Discordant population structure inferred from male- and female-type mtDNAs from *Limecola 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 insight 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 (*Limecola balthica*) to test whether *cox1m* and *cox1f* genes bear the marks of similar phylogeographic histories. Both markers were sequenced for 313 male individuals sampled from the Baltic Sea to the Gironde Estuary (Southern France). Haplotype 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 = 0.447 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 selective pressure 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 are able to 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). Therefore, DUI offers tremendous potential for genetic incompatibilities to develop, in particular, in inter-populational hybrids, as a network of cyto-nuclear interactions exists (Saavedra et al 1996): mito-genetic incompatibilities can be expressed not only between the F-type and nuclear genes (in somatic tissues and oocytes in females), but also between the M-type and nuclear genes in sperm. Recombination between M- and F-types can further complicate mito-nuclear dynamics (reviewed in Zouros 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. While significant efforts have been made in the recent years to comprehend how DUI works and how it evolved (*e.g*. Breton et al 2007, 2014; Zouros, 2013, Zouros 2020), little information is available on how it might participate to reproductive isolation.

Thus, 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.

Limecola balthica (previously known as *Macoma balthica*, Huber, 2015), 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 *L. balthica* co-occur in North Atlantic: a Pacific lineage (*L. balthica balthica*) present in the Baltic Sea and the White Sea, and an Atlantic lineage (*L. balthica 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 *L. balthica 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 *L. balthica*.

Here, we present sharp differences in genetic diversity, divergence and population genetic structure between F- and M-type mtDNAs of *L. balthica* 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 *L. balthica.*

Materials and Methods

Sampling

Individuals were collected from a total of 17 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 Umea (Swedish Baltic Sea) (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 if necessary. 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 determined with a dissecting microscope (x100 to x400). For each individual, two types tissue samples were collected, a gonadic sample and a somatic sample (mantle). All tissue samples were flashfrozen 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 and Mont Saint Michel, 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.

Polymerase chain reaction (PCR) amplifications of the female (*cox1f*) and male (*cox1m*) mitochondrial DNA regions were performed using specific primers (Cox1m: cox1m14641F ATAGCTGGCCTGGTGTTTAGG, cox1m15560R

TTGGACCCTTTCGAGCCAAG; Cox1f: cox1f5343F 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. Full length *cox1m* (676 bp) was called *cox1m-long* hereon and trimmed *cox1m* sequences at the same length as *cox1f* (479bp) to compute further comparative analyses was referred to *cox1m* hereon.

Haplotype sequences and their frequencies from *cox1-long* 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-long*, median-joining networks (Bandelt et al., 1999) were constructed with PopART v1.7 software (Leigh & Bryant, 2015).

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 $\square_{ST}$ 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 compare polymorphism and divergence among female and male mitotypes. The test was conducted in DNAsp (Librado and Rozas 2009) 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 using three different methods: first, 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)

$$n_e = CN_e\mu + 1$$ (not $n_e = Cn_e\mu$ 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)

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

This method solely relies on the estimate of haplotype diversity Hd and the mutation rate μ. The marker-specific mutation rates were estimated together with the molecular clocks as described thereafter in the paragraph. Second, π_a/π_s was used as a proxy Ne since small Ne are expected to exhibit a higher segregating mutation load (high π_a/π_s) (Galtier and Rousselle 2020).

Last, we used Coalescent Bayesian Skylines to estimate differences in demographic history between the male and the female mitochondrial makers, both sampled from male individuals. This method is based on the tree topology, from which is extracted the rate of coalescent event and hence Ne (Drummond et al 2005). For this, we focused on a southern group composed of Arcachon, Fouras, Aytré and Saint Brévin, and on a northern group composed of Kruiningen, Crildumersiel, Wilhelmshaven and Sylt. In both case, populations are genetically homogeneous (Tables S2 and S3), unambiguously outside of the Finistère/Cotentin hybrid zone. Sample sizes were 102 and 42 males, respectively. First, we estimated a molecular clock for the male and female *cox1* gene by calculating the genetic distance among sequences of *L. balthica* and *Donax*, another Tellinoidea characterized by DUI (Theologidis et al 2008; Gusman et al 2016). *For L. balthica*, we used m and f haplotypes from Umea and Arcachon as extremes on the haplotypes networks (Fig 1). For *Donax*, we used GenBank records for *D. fab*a (AB040843 and AB040844 for m and f *cox1*, respectively) and for *D. cuneatus* (AB040841 and AB040842 for m and f *cox1*, respectively). The mean genetic distance (Kimura 2-parameters distance, Kimura 1980) between all *Limecola* / *Donax* sequence pairs were used to calculate a marker-specific molecular clock, given a divergence time of 90 to 140 My between the two genera (as in Luttikhuisen et al 2003, based on fossil dating in Pohlo 1982). To run the Bayesian skyline analysis, we followed the protocol of Müller and du Plessis (Barido-Sottani et al 2018). For each dataset (male and female) and for each extreme of the estimated molecular clocks, we ran BEAST v2.6.3 (Bouckaert et al 2019) using a GTR+G+I site model, a strict clock model, 800M generations with a pre-burnin of 80M generations. The number of dimensions (groups of coalescent events) was set to 3 after test runs with 3 to 5 dimensions. Other priors were left at their default values. Convergence was checked in Tracer v1.7.1. Ne estimates are presented

as median values for the retained runs for the low mutation rate, unless otherwise noted. Generation time for *L. balthica* is 2 yrs (Beukema et al 2001).

Results

A total of 666 individuals from 17 sampling sites of the French Atlantic coast were dissected and optical microscopy sexing reported 248 males, 185 females and 233 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 undiffereciated individuals.

Both *cox1m-long* and *cox1f* were successfully sequenced for 313 male individuals from 17 sampling sites (Table 1). Sampling size per site ranged from 2 to 33 overall sampling sites and from 28 to 33 when only considering sampling sites in France, apart from Arcachon (N=10). We used *cox1m-long* for haplotype networks (Fig 1) and haplotype geographical frequencies (Fig 2). All other analyses were performed on *cox1m* rather than *cox1m-long* to compare M and F markers in a strict, quantitative fashion.

Among the 313 studied individuals, 37 and 81 haplotypes were found for *cox1f* and *cox1m-long*, respectively. Among these, 57% were singletons at cox1f and 80% at *cox1m-long*. Median joining networks showed a similar geographical pattern with three main haplogroups encompassing mainly the northern, central and southern samples (Fig 1 A and C). A second *cox1m-long* central haplogroup (IIb) containing 6 haplotypes was observed and only found in the hybrid zone (MSM: Mont Saint Michel and BRI: Saint Brieuc). Nonetheless, the networks revealed important differences between markers. First, *cox1m-long* not only presented a higher diversity, but also a higher divergence level: a maximum of 24 and 40 mutation steps separated the most divergent haplotypes for *cox1f* and *cox1m-long*, respectively. Haplotype networks and frequencies for the truncated *cox1m* dataset are presented in Fig S1 and revealed similar pattern of high diversity (64 haplotypes) and divergence (9 mutation steps were estimated between the main central haplotype and the northern one for *cox1f* against 18 for *cox1m*). We estimated divergence rates of 0.47 s/s and 0.24 s/s for the m and f datasets, respectively. This difference is significant based on the relative-rates test (100% significant comparisons out of 2368 https://github.com/lyy005/Relative-Rate-Test). This resulted in molecular clocks of 0.0033 and 0.0017 s/s/My for *cox1m* and *cox1f*, respectively, considering a 140My divergence

between *Limecola* and *Donax*; 0.0052 and 0.0027 s/s/My for *cox1m* and *cox1f*, respectively, considering a 90 My divergence between these taxa.

The relative frequencies of the haplogroups illustrated in Fig 2 showed different patterns. The *cox1m-long* haplogroup I was present only in Baltic and North Sea samples while the *cox1f* haplogroup I encompassed individuals from the Baltic Sea to the English Channel (hybrid zone). Also, the haplogroup II of *cox1f* was shared among the Baltic Sea, North Sea, the English Channel and Atlantic samples whereas the main central haplogroup (IIa) of *cox1m-long* 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-long*, the Cotentin peninsula represented a sharp transition zone between the haplogroups IIa and III 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 III 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 (MAH) sampling site was mildly differentiated from Saint Brévin (BRE) and Aytré (AYT) (pairwise Φ_{ST} of 0.13 and 0.08 respectively, table S3). Also, the relative frequency of the 3 haplogroups was 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 presented in Table 1 and used in subsequent analysis (North, Center and South). 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 I at *cox1f* and the haplogroup III at *cox1m*, no individual shared the *cox1f* haplogroup III and the *cox1m* haplogroup I. Also, only 4 individuals presented both the *cox1f* haplogroup III and the *cox1m* haplogroup IIa,

suggesting non-random mating or lethal haplotype combinations. Noteworthily, all these individuals were found in the hybrid zone sampling sites. 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 0.055 and a range of 0 to 0.0141 for *cox1f*; a mean of 0.0038 and a range 0.0004 to 0.025 for *cox1m*), the median values of the nucleotide diversity (0.61 and 0.20 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.

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 is 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). 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.

Two of the three methods used to infer effective population size converged to suggest that contemporary N_{eM} is higher than N_{eF} at northern sampling sites, while the opposite holds at southern sites (Zouros N_{eF}/N_{eM} ratio, Table 1; Bayesian Skyline Plot, Figure 4). Contemporary N_{eF}/N_{eM} calculated using Zouros' method and BSP at time=0 were 0.91 and 0.27 within the northern group and 5.09 and 1.07 within the southern group, respectively (0.13 and 1.09 for high mutation rates, Figure S2). π_a/π_s estimates, on the other hand, were always higher for *cox1m*, suggesting a smaller Ne for this marker compared to cox1f regardless of the group considered.

BSP further suggested that historical N_e dynamics differed between markers (Figure 4 for the high mutation rate and figure S2 for the low mutation rate). Southern populations exhibited N_e ranging between 1 and 4.5 M individuals with similar median values (2.3 and 2.5 M for *cox1m* and *cox1f*, respectively), with similar population growth that started about 200,000 years ago for *cox1f* (about 122,000 years ago based on the fastest mutation rate). ESS values for the estimated parameters are excellent for the southern group (e.g. posterior ESS between 270 and 422). Northern M-type and F-type datasets exhibited significantly different patterns, with similar N_e for *cox1f* and *cox1m* prior to about 155,600 years ago (about 107,300 for the highest mutation rate), followed by an exponential N_e increase for *cox1m* only. ESS for the posterior were lower than for the southern group, but acceptable (110 to 154).

Discussion

Investigating genetic structure of the DUI species *Limecola balthica* 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 *L. balthica*) 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 historical (*e.g.* past expansion inferred for *cox1m* in the north) and 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, south of Finistère (Fig 2). While anecdotal data support the presence of *L. balthica* 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 *L. balthica* are scarce, and our field surveys failed at identifying populations between Mont-Saint-Michel Bay and Saint Vaast, 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 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 scenario of isolation with migration using the present dataset without success). We can nevertheless note that divergence of populations on either side of the Finistère-Cotentin break occurred between 0.11 to 2.6 Myr ago (Luttikhuisen et al 2003), while population expansion on either side of the break took place 107 to 200 k years ago (depending on the marker and on the clock; table 1, Figures 4, S2). We can therefore tentatively speculate that these populations diverged during the penultimate glacial maximum occurring in the NE Atlantic 140,000 years ago (Martin-Garcia 2019) and expended with secondary contact after that event.

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 *L. balthica* 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 *L. balthica 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* call for further investigation of incompatibilities involving the male mitogenome specifically.

Interestingly, significant differentiation with *cox1f* was detected between Pont Mahé (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 φ_{ST} values between sampling sites that were common between our study and Becquet's. F_{ST} and φ_{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* IIb haplogroup, branching from the southern haplogroup III, is private to the two populations sampled between Finistère and Cotentin (Saint Brieuc and Mont-Saint-Michel; Figs 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 observed in the south, is a stepwise colonization of the south from the north and the loss of IIb 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 *L. balthica*, 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 *L. balthica*, 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 *L. 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 313 individuals distributed in 17 sampling sites for (A) the *cox1f* gene (479bp) and (C) the *cox1m-long* gene (676bp). Haplotype associations among haplogroups are represented with a tanglegram (B) with bold lines highlighting individuals sharing distant haplogroups

Figure 2: Geographical distribution of the *cox1f* and *cox1m-long* 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-long*. 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-long* respectively.

Figure 4: Bayesian skyline plots for North and South subpopulations illustrating effective population size (N_e) variation through time for both markers. The lines represent the mean effective population size, and the colored area represent 95% higher and lower confidence interval of posterior probabilities Here the 90 My divergence time with the *Donax* genus is considered (5.19×10^{-2}substitution/site/My).

Figure 5: Comparison of pairwise Φ_{ST} estimated at the *cox1f* gene between common sampling sites studied in Becquet et al (2012) (males and females) and 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, 10000 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}**
	Umea (UME)	2	6	2	1.41	-	-	12	2	2.5	-	-	-
	Lomma (LOM)	6	7	3	0.81	-	-	4	3	0.28	-	-	-
	Mecklenburg bight (MEC)	3	4	3	0.61	-	-	10	3	1	-	-	-
	Sylt (SYL)	7	7	4	0.91	-	-	5	4	0.38	-	-	-
	Wilhelmshaven (WIL)	3	0	1	0.00	-	-	1	3	0.13	-	-	-
North	Crildumersiel (CRI)	10	6	2	0.77	0.53	2.10	14	9	0.70	0.98	-1.47	0.04
	Kruiningen (KRU)	22	11	7	0.77	0.69	0.11	10	22	0.23	0.65	-2.00*	2.30
	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**	*0.91*
	Le Crotoy (CRO)	28	13	7	0.77	0.58	-0.24	14	14	0.38	0.88	-1.68*	0.36
Center	Saint Vaast (VAA)	31	11	5	0.33	0.24	-1.63*	11	9	0.27	0.75	-1.69*	0.20
	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**	*0.33*
	Mont Saint Michel (MSM)	31	8	4	0.22	0.24	-1.60*	5	7	0.11	0.35	-1.51*	1.12
Hybrid/Finistère	Saint Brieuc (BRI)	30	15	9	0.76	0.99	0.17	4	5	0.08	0.36	-1.58*	337.57
	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**	*4.92*
	Pont Mahé (MAH)	30	8	6	0.18	0.36	-1.86*	5	6	0.07	0.31	-2.00*	2.40
	Saint Brevin (BRE)	29	9	8	0.71	0.49	-0.25	8	9	0.13	0.53	-2.14*	1.63
	Noirmoutier (NOI)	8	3	4	0.23	-	-	1	2	0.05	-	-	-
	Aytré (AYT)	33	8	6	0.43	0.55	-0.18	5	6	0.07	0.28	-1.88*	6.03
South	Fouras (FOU)	30	8	7	0.29	0.50	-1.12	6	6	0.08	0.31	-2.10*	4.27
	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**	*5.09*
	Arcachon (ARC)	10	5	3	0.23	0.38	-1.74*	1	2	0.04	0.2	-1.11	4.70
	All	**313**	**37**	**37**	**0.85**	**0.76**	**-1.13**	**55**	**64**	**0.42**	**0.70**	**-2.28***	**2.60**

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

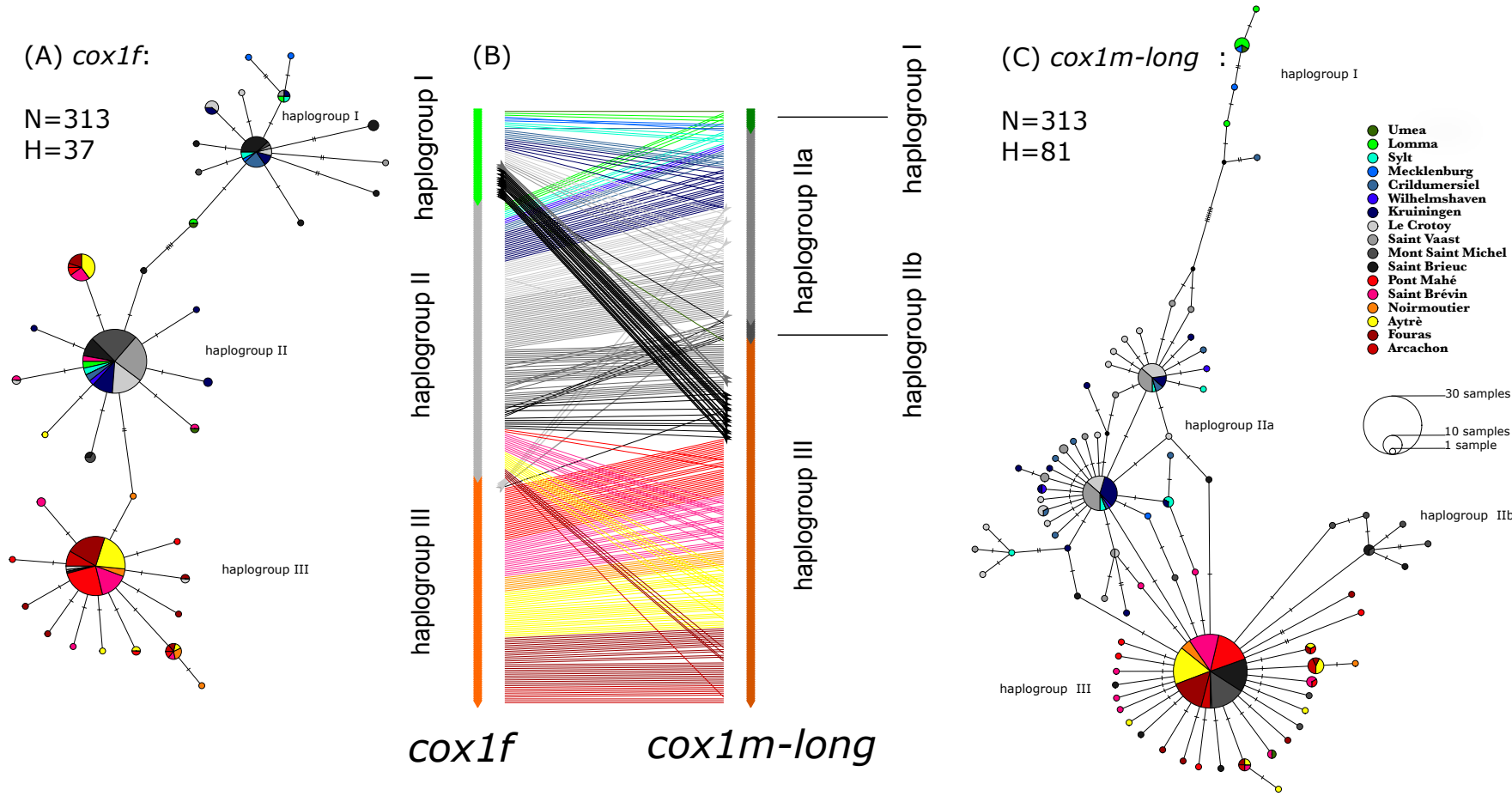
Table 3: McDonald and Kreitman contingency table: the number of non-synonymous and synonymous substitutions within and among mitotypes for the two *L. balthica* genetic lineages, the neutrality index (NI) and the results from the Fisher Exact and G tests.

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,

Table 4: Measures of nonsynonymous to synonymous (i) nucleotide diversity within each mitotype (π_a, π_s) and (ii) number of substitutions among mitotypes (K_a, K_s) using the Jukes and Cantor correction

		Intra mitotype ($10^{2)}$			Inter-mitotype		
		π_a	π_s	ratio	K_a	K_s	ratio
North					0.201	2.008	0.100±0.028
	f	0.000	2.779	0.000			
	m	0.026	1.401	0.018			
South					0.201	2.086	0.097±0.031
	f	0.011	1.421	0.008			
	m	0.005	0.348	0.015			
All					0.201	2.063	0.098±0.029
	f	0.008	2.733	0.003			
	m	0.012	1.037	0.011			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H=37
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haplogroup II
haplogroup III
(B)
haplogroup I
haplogroup II
haplogroup III
haplogroup IIa
haplogroup IIb
haplogroup I
haplogroup III
cox1f
cox1m-long
(C) cox1m-long :
N=313
H=81
haplogroup I
haplogroup IIa
haplogroup IIb
haplogroup III
Umea
Lomma
Sylt
Mecklenburg
Crildumersiel
Wilhelmshaven
Kruiningen
Le Crotoy
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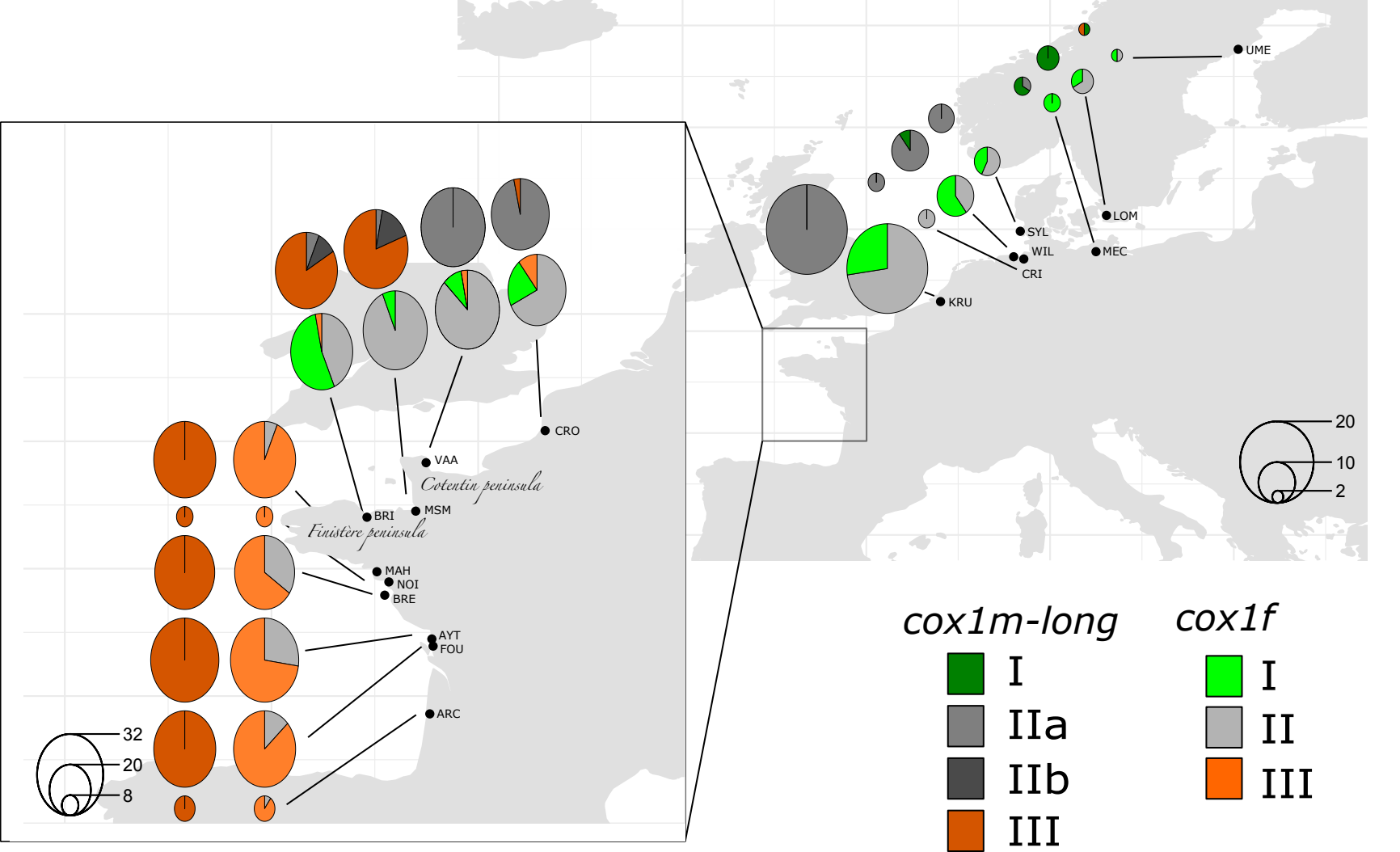
UME
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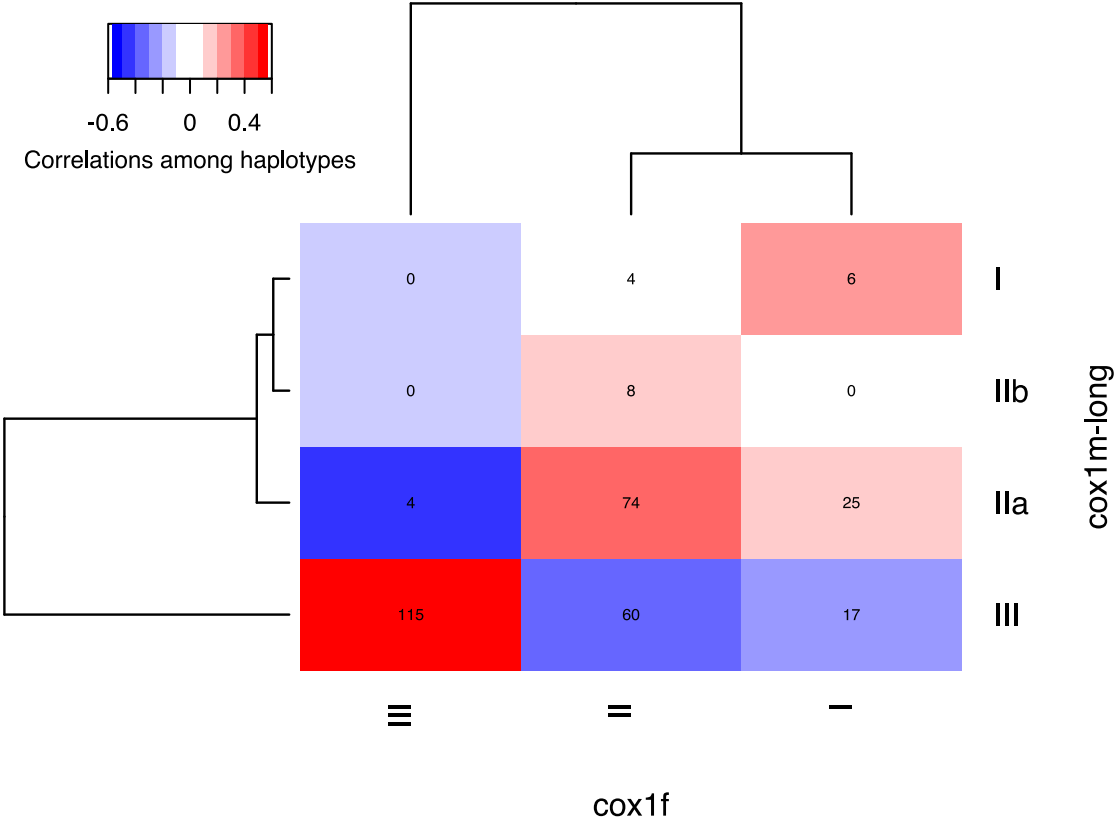
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0.4
Correlations among haplotypes
0
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74
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I
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cox1m-long
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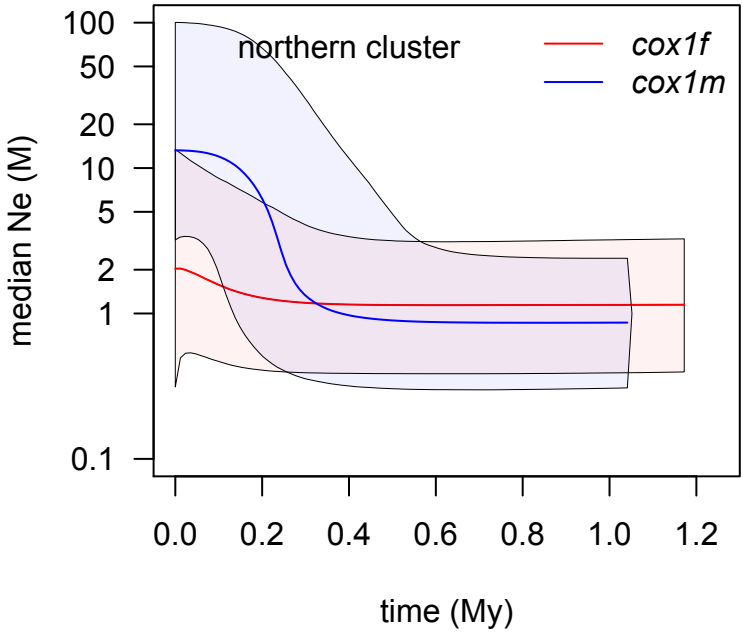
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cox1f
cox1m
median Ne (M)
100
50
20
10
5
2
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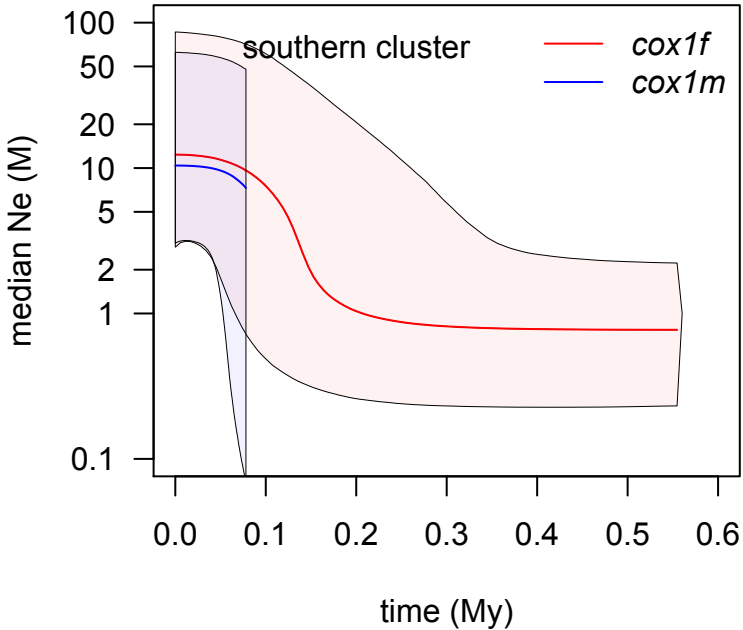
1.2
time (My)


southern cluster
cox1f
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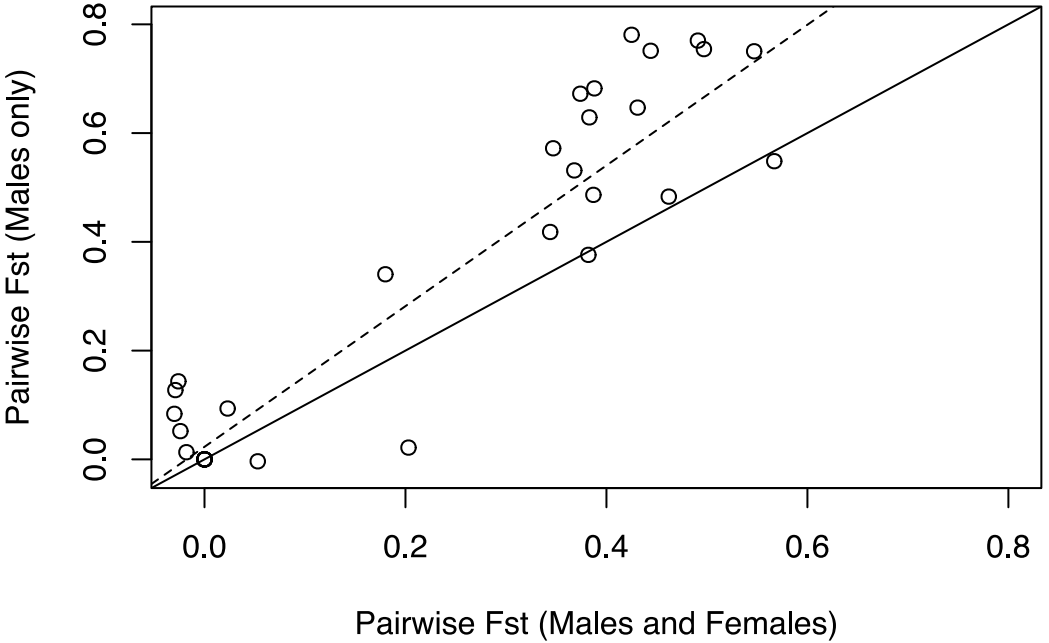
time (My)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Pairwise Fst (Males and Females)