. The haplogroup b1a was composed of haplotypes found in majority at the south of the Finistère (Fig 2) and samples from either side of the Finistère were highly divergent (pairwise Φ_{ST} ranging from 0.41 to 0.79, Table S3). On both sides of the Finistère peninsula, significant population substructure was detectable: the Pont-Mahé Bay (MAH) sampling site was mildly differentiated from Saint-Brevin-les-Pins (BRE) and Aytré (AYT) (pairwise Φ_{ST} of 0.13 and 0.08 respectively, table S3). Also, the relative frequencies of the 3 haplogroups were variable in the north of the Finistère peninsula, resulting in a patchy distribution of the genetic diversity (Fig 2). Saint-Brieuc (BRI) for instance was highly differentiated from all other northern sites except from MSM (Mont-Saint-Michel). Based on these results, genetically homogenous sampling sites were merged in 3 geographical groups (Table 1) and used in subsequent analysis (North, Center and South). Sampling sites within the hybrid/Finistère group showed genetic differentiation only for *cox1m*.

Haplotypes from the extreme haplogroups were rarely shared within one individual (Fig1 B), and while 17 individuals exhibited the haplogroup b2/b3 at *cox1f* and the haplogroup m3 at *cox1m*, only 3 individuals shared the *cox1f* haplogroup b1b and the *cox1m* haplogroup m1. Also, only 1 individual presented both the *cox1f* haplogroup b1b and the *cox1m* haplogroup m2, suggesting non-random mating or lethal haplotype combinations. Noteworthily, most of these individuals were found in the hybrid zone sampling sites (18 out of 22). These results were confirmed by the significant linkage disequilibrium test carried out among haplogroups for phased data (T_2 test df=6, $p<0.001$). The heatmap presented in Figure 3 illustrates the frequency of all the possible haplogroup associations and the associated correlation values.

The level of genetic diversity also differed between the two markers (Table 1). While the average nucleotide diversity (π) and its range across sampling sites were similar (a mean value of 0.0047 and a range of 0 to 0.0091 for *cox1f*; a mean of 0.0019 and a range 0.0004 to 0.007 for *cox1m*), the median values of the nucleotide diversity (0.38 and 0.12 for *cox1f* and *cox1m* respectively) showed that the nucleotide diversity is significantly greater for *cox1f* than for *cox1m* (Wilcoxon rank test V= 65 p= 0.002). We observed the lowest nucleotide diversities in southern sampling sites for *cox1m* compared to *cox1f*. The haplotype diversity was not significantly different between the two markers (Wilcoxon rank test, V = 35, p = 0.89) but the haplotype diversity showed a significant negative southward trend (a = -0.07, R^2= 0.72) only for *cox1m*. Finally, Tajima's D also differed among the two markers. When considering the three geographical groups, significant negative values were found only for *cox1m* indicating the possibility of population expansion after a bottleneck event or selective sweep. For *cox1f*, on the other hand, no sign of deviation from neutral expectations was detected at the population level. The proportion of singletons at the scale of sample sites (Figure 4) showed a higher number of rare frequency mutations in *cox1m* compared to *cox1f*, which combined with negative D values, could indicate a recent expansion in population size.

Consistently with the haplotype networks, the results of the AMOVAs suggested different geographic structuration patterns for *cox1m* and *cox1f* (Table 2). The best hierarchical model based on the F_{SC}/F_{CT} ratio (least variation within and highest between groups) was different for the two markers: for *cox1f* the best model was two groups separated by the Finistère peninsula (KRU, CRI, CRO, VAA, MSM, BRI *vs* MAH, BRE, AYT, FOU, ARC) whereas for *cox1m*, the best model was also two groups of subpopulation but with the Cotentin peninsula as a major geographical break (KRU, CRI, CRO, VAA) *vs* (MSM, BRI,

MAH, BRE, AYT, FOU, ARC). The result of the AMOVAs (Table 2) showed similar level of among subpopulation genetic variation relative to the total variation for both markers (*cox1m*: 42% and Φ_{ST}= 0.58, p<0.001; *cox1f*: 44% and and Φ_{ST}= 0.55, p<0.001). The level of differentiation among groups was higher for *cox1m* compared to *cox1f* (Φ_{CT}= 0.57, p<0.001 and Φ_{CT}= 0.49, p<0.001 for *cox1m* and *cox1f*, respectively). Finally, within group genetic structure was detected only for *cox1f* (Φ_{SC}= 0.01, p=0.062 and Φ_{SC}= 0.13, p<0.001 for *cox1m* and *cox1f*, respectively). Genetic differentiation analyses based only on haplotype frequencies (conventional F-statistics) were also carried out and gave similar results (not shown).

McDonald and Kreitman tests were carried out in two genetically homogeneous groups for both markers: "North" and "South" composed of individuals from KRU, SYL, WIL and CRI sampling sites and from AYT, ARC, BRE and FOU respectively (Table 3) and overall samples. In both groups, the MK tests revealed that the divergence between male- and female-type *cox1* gene showed a significant departure from neutral expectations with a large excess of fixed non-synonymous mutations (Table 3). Also, the very low π_a/ π_s within each mitotype in both genetic groups showed that the two genes (*cox1m* and *cox1f*) are under strong purifying selection. Non-synonymous nucleotide diversity in *cox1m* was slightly higher than in *cox1f* in the North group and overall samples but not significantly, suggesting selection pressure acting on *cox1* gene was similar among mitotypes.

The effective population size ratio (N_{eF}/N_{eM}) suggested that N_{eF} was more than two times higher than N_{eM} at southern sites (0.16 within the northern group and 2.26 within the southern group).

Discussion

Investigating genetic structure of the DUI species *Macoma balthica rubra* across a hybrid zone in the English Channel revealed discordant phylogeographic patterns for the female- and male-type mitochondrial *cox1* gene. Because F- and M- mtDNA were compared among homologous regions within heteroplasmic males, variability can be strictly attributed to heredity rather than inter-individual or inter-genic differences. This variability resides in (1) a latitudinal gradient in haplotypic diversity for *cox1m* but not *cox1f*, (2) sharper deviation from neutral expectations of nucleotide diversity levels for *cox1m* relative to *cox1f* that can be attributed to historical demographic changes and / or relaxed purifying selection at the former

locus, and (3) discordant geographic clines for M- and F- mtDNAs. Below we discuss these differences considering neutral and selective processes that may have caused them.

Genetic diversity: differential latitudinal variation between m and f genes

Male-type mtDNA was almost up to twice as polymorphic as its female counterpart, with more haplotypes, more haplotypes represented by a single male and more segregating sites. Higher polymorphism in the M-type mtDNA, both within and between species (Skibinski et al 1999), is a common feature among DUI species, as reported in *Polititapes rhombroides* (Chacon et al 2020), *Pyganodon grandis* (Krebs 2004) and *Mytilus sp.* (Ort and Pogson 2003; Smietanka et al 2009, 2013, 2017). Higher frequency of rare M-type haplotypes (80% of singletons compared to 57% among F-type haplotypes in *M. b. rubra*) is another largely shared feature among DUI species (Chacon et al 2020, Ort & Pogson 2007, Smietanka et al 2009, 2013, 2017). Major determinants of genetic diversity in animals are linked selection, mutation, and effective population size (Ellegren and Galtier 2016). In DUI bivalves, polymorphism might result from different selection pressures on male- and female-type mitogenomes (*e.g.* Milani et al 2012): female-type mitochondria ensure proper mitochondrial function at the scale of the individual whereas male-type mitochondria, which solely co-occurs in heteroplasmic individuals with the F-type mitochondria (Skibinski et al 1994 and Zouros et al. 1994) have to effectively function exclusively in the male germ line and gametes (different 'arenas of selection' *sensu* Stewart et al 1996). According to the male-driven evolution hypothesis, higher germ cell divisions in males (Shimmin et al 1993) could result in an enhanced replication rate of the M-type mitochondria during spermatogenesis, providing more opportunities for mutations to accumulate (Rawson and Hilbish 1995 and Stewart et al 1995). In addition, oxidative damage might be important in sperm (Zouros 2013), and/or selection pressures on chaperonins and DNA repair genes might be relaxed. We can also expect sex-linked demographic differences to influence polymorphism, if the effective population size differs between males and females; this is supported by our dataset to a large extent at contemporary time scales. Most hypotheses on the role of selection, mutation and effective population size in shaping genetic diversity in DUI bivalves remain to be empirically tested.

However, counter examples do exist. For instance, Beauchamp et al (2020) found more F-type haplotypes than M-type haplotypes in *Pyganodon grandis* (11 and 7, respectively) and *P. lacustris* (20 and 6, respectively). Smietanka et al 2013 (87 F- and 76 M-type haplotypes in *Mytilus trossulus*) and Riginos et al 2004 (132 F- and 56 M-type

haplotypes in *Mytilus edulis*) reported the same pattern, although the latter results might be due to the difficulty of amplifying the male-type mtDNA.

Although often presented as such in the literature, higher polymorphism in males is therefore not a universal feature of the population genetics of DUI species. When genetic diversity was measured using nucleotide diversity π, the two markers differed significantly along the geographical area sampled with a significant positive trend observed between π and latitude at the M-type marker. While it varied between sites, π at *cox1f* displayed no geographic pattern.

Estimating π at *cox1f*, regardless of individual sexes, Becquet et al (2012) found that Southern populations, near the species range limit (brought here to Arcachon Basin), were more diverse than northern populations both at mitochondrial and nuclear markers. This pattern was attributed to phylogeography, the Bay of Biscay being a glacial refugium for other species (eg. Hewitt 1999; Gomez and Lunt 2007). Contrasting diversity patterns between F-type and M-type mtDNA observed in the present study bear similarities with predictions of population characteristics at the border of the species range (Holt et al 2003, reviewed in Dawson et al 2010). Predictions associated with the hypothesis of genetic impoverishment (H1: few northern migrants enter southern peripheral populations) are mostly met at *cox1m*, while the migration load hypothesis (H2: northern migrants bear alleles that are maladaptive in southern environments) seems to better fit the *cox1f* dataset. Gene flow between populations towards the range limit should be lower under H1 and higher under H2. While populations across the Cotentin were more differentiated at *cox1m* than populations across the Finistère at *cox1f*, pairwise Φ_{ST} were higher between southern populations at *cox1f* than *cox1m*. Genetic diversity should be low (decreasing toward the range limit) under H1 and high under H2, which is met for *cox1m* and *cox1f*, respectively. These patterns support the hypothesis that different selection pressures act on M-type and F-type mt genes, and therefore on male gametes vs. male adults and female at all life stages. This seems plausible (and testable) if we consider that genetic impoverishment occurs at early life stages in males (gamete and/or early zygote survival) and migration load occurs at all stages, such as was previously hypothesized for mitochondrial function (Pante et al 2012, 2019).

Discordance of geographic breaks between sex-specific mitogenomes

Both male and female mtDNAs displayed significant segregation of haplotypes in space. As previously described by Becquet et al (2012), southern populations were significantly differentiated from northern ones based on *cox1f*, and a geographical break between these two groups was observed at the Finistère peninsula. Sampling done for the present study narrows the gap between northern and southern populations by about 50 km thanks to the addition of the site of Saint-Brieuc Bay, however we failed at sampling any large population between the latter and Pont-Mahé Bay, south of Finistère (Fig 2). While anecdotal data support the presence of *M. b. rubra* near Brest (on the banks of Le Faou River; Hily 2013), we speculate based on field observations (Becquet et al 2012, Le Mao et al 2020 and this study) that the Finistère intertidal seem largely inhospitable to this species due to the scarcity of mud flats. It therefore appears as a credible physical barrier to genetic connectivity, and it has been indeed identified in the past as a genetic transition zone for other marine benthic-pelagic species confined to estuaries and bays (muddy-fine sediment species Jolly et al 2005, 2006).

Likewise, *cox1m* haplotypes strongly segregated in space, separating southern and northern populations at the Cotentin peninsula. This peninsula is located about 50 km farther East of the Finistère peninsula. As for the Finistère, sandy mud flats hospitable to *M. b. rubra* are scarce, and our field surveys failed at identifying populations between Mont-Saint-Michel Bay (MSM) and Saint-Vaast-la-Hougue (VAA), although anecdotal presence of the species was recorded farther along the Cotentin coast and was reported to be present but rare on the Normand-Breton Gulf (Le Mao et al 2020). Along with the scarcity of seemingly appropriate habitat and patchiness of small populations in the intertidal zone of Cotentin, we observed significant differences in gonadal development and spawning phenology between populations on either side of the peninsula. While individuals with full gonads and well-developed gametes were sampled between the 4th and the 23rd of April 2018 from Fouras to Mont-Saint-Michel Bay (some individuals having even spawned in the field at MSM and Saint-Brieuc), gonads from Saint-Vaast-la-Hougue (VAA) and Somme Bay individuals were still in early stages of development by April 19, 2018 (most gametes being still undifferentiated). These observations were on par with previous reports suggesting spawning asynchrony across the Cotentin Peninsula, with spawning occurring in early April in Aytré (Saunier 2015) and August in Somme Bay (Ruellet 2013). In the great scallop, *Pecten maximus*, different spawning phenologies were described across the Cotentin peninsula (Lubet et al 1995). As for the Finistère, the Cotentin peninsula was previously identified as a barrier to gene flow (Jolly et al 2005, Quéméré et al 2016, Handal et al 2020). It is also the southern

biogeographical boundary of the northern cold temperate Boreal region (Dinter 2001) with distinct hydrologic and oceanographic features from the western part of the English Channel (Dauvin 2012)

There are, therefore, multiple exogenous (habitat availability; oceanographic currents, *e.g.* Hily 2013, Dupont et al 2007, Fievet et al 2007) and endogenous (asynchrony in reproductive phenology and genetic incompatibilities, discussed below) barriers that could contribute to the genetic differentiation of populations across these two peninsulas. Whether these breaks are involved in primary intergradation or secondary contact (by geographically stabilizing endogenous genetic barriers; Bierne et al 2011) remains to be answered (we attempted to test scenarii of isolation with migration using the present dataset without success). The fact that each of these barriers is sex-specific is quite original for a marine invertebrate. In the DUI literature, studies have reported that genetic differentiation across populations is stronger for M-type mtDNA than for F-type mtDNA (Liu et al 1996, Skibinski et al 1999, Riginos et al 2004), and some authors reported discordant genetic structure between markers. The freshwater mussels *Lampsilis siliquoidea* displayed population genetic structure at *cox1f* but none at *cox1m* (Krebs et al 2013). Conversely, Riginos et al (2004) found weak connectivity at F-type mtDNA but none at M-type mtDNA in *Mytilus edulis* across the Atlantic Ocean. At the entrance of the Baltic, rampant introgression of *M. edulis* F-type mtDNA was observed, compared to a sharp cline at M-type mtDNA concordant with M7 lysin (a nuclear gene involved in fertilization; Stukas et al 2009). This makes the population structure described here for *M. b. rubra* noteworthy in the sense that the phylogeography of F- and M-type mt markers do not simply differ in amplitude or resolution (due to significant differences in mutation rates, μM being about twice μF) but differ in the geographical position of the haplotypic cline separating southern and northern *M. b. rubra*.

While a genetic break located around the Finistère peninsula corresponds to previous results by Becquet et al (2012) for *cox1f* and eight nuclear microsatellites, the Cotentin was not detected as a barrier in that study. However, spatial structure was detected at the nuclear *atp5c1* gene (encoding the gamma subunit of the FO/F1 ATP synthase protein complex) between Mont-Saint-Michel and Somme Bay (Saunier 2015). Non-synonymous point mutations separating southern and northern populations are located in an inter-peptide interaction domain of the gamma subunit, suggesting mito-nuclear incompatibilities (Pante et al 2019). The concordance of *atp5c1* and *cox1m* calls for further investigation of incompatibilities involving the male mitogenome specifically.

Interestingly, significant differentiation with *cox1f* was detected between Pont-Mahé Bay (MAH) and the Saint Brévin (BRE) and Aytré (AYT) populations (Table S3). These two groups, separated by the Vilaine River, were identified as significantly differentiated based on microsatellites, but not *cox1f* in the study of Becquet et al (2012), which was based on individuals from both sexes, while our study focused on males exclusively. We may therefore benefit from higher statistical power to detect differences in differentiation across the Vilaine River. This implies that genetic structure among males at *cox1f* is higher than in females. Although we did not include females in our study, we did compare pairwise $\square_{ST}$ values between sampling sites that were common between our study and Becquet's. F_{ST} and $\square_{ST}$ were both significantly higher in the dataset composed of males exclusively, compared to the dataset composed of males and females (Figure 5). This pattern can be attributed to stochastic genetic variation (effects of mutations and drift) and/or differences of gene flow between sexes. The geographical discordance observed at the Finistère and Cotentin peninsulas for *cox1f* and *cox1m* are also reminiscent of sex-level differences. Indeed, in studies using female-transmited mtDNA and male-transmitted Y chromosome to investigate population structure, sex-biased asymmetries are often cited as a determinant of discordant geographic patterns (*e.g.* Boissinot et al 1997, Trejo-Salazar et al 2021). Sex-level differences in gene flow are scarcely reported but exist in bivalves, as in the protandrous pearl oyster *Pinctada mazatlanica*, which effective sex-ratio is strongly biased towards males (Arnaud-Haond et al 2003).

Interestingly, the *cox1m* displayed haplotypes branching from the southern haplogroup m3, private to the two populations sampled between Finistère and Cotentin (Saint-Brieuc and Mont-Saint-Michel; Figures 1-2). This suggests genetic isolation of these populations and subsequent retention of these private haplotypes in the Gulf. This pattern supports a (re)colonization scenario from the south to the Gulf (fitting "concordant" simulated networks for colonization from a refugium; Maggs et al 2008). An alternative scenario, unsupported by the genetic diversity levels estimated in the south, is a stepwise colonization of the south from the north and the loss of theses haplotypes in the south either from drift or selection.

Other factors could explain the M- vs. F-mtDNA discordance, such as mtDNA introgression and heterogeneity in its rate, hybrid zone movement and drift (Barton and Hewitt 1985, Bierne et al 2003, Toews et al 2012). Patterns of asymmetric introgression of alleles are commonly observed in hybrid zones (Barton and Hewitt 1985) and were observed in the Bay of Biscay between *Mytilus edulis* and *M. galloprovincialis* (Rawson and Hilbish

1998). In *M. b. rubra*, asymmetrical gene flow from North to South was detected at *cox1f* (Becquet et al 2012, this study), and *atp5c1* (Saunier 2015, Pante et al 2019). At that same locus, Gagnaire et al (2012), looking at divergence and selection among populations of the eels *Anguilla rostrata* and *A. anguilla* (two hybridizing sister species) also found evidence of unidirectional introgression from the former to the later. At *cox1m*, little introgression was detectable in *M. b. rubra*, but nevertheless sensibly higher from north to south.

Hybrid zone movement has been recognized for long, as a manifestation of variation in population density, dispersal rate, individual fitness, effects of allele frequencies on population structure, and spatially- or frequency-dependent selection (Barton and Hewitt 1985). It has been proposed that the northeastern Atlantic *Mytilus* hybrid zones move parallel to warming sea surface temperatures (Hilbish et al 2012). While a similar phenomenon may occur in Baltic tellins, suggesting that genotype-environment correlations could affect cline geography (Ducos et al unpublished), neutral forces may be sufficient to result in discordant M-type and F-type mtDNA cline centers and width. Indeed, as detailed above, both the Finistère and the Cotentin peninsulas are characterized by low population densities and oceanic currents disrupting along-coast dispersal. While mitochondrial recombination is expected to be rare (or at least, rarely transmitted to offspring; Passamonti et al 2003), looking at whole mitogenomes as well as nuclear genes of mitochondrial function vs. other (putatively neutral) nuclear markers may help shed light on the degree to which the patterns detected at *cox1* and atp5c1 depart from neutral expectations.

Whether the asymmetric, discordant clines in *M. balthica* observed here are due to cline movement, differential gene flow, population structure, or selection (Barton and Hewitt 1985) remains a fascinating avenue for future research. In particular, introgression across the hybrid zone in the English Channel calls for a test of asymmetric fitness of inter-lineage crosses (e.g., Turelli et al 2007). An involvement of mito-nuclear incompatibilities could explain the observed asymmetric allele frequencies if northern hybrids with southern mitochondria are less fit than southern hybrids with northern mitochondria.

Data Accessibility

Cox1f and *cox1-long* sequences were submitted to Genbank (accession N° OM855617 - OM855929 and OM856027 - OM856339 respectively). Rcode is available at https://github.com/SabLeCam/Cox1_DUI .

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Legends

Figure 1: Median joining networks constructed from the haplotypes found among the 302 individuals distributed in 14 sampling sites for (A) the *cox1f* gene and (C) the *cox1m* gene. Haplotype associations among haplogroups are represented with a tanglegram (B) with bold lines and arrows highlighting individuals sharing distant haplogroups

Figure 2: Geographical distribution of the *cox1f* and *cox1m* haplogroups: haplogroup frequencies are represented for each marker in all the sites with a pie chart. The size of the pie chart indicates the number of individuals analyzed.

Figure 3: Heatmap representing linkage disequilibrium among haplogroups of *cox1f* and *cox1m*. Color key is based on correlation values among haplogroups and cell value represent the observed frequencies of each possible association. Dendogram based on pairwise distance between main haplotypes of each haplogroup using the Tamura-Nei nucleotide substitution model and a gamma correction of 0.1150 and 0.1450 for *cox1f* and *cox1m*, respectively.

Figure 4: The proportions of singletons per sample site (n>=10) for *cox1m* in blue and *cox1f* in red together with trendlines (dashed lines, third order polynomial) and R-squared goodness-of-fit measures.

Figure 5: Comparison of pairwise Φ_{ST} estimated at the *cox1f* gene for study sites that were both sampled by Becquet et al (2012) (males and females) and for the present study (males only). The plain line represents the x=y axis and the dash line, the linear regression (y = 1.27 x + 0.03, df = 62, R^2_{adj}=0.74, p<0.001). A Mantel test revealed a significant correlation between the two datasets (r=0.786, p=0.0084).

Table 1: Genetic diversity indices at *cox1f* (479bp) and *cox1m* (479bp) loci for each sampling site: n: number of individuals, nsites: number of polymorphic sites, H: number of haplotypes, π: nucleotide diversity, Hd: Haplotype diversity and D: Tajima's D coefficient. N_{eF}/N_{eM}: the ratio of female to male effective population size * *indicates significant values (<0.05, 10,000 permutations), Na: not available*

Geographic zone	Sampling site		*cox1f*					*cox1m*					
		n	**nsites**	**H**	**π(10²)**	**Hd**	**D**	**nsites**	**H**	**π(10²)**	**Hd**	**D**	**N_{eF}/N_{eM}**
North	Sylt (SYL)	7	7	4	0.91	-	-	5	4	0.38	-	-	-
	Wilhelmshaven (WIL)	3	0	1	0.00	-	-	1	3	0.13	-	-	-
	Crildumersiel (CRI)	10	6	2	0.77	0.53	2.10	14	9	0.70	0.98	-1.47	0.02
	Kruiningen (KRU)	22	11	7	0.77	0.69	0.11	10	22	0.23	0.65	-2.00*	1.11
	all	*42*	*11*	*7*	*0.75±0.43*	*0.64±0.06*	*0.75*	*23*	*16*	*0.36±0.24*	*0.79±0.05*	*-2.26**	*0.44*
Center	Le Crotoy (CRO)	28	13	7	0.77	0.58	-0.24	14	14	0.38	0.88	-1.68*	0.17
	Saint-Vaast-la-hougue(VAA)	31	11	5	0.33	0.24	-1.63*	11	9	0.27	0.75	-1.69*	0.10
	all	*59*	*14*	*9*	*0.52±0.31*	*0.42±0.08*	*-0.79*	*20*	*20*	*0.32±0.21*	*0.81±0.04*	*-1.99**	*0.16*
Hybrid/Finistère	Mont-Saint-Michel (MSM)	31	8	4	0.22	0.24	-1.60*	5	7	0.11	0.35	-1.51*	0.54
	Saint-Brieuc (BRI)	30	15	9	0.76	0.99	0.17	4	5	0.08	0.36	-1.58*	160.40
	all	*61*	*16*	*10*	*0.74±0.42*	*0.58±0.07*	*-0.28*	*8*	*10*	*0.09±0.09*	*0.35±0.08*	*-1.91**	*2.37*
	Pont-Mahé (MAH)	30	8	6	0.18	0.36	-1.86*	5	6	0.07	0.31	-2.00*	1.16
	Saint-Brevin (BRE)	29	9	8	0.71	0.49	-0.25	8	9	0.13	0.53	-2.14*	0.79
	Noirmoutier (NOI)	8	3	4	0.23	-	-	1	2	0.05	-	-	-
South	Aytré (AYT)	33	8	6	0.43	0.55	-0.18	5	6	0.07	0.28	-1.88*	2.90
	Fouras (FOU)	30	8	7	0.29	0.50	-1.12	6	6	0.08	0.31	-2.10*	2.05
	all	*63*	*11*	*10*	*0.36±0.23*	*0.52±0.07*	*-0.89*	*9*	*9*	*0.08±0.08*	*0.29±0.07*	*-2.15**	*2.45*
	Arcachon (ARC)	10	5	3	0.23	0.38	-1.74*	1	2	0.04	0.2	-1.11	2.26
	All	***302***	***34***	***34***	***0.81±0.45***	***0.76±0.02***	***-1.06***	***53***	***61***	***0.29±0.20***	***0.68±0.03***	***-2.44****	***1.38***

Table 2: Analysis of molecular variance (AMOVA) to partition the genetic variation among hierarchical geographical scales. Results are presented for both markers, with different options of hierarchical grouping of subpopulations.

			cox1f			*Cox1m*		
Number of groups	Groups		Among groups relative to total variance	Among subpopulations within groups	Among subpopulations relative to total variance	Among groups relative to total variance	Among subpopulations within groups	Among subpopulations relative to total variance
3	(CRI,KRU) (CRO,VAA,MSM,BRI) (MAH,BRE,AYT,FOU,ARC)	%var	44.71	7	48.29	22.2	22.54	55.26
		Fixation indices	Φ_{CT}=0.447***	Φ_{SC}=0.517***	Φ_{ST}=0.126**	Φ_{CT}=0.221^{ns}	Φ_{SC}=0.290***	Φ_{ST}=0.447***
2	(CRI,KRU,CRO,VAA) (MSM,BRI,MAH,BRE,AYT,FOU, ARC)	%var	16.98	31.02	52	57.36	0.56	42.08
		Fixation indices	Φ_{CT}=0.170^{ns}	Φ_{SC}=0.374***	Φ_{ST}=0.480***	Φ_{CT}=0.574***	Φsc=0.013^{ns}	Φ_{ST}=0.579***
2	(CRI,KRU,CRO,VAA,MSM,BRI) (MAH,BRE,AYT,FOU,ARC)	%var	49.14	6.4	44.46	21.88	4.18	53.94
		Fixation indices	Φ_{CT}=0.491***	Φ_{SC}=0.126***	Φ_{ST}=0.555**	Φ_{CT}=0.212*	Φ_{SC}=0.309***	Φ_{ST}=0.460***

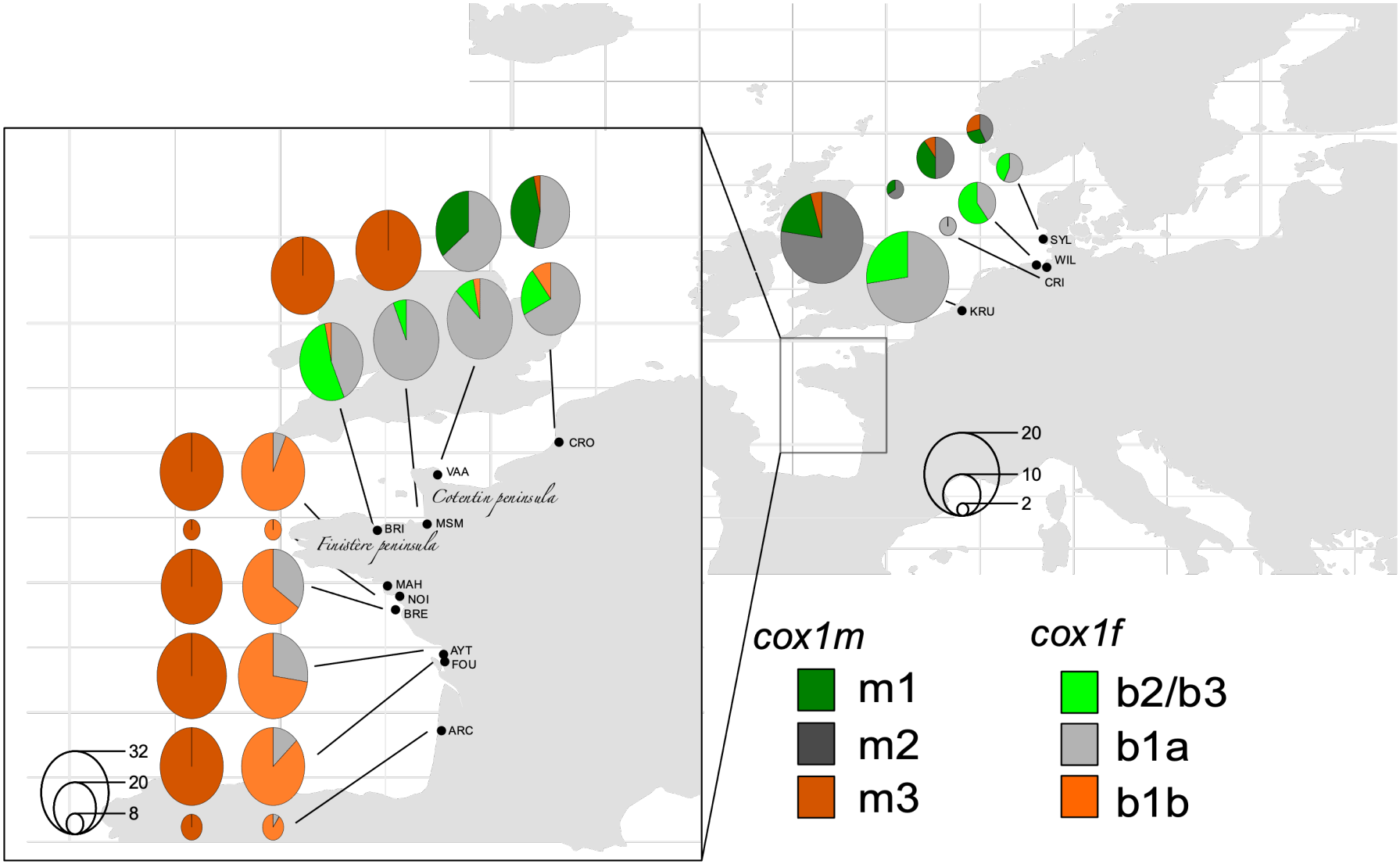
*P>0.05, **P>0.01,***P < 0.001, ns: non significant

Table 3: McDonald and Kreitman contingency table: the number of non-synonymous and synonymous substitutions within and among mitotypes for the two *M. balthica rubra* genetic lineages, the neutrality index (NI) and the results from the Fisher's Exact test and G test.

Group	*cox1f* vs. *cox1m* segregating sites	Polymorphic	Fixed	NI	Fisher Exact Test Pvalue	G test
North						
	Non-synonymous	2	56	0.083	<0.0001	20.165***
	Synonymous	32	74			
South						
	Non-synonymous	2	54	0.107	<0.0002	15.111***
	Synonymous	29	84			

***P < 0.0001

30 samples
3 samples
SYL
CRI
WIL
KRU
CRO
VAA
MSM
BRI
MAH
BRE
NOI
AYT
FOU
ARC
cox1f
N=302
H=34
haplogroup b1a
haplogroup b1b
haplogroup b2_b3
haplogroup_m1
haplogroup_m2
haplogroup_m3
cox1m
N=302
H=61


CRO
VAA
Cotentin peninsula
BRI
MSM
Finistère peninsula
MAH
NOI
BRE
AYT
FOU
ARC
32
20
8
SYL
WIL
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KRU
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cox1m
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m2
m3
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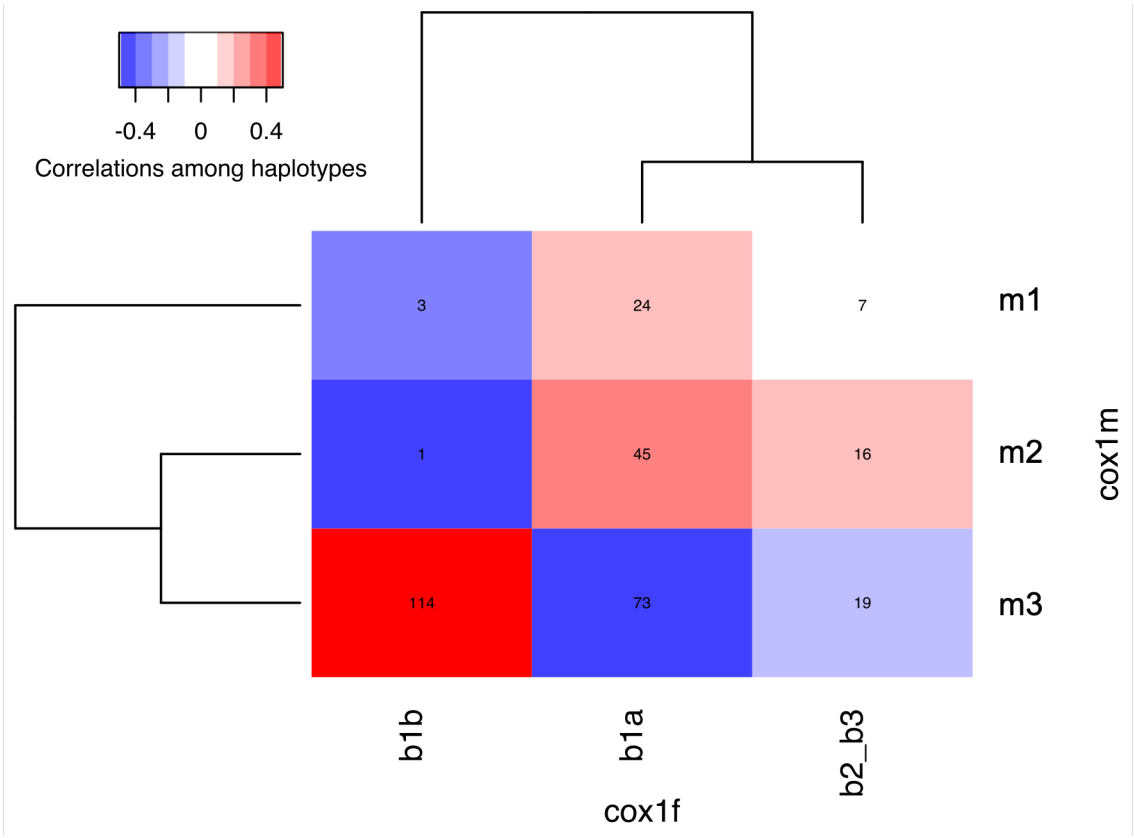
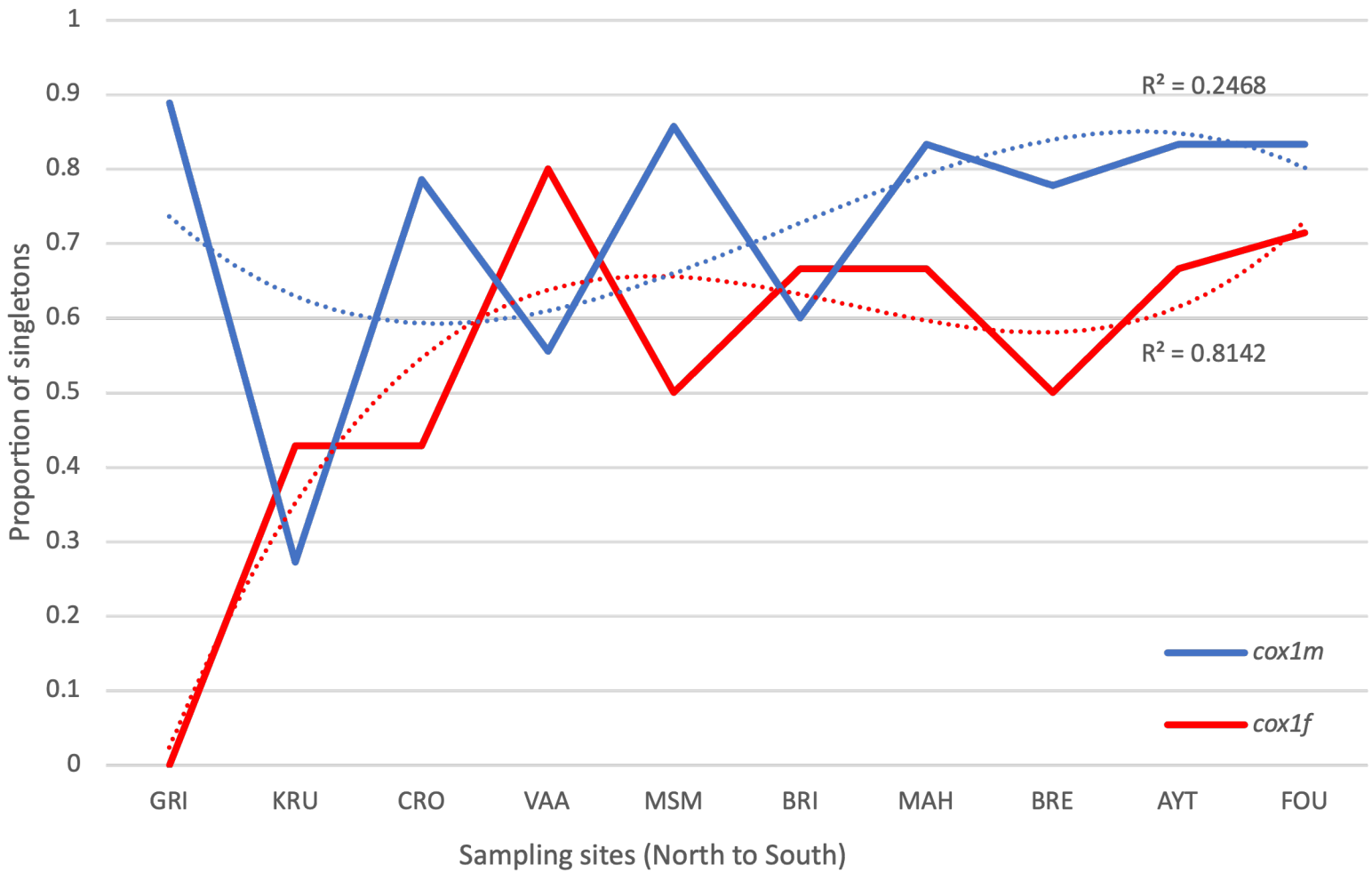
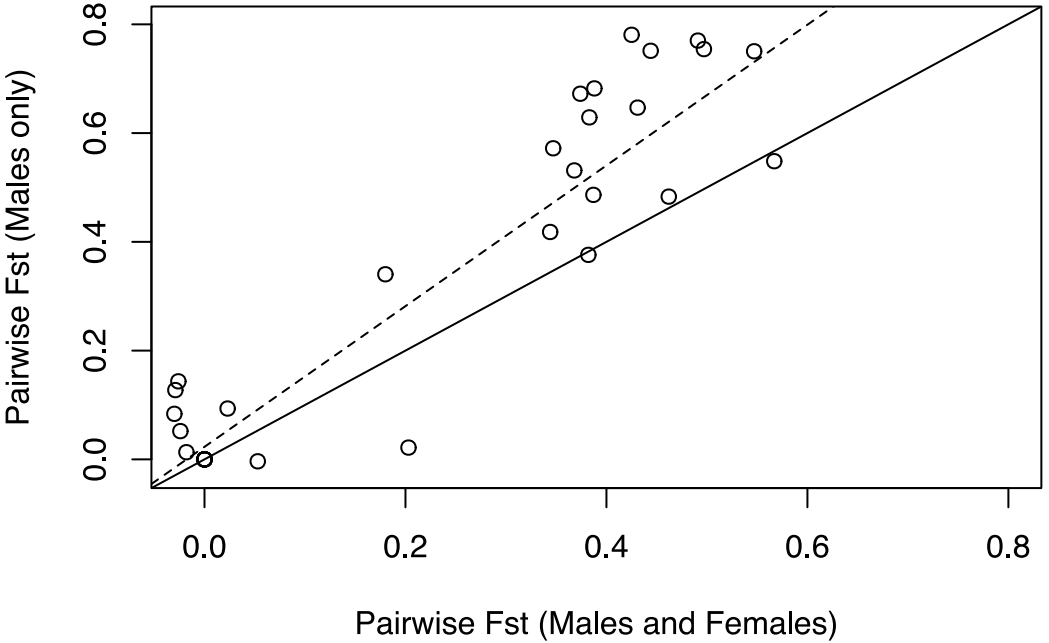
b2/b3
b1a
b1b


-0.4
0
0.4
Correlations among haplotypes
3
24
7
1
45
16
114
73
19
m1
m2
m3
cox1m
b1b
b1a
b2_b3
cox1f


1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Proportion of singletons
R² = 0.2468
R² = 0.8142
cox1m
cox1f
GRI
KRU
CRO
VAA
MSM
BRI
MAH
BRE
AYT
FOU
Sampling sites (North to South)


Pairwise Fst (Males only)
0.0
0.2
0.4
0.6
0.8
0.0
0.2
0.4
0.6
0.8
Pairwise Fst (Males and Females)