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1 GLACIAL REFUGIUM VERSUS RANGE LIMIT: CONSERVATION GENETICS
2 OF *MACOMA BALTHICA*, A KEY SPECIES IN THE BAY OF BISCAY (FRANCE)

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Abstract: The bivalve *Macoma balthica* (L.) is a key species of intertidal mudflats in France and Europe. Its natural range has experienced a contraction along the European coastline towards the northeast during the past five decades. This southern boundary shift seems to be correlated with the increased sea surface temperature in the Bay of Biscay (France), a major glacial refugium during the LGM (Last Glacial Maximum, 18000 years ago). In this study, we used one mitochondrial marker (COI) and eight nuclear microsatellite markers to reveal patterns consistent with populations that are close to a glacial refugium. Meridional populations exhibit high genetic diversity, contrary to what is expected from populations at the edge of a species range. In addition, we highlight a barrier to gene flow in the Bay of Biscay populations, near Brittany. So considering (i) the previously-reported sensitivity of *M. balthica* to elevated temperatures, (ii) the genetic isolation of the southernmost populations, and (iii) the importance of this species in the trophic web, the population ecology and genetic structure of this species should be monitored in the context of global warming.

Keywords: Refuge zone, Global warming, Boundary shift, *Macoma balthica*, Microsatellites, Hybrid zone.

1. INTRODUCTION

Populations located near the boundary of their distribution area theoretically present structural and genetic characteristics that may limit their adaptive capacity (Eckert et al., 2008). Indeed, near the species range limit, populations are not at their optimum fitness level and most of the time exhibit a reduced growth rate (Brown et al., 1995; Thomas and Kunin, 1999). As a consequence they are often small, fragmented populations, vulnerable to genetic drift and exposed to the Allee effect and a reduced mutation rate (Courchamp et al., 1999; Alleaume-Benharira et al., 2006). Moreover, asymmetric gene flow from central populations toward those on the periphery restricts opportunities to develop local adaptations. This process, known as migration-selection balance, results in chronic maladaptation (Ronce and Kirkpatrick, 2001; Bolnick et al., 2008). Furthermore, environmental changes associated with supposedly low genetic diversity in populations close to the species range limit might increase the risk of extinction for such populations (Lawton, 1993; Vucetich and Wayne, 2003). The dynamic equilibrium between extinction and recolonization in these areas is more fragile, and renewal of populations more difficult (Lande et al., 2003). Under these conditions, the rate of environmental change is of direct importance because it is the main limitation to the development of an adaptive process.

In recent decades, the anthropogenic pressures applied to natural populations have dramatically increased (Vitousek et al., 1997; Walker and Kendrick, 1998), accelerating habitat fragmentation, change of species range and maladaptation. Moreover, during the last 30 years these effects have been accelerated by global warming (Bell and Collins, 2008) and are thus too recent and too fast for adaptation to have occurred. Indeed, stressors related to human activities (e.g. pollution, loss and fragmentation of habitat, release of invasive species) that lead to an interruption of connectivity within and between populations are synergistic with the increase in

temperature (Gordon, 1998; Schroter et al., 2005). Thus, between 1906 and 2005, the mean temperature of the earth surface increased by 0.74 °C, partially due to the greenhouse effect (GIEC, 2007), and in the marine environment the average temperature of surface waters increased by 0.23 °C per decade between 1977 and 2007 in the North East Atlantic (elevation of the surface water temperature in the Bay of Biscay in France, Goikoetxea et al., 2009). Parmesan and Yohe (2003) showed that, for more than 400 species, this phenomenon resulted in a shift in the distribution range coherent with the expected response to global warming: shifts of species range towards the pole, higher altitudes for terrestrial species or greater depths for aquatic species (see also Dulvy et al., 2008; Wetthey and Woodin, 2008). The response to such environmental pressures in populations located near their range limit is not generally known (Walther et al., 2002) but the consequences of species range shifts on the functioning and structure of ecosystems might be serious, particularly when key species are concerned.

As an example, the Baltic clam, *Macoma balthica* (Linnaeus, 1758) (Bivalvia: Tellinidae), is an infaunal tellinid bivalve commonly present in marine and estuarine soft-bottom habitats of the northern hemisphere. As an important prey for migratory birds (Piersma and Beukema, 1993), macro-invertebrates (Edjung and Bonsdorff, 1992) and fish (Mattila and Bonsdorff, 1998), *M. balthica* occupies an important place in the trophic webs of the European coasts (Philippart et al., 2003). It is usually found in tidal and subtidal sandy and muddy bottoms to a maximal depth of 30 m (Scarlatto, 1981; Hummel, 1985). In Europe, it is currently widely distributed from the eastern Pechora Sea (northern range limit) in Russia (Hummel et al., 1997b) to the Gironde estuary (southern range limit) in France (Bachelet, 1980). Nevertheless, during the past five decades, the natural range of *M. balthica* has undergone a significant shift towards the northeast (Hummel et al., 2000). Abundant along the Atlantic coasts of the Iberian

Peninsula more than 40 years ago (Otero and Milan, 1970), it has now completely disappeared from this area.

Several evolutionary studies with contradictory results have already been performed on *M. balthica*. Väinölä (2003) used allozymes to show the existence of two groups of populations, one large homogeneous NE Atlantic population (including the Norwegian Sea, the North Sea and the coasts of the British Isles), and one structured meta-population with a high genetic diversity and a large number of alleles in the northern part of the natural range of the species (the Pacific ocean, the Baltic Sea and the White Sea). These two groups are supposedly two subspecies coexisting in Europe: the Pacific lineage *M. balthica balthica* and the Atlantic lineage *M. balthica rubra* (Väinölä, 2003). Mitochondrial markers confirmed these results (Luttikhuisen et al., 2003; Nikula et al., 2008) but also revealed a very specific mitochondrial pattern in the southernmost population of *M. balthica* (i.e. Gironde estuary, France), with haplotypes that are quite divergent from the other European and Pacific populations. In contrast, Hummel et al. (1995, 1997b) and Strelkov et al. (2007) did not find any isolation of French Atlantic populations (including the Gironde and the Loire estuary) from other populations using allozymes. The French populations were indeed similar to the Norwegian Sea and western Barents Sea populations.

Thus, depending on the genetic markers used, studies have revealed two contrasting patterns for the populations in the Bay of Biscay: either (i) no differentiation from the northern populations and a low genetic diversity, which is to be expected for populations located near the species range limit, or (ii) a significant differentiation and divergence from the northern populations. The Bay of Biscay represents the current southern range limit of *M. balthica* but it was also one of the four major Pleistocene refuges in Europe during the Last Glacial Maximum (LGM) 18,000 years ago (Gómez and Lunt, 2007). As the ice retreated, populations recolonized northward by successive

founder-events (Hewitt, 1999). These populations might have come into secondary contact with Pacific populations recolonizing the Atlantic coasts from the North. In these circumstances, genetic diversity is expected to decrease from the refuge zone populations to the newly established populations (Petit et al., 2003).

From a conservation standpoint and given the theoretical vulnerability of populations at the range limit, it is therefore of importance to have a proper description of their precise distribution, genetic diversity and structure to infer their potential for adaptation under shifting selective environments (e.g., climate change, anthropogenic pressure; Moritz, 1994; Crandall et al., 2000). In this context, we conducted a mitochondrial analysis of 12 populations sampled along the French coastline and 6 populations sampled in the North Sea, the English Channel and the North Atlantic Ocean to assess historical processes. Contemporary features such as recent demographic history and gene flow were investigated using a set of newly-developed microsatellite markers (Becquet et al., 2009) with an emphasis on populations located at the edge of the current natural range of *M. balthica*.

2. MATERIALS AND METHODS

2.1 Sampling

Adult specimens of *M. balthica* were collected between 2003 and 2007 at 18 locations ranging from the Gironde estuary (known southern limit) to the Barents Sea (Murman population, see Fig. 1 and Table 1 for precise locations and sample sizes). Specimens were stored at -20 °C or in 95 % ethanol.

2.2 Mitochondrial COI sequencing of PCR products

Total DNA was extracted from less than 15 mg of muscle using the DneasyTM Tissue Kit (Qiagen, Germany). Amplifications of a COI gene fragment

(Cytochrome Oxidase Subunit 1) were performed using the set of primers described in Luttikhuisen et al. (2003). Amplifications were carried out in a total volume of 50 μ L consisting of 1X PCR buffer, 1.85 mM $MgCl_2$, 125 μ M dNTPs, 0.25 μ M of each primer, 1.6 U of Taq DNA polymerase (Red Hot $\text{\textcircled{R}}$ *TAQ* DNA Polymerase, Abgène) and about 10 ng of template DNA. A MJResearch PTC 100 Thermal Cycler was used with the following cycling profile: 3 min of initial denaturation at 95 $^{\circ}C$, followed by 35 cycles of denaturation at 95 $^{\circ}C$ for 45 s, annealing at 60 $^{\circ}C$ for 60 s and extension at 72 $^{\circ}C$ for 90 s. A final extension step was carried out for 5 min at 72 $^{\circ}C$. Double-stranded PCR products were cleaned using MultiScreen-PCR MANU03010 plates (Millipore). The sequencing was performed by the Genoscreen corporation (Campus Pasteur - 1 rue du Professeur Calmette - 59000 Lille - France) using an ABI PRISM $\text{\textcircled{R}}$ 3730 XL automated DNA Sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA). Sequence data were aligned using Clustal W (Thompson et al., 1994) and ambiguities were manually checked, comparing each sequence with its complementary fragment using BioEdit (Hall, 1999).

2.3 Microsatellite amplification and genotyping

PCR amplifications of 8 microsatellite loci were conducted using the primers described in Becquet et al. (2009). The protocol for DNA amplification consisted in a touch-down PCR procedure: 3 min of an initial denaturation step at 95 $^{\circ}C$ followed by 10 cycles of denaturation at 95 $^{\circ}C$ for 45 s, annealing at temperatures decreasing by 1 $^{\circ}C$ per cycle from T_{initial} to $T_{\text{annealing}}$ (T_{initial} varied between 68 $^{\circ}C$ and 62 $^{\circ}C$ depending on primers, Becquet et al., 2009) for 45 s and extension at 72 $^{\circ}C$ for 45 s, followed by 30 cycles at 95 $^{\circ}C$ for 45 s, annealing at a final $T_{\text{annealing}}$ for 45 s and extension at 72 $^{\circ}C$ for 45 s. A final extension step was carried out for 7 min at 72 $^{\circ}C$. The PCR products were screened on 6.5 % polyacrylamide gels using a Li-Cor NEN Global IR2 DNA sequencer. Allele sizes were determined using a known DNA

sequence with SAGA-GT software (v. 3.1: Automated microsatellite Analysis Software, LI-COR Biosciences).

2.4 COI sequence analyses

The haplotype number H , haplotype diversity H_d (Nei, 1987), the number of polymorphic sites S , and nucleotide diversity π (i.e. average number of nucleotide differences between pairs of sequences) were calculated for each population using the DnaSP v.5 software (Librado and Rozas, 2009). In order to test the assumption that populations at the range limit are less diversified than core populations, we defined two sets of populations separated by Finistère (Brittany, Fig. 1): the 9 southernmost locations were labelled as the ‘Bay of Biscay’ set (populations 1 to 9, Table 1) and the remaining 9 sampling sites were labelled as the ‘Core populations’ set (populations 10 to 18, Table 1). We examined the genetic structure of the overall and regional population by performing an analysis of molecular variance (AMOVA, Excoffier et al., 1992) as implemented in ARLEQUIN v.3.11. (Excoffier et al., 2005). Fixation indices (Wright, 1951) analogous to the F_{ST} , F_{CT} and F_{SC} parameters, were calculated to analyze genetic differentiation between populations over the whole study area (Φ_{ST}), among groups of populations within the species range (Φ_{CT}) and among populations within groups (Φ_{SC}) as defined in Excoffier et al. (1992). Φ -statistics take into account both haplotype frequencies and the molecular distances between haplotypes. Statistical significance of the Φ indices was tested using a non-parametric permutation procedure implemented in ARLEQUIN v.3.11. Moreover, one-sided tests to compare mean haplotype numbers of the two sets and mean nucleotide diversities were performed in R v. 2.11.1 (R Development Core Team, 2010) using either the Student t -test or the Wilcoxon test when parametric assumptions were not met.

Phylogenetic analyses at the intra-specific level were performed with the Network software v. 4.0.0.0 (Bandelt et al., 1999) that builds haplotypic networks based

on the median-joining algorithm (Cassens et al., 2003). This process combines both minimum spanning trees and maximum parsimony approaches to simplify the complex branching pattern and represent all the most parsimonious intra-specific phylogenies. Such networks describe the evolutionary relationships (based on mutational events) between the different haplotypes observed over the whole dataset. Finally, for each population we calculated the D parameter defined by Tajima (1989) and estimated the significance of deviations from zero in DNAsp v.5 (Librado and Rozas, 2009). Geneland v. 4.0 (Guillot et al., 2005a, 2005b, 2012) was used to infer spatial population structure. Geneland incorporates geographical information as a weak prior, with the assumption that most populations exhibit some degree of spatial structuring, and that the joint probability of any two individuals belonging to the same population decreases with the geographical distance between them. For each run, the number of groups (K) at Hardy-Weinberg equilibrium is assessed using maximum-likelihood estimations. Then, K is fixed at its most likely value and the posterior probability of belonging to each class is computed for each individual of the dataset. Finally, maps of posterior probabilities are generated. A total of 30 independent runs were computed in order to check for the convergence of MCMC computations with the following parameters: length of MCMC = 150,000 steps, thinning = 10, maximum rate of Poisson process = 550, maximum seed number for the Poisson-Voronoi tessellation = 1,650.

2.5 Microsatellite analyses

2.5.1 Genetic diversity within localities

Pairwise linkage disequilibrium among loci was tested using GENEPOP version 4.0.10 (Rousset, 2008), and significance levels were evaluated using a Markov-chain randomization procedure (MCMC) with 10,000 dememorization steps, 5,000 batches and 10,000 iterations per batch. The number of alleles per population and locus (N_{all})

and the observed (H_o) and expected (H_e) heterozygotes under Hardy-Weinberg equilibrium were calculated using Genetix v. 4.05.2. (Belkhir et al., 1996-2004). Allelic richness (R_{all}) was computed using FSTAT v. 2.9.3.2 (Goudet, 2002) to account for the differences in sampling sizes across populations. Mean and overall N_{all} , R_{all} , H_o and H_e were also computed for each set of populations (Bay of Biscay and Core population sets). As for the mitochondrial dataset, one-sided Student or Wilcoxon tests were performed in R v. 2.11.1 (R Development Core Team, 2010) to compare mean values for each population set.

2.5.2 Genetic differentiation and relationships among localities

Population assignment tests were conducted using GeneClass v. 2.0 (Piry et al., 2004). We followed the Bayesian approach of Rannala and Mountain (1997), and incorporated the exclusion-simulation significance test of Cornuet et al. (1999).

In addition to Geneland, two other Bayesian clustering programs, Structure and TESS, were used to infer structure among populations. Structure explores the number of populations in a dataset by optimizing Hardy-Weinberg equilibrium within putative groups, without taking geographical information into account. As Geneland, TESS uses geographical information in membership assignment. The three programs have different sensitivities to population structure and admixture levels (Chen et al., 2007; Guillot 2009; François and Durand 2010), and their comparative use allows weighing of the relative importance of geography.

Structure v. 2.3.2 (Pritchard et al., 2000; Falush et al., 2003, 2007; Hubisz et al., 2009) was used to test 1 to 10 clusters. We ran 10 independent simulations for each K using the admixture model, with a burnin period of 50,000 steps, and 200,000 post-burnin steps. The most likely number of clusters was inferred based on examination of $\ln P(D|K)$, as recommended by Pritchard et al. (2000). Scenarios with three and four

clusters were equally likely; however, examination of population assignment plots suggested that the presence of three clusters in our dataset was more probable. Geneland analyses were performed as for the mitochondrial dataset (details above). TESS v. 2.3 (Durand et al., 2009) was run with the conditional auto-regressive (CAR) admixture model, using 12,000 MCMC steps and a burnin period of 2,000 steps. Two to 10 clusters were tested, with 10 replicate runs of each K_{\max} . The spatial interaction parameter was set to 0.6 and the degree of trend was linear. The most likely K_{\max} was selected based on the Deviance Information Criterion (DIC), by minimizing its value and its variance, and by examining plots of individual membership probabilities. The most likely run among 10 replicates was then selected based on DIC.

3. RESULTS

3.1 Spatial population structuring

A 313-bp fragment of the COI gene was sequenced for 424 individuals sampled at 17 locations (Table 1, Fig.1). We identified 20 polymorphic sites defining 19 haplotypes (Genbank accession numbers: HM756170 to HM756189). The genealogical relationships among haplotypes are depicted in the minimum-spanning network presented in Fig. 2. The network is structured in three major clades organized around the 4 most frequent haplotypes (the combined frequencies for H1 to H4 was 94.7%). Three of these four haplotypes (H1 to H3) are separated by a single mutation, while haplotype H4 was found 3 mutations apart from the 3 others. The other 15 haplotypes were found at low frequencies and are equally spread around the 3 major clades. The overall structure of the network is consistent with the geographic locations of sampling sites: haplotypes H1 and H2 were found within the Bay of Biscay populations only, haplotype H3 was found mostly in the English Channel and the North Sea, while the most divergent haplotype H4 is characteristic of the populations of Ireland and Murman

(Fig. 2 and Fig. 3). For all sampling sites except WAD and IRE, Tajima's D did not significantly deviate from the standard neutral model ($p > 0.1$, Table 2). We therefore cannot reject the hypothesis of demographic equilibrium and selective neutrality (for the mitochondrial locus studied) in most European populations.

The mitochondrial pairwise F_{ST} estimates (Table 3) were found to be elevated and highly significant when comparing populations from the Bay of Biscay set (populations 1 to 9) with populations from the Core set (populations 10 to 18, $0.325 < F_{ST} < 0.938$, $p < 0.001$). This clustering was also significant according to the AMOVA results: the overall high genetic structure ($\Phi_{ST} = 0.613$, $p < 0.0001$) was mostly due to a large genetic differentiation between these 2 groups ($\Phi_{CT} = 0.587$, $p < 0.0001$) while the genetic differentiation among populations within these groups appeared to be low ($\Phi_{SC} = 0.0613$, $p < 0.0001$).

Assignment analyses with microsatellite markers revealed a high rate of self-recruitment in all populations from the Bay of Biscay and the Core population sets. Auto-assignment proportions ranged from 54 % (Aiguillon) to 100 % (Murman) with a mean value of 70 % (Fig. 4).

Spatial population clustering inferred from Structure, TESS and Geneland analyses led to contrasting mitochondrial and nuclear patterns. Mitochondrial data, analysed using Geneland, revealed significant clustering among (1) Bay of Biscay populations, (2) core populations from Brittany to Germany, and (3) Ireland and Murman populations (Fig. 5, Fig. S1). Microsatellite data, analysed with Geneland, Structure and TESS, could be split into three clusters. Bay of Biscay populations were separated by the Loire estuary and formed two groups (1st group: populations 1-6; 2nd group: populations 7-9; $F_{ST} = 0.044$, Table S1). A second genetic break was detected at the tip of Finistère (F_{ST} ranges between 0.021 and 0.035, Table S1). In addition, in a four-cluster scenario (data not shown), TESS further split core populations into two strongly-admixed groups: (1)

populations from Brittany to Germany, and (2) populations from Ireland and Murman.

3.2 Molecular diversity

Overall (except for the monomorphic population of Murman) the numbers of haplotypes (H) and segregating sites (S) ranged from 2 to 5 (mean = 3.35, sd =1.32) and from 2 to 7 (mean = 3.71, sd =1.65), respectively (Table 2). The mean haplotype number (see Table 2) was significantly higher in the Bay of Biscay set than in the Core set (one-sided Wilcoxon test, $W = 55$, $p = 0.033$). The same result was obtained for nucleotide diversity (one-sided Wilcoxon test, $W = 57$, $p = 0.023$) with an even more striking difference between the population sets: the mean nucleotide diversity within the Bay of Biscay set ($\pi = 0.03153 \pm 0.02646$) was found to be more than ten fold greater than within the Core set ($\pi = 0.00275 \pm 0.00192$, Table 2).

The multilocus genotype (8 microsatellite markers) was obtained for 545 individuals collected at 18 sampling sites (Table 1). No linkage disequilibrium was detected between loci (pairwise exact tests, $p > 0.2$). Overall, the mean number of alleles (N_{all}) ranged from 6.62 (Bonne Anse) to 11.25 (Aytré) with a total of 20.87 over the whole dataset (Table 2). Most of the differences observed across populations were due to varying sample sizes since all allelic richness values (R_{all}) were similar, ranging from 4.0295 (Seine) to 4.7716 (Aiguillon) (overall population mean and standard deviation = 4.39 ± 0.23). However, a highly significant difference was found when comparing mean R_{all} values for both population sets (one-sided two-sample Student t -test, $t = 5.933$, $df = 16$, $p = 1.051e^{-5}$): a greater mean R_{all} value was found within the Bay of Biscay set ($R_{all} = 4.58 \pm 0.15$) than within the Core set ($R_{all} = 4.21 \pm 0.12$, see also Fig. 6).

The same pattern was observed for both the observed (H_o) and the expected (H_s) heterozygosities (see Fig. 7). While H_o and H_s showed little variation across the whole

dataset (the mean and standard deviation across the 18 populations was 0.366 ± 0.0425 and 0.6325 ± 0.0347 for H_o and H_s respectively, Table 2), both indices were found to be significantly greater in the Bay of Biscay set than in the Core set ($t = 2.513$, $df = 16$, $p = 0.012$ for H_o and $t = 2.246$, $df = 16$, $p = 0.020$ for H_s). Heterozygote deficiency was high for all populations (mean F_{IS} value and standard deviation of 0.424 ± 0.054). The same levels of heterozygote deficiency were found in both sets (mean F_{IS} and standard deviation = 0.4187 ± 0.0491 and 0.4305 ± 0.0603 for the Bay of Biscay set and for the Core set, respectively) and a two-sided Student t test showed no difference between the set means ($t = 0.457$, $df = 16$, $p = 0.654$).

4. DISCUSSION

4.1 Genetic structure

4.1.1 High gene flow at macro-scale

The first stage of development of *M. balthica* consists of a 2 to 5 week period of larval pelagic life, during which larvae can be carried over long distances by ocean currents before they settle (Beukema and De Vlas, 1989; Caddy, 1967; Drent, 2002). As a consequence, in the absence of physical or genetic barriers, populations are supposed to be highly connected by gene flow (Caley et al., 1996). However, the detection of first generation migrants with microsatellite markers showed the existence of a high self-recruitment inside each population sampled along the coasts of Europe. This result is congruent with those obtained with many marine benthic organisms for which evidence of a high proportion of self-recruitment at local spatial scales has accumulated (Cowen et al., 2000; Armsworth et al., 2001). Hence, a larval dispersion model tested on the marine polychete *Pectinaria koreni* confirmed that a strong larval retention in a bay led to self-recruitment (Ellien et al., 2000); marine ecologists have recently argued for a

paradigm shift, advocating the preponderance of self-recruitment (Hellberg et al., 2002; Swearer et al., 2002). Among the many parameters potentially involved in self-recruitment, the literature emphasizes the importance of larval behaviour and mortality as well as oceanographic features on the effective dispersal distance of marine species (Kingsford et al., 2002; Siegel et al., 2003; Toonen and Tyre, 2007).

In spite of this high self-recruitment, the genetic structure analysis of natural populations of *M. balthica* with a mitochondrial marker showed a high level of gene flow at the European scale. Indeed, only 3 clades separated by a few mutations (1 to 3) were detected with mtDNA. However, the genetic structure analysis revealed a clear gene flow rupture between the Bay of Biscay population set (i.e. populations 1 to 9) and the core population set located in the North (Fig. 1) with a high genetic differentiation (Table 3, $0.325 < F_{ST} < 0.938$). This reproductive isolation between the Bay of Biscay populations and the other European populations is in agreement with previous results obtained by Luttkhuisen et al. (2003) and is probably due to the marine biogeographic boundary located around Brittany. As underlined by Leppäkoski et al. (2002), the effects of the Gulf Stream along the European coastline result in a strong biogeographic barrier around Brittany for a variety of marine phyla, limiting species exchange between the northern and southern areas. This gene flow rupture between populations located on both sides of the tip of Brittany has already been established for several marine invertebrate species, including *Pecten maximus* (Wilding et al., 1997), *Mytilus* sp. (Bierne et al., 2003a), *Crepidula fornicata* (Dupont et al., 2003) and *Pectinaria koreni* (Jolly et al., 2005).

4.1.2 Discordance between mitochondrial and nuclear genetic structure.

Within the Bay of Biscay, the genetic structure inferred from the results with the two types of markers (mtDNA and nuclear) differed significantly. The mitochondrial

structure revealed almost identical haplotype frequencies for all sampling sites within the Bay of Biscay (high frequency of clade 1 haplotypes, very low frequency or absence of clade 2 and 3 haplotypes, Fig. 1 and Fig. 3) with no significant pairwise differentiation between populations (Table 3), a pattern confirmed with the Bayesian analysis performed on mitochondrial data with Geneland. These results suggest that all sampling sites in the area belong to a unique panmictic population. On the contrary, the Bayesian simulations performed with microsatellite markers showed a strong and well supported genetic break within the Bay of Biscay, focused on the Loire estuary (i.e. between populations 6 and 7, Fig. 5). This gene flow rupture was not detected in previous studies, probably because of the limited resolution of the markers used (mitochondrial marker in Luttikhuisen et al. 2003 and Nikula et al. 2007 and allozymic markers in Hummel et al. 1997b) and because of the low number of populations sampled within the Bay of Biscay.

Local hydrodynamics during the larval stage might explain this genetic barrier within the Bay of Biscay. Indeed, a postzygotic isolation might exist within the semi-closed Bay of Marennes-Oléron and, on a wider scale, within the Pertuis Charentais area (i.e. populations 1 to 6) where a 1300 km² semi-closed area is delineated by two 30 km-long islands only a few kilometres apart. This assumption is supported by the high self-recruitment detected in this study (Fig. 4). Larval retention might be reinforced for populations 1 to 6 as waters from the Gironde and Loire estuaries rarely mix, leading to the formation of eddies and up-wellings that favor larval retention (Lazure and Jegou, 1998). The incongruence between mitochondrial and nuclear data, however, would suggest that this barrier is too recent to be detected with mitochondrial markers, which tend to evolve more slowly than microsatellite ones (e.g. Brown et al., 1979; Estoup and Angers, 1998). This hypothesis is unlikely, as the Loire estuary was already present in the Pleistocene, when profound structuration of mitochondrial lineages occurred.

The nuclear genetic differentiation inside the Bay of Biscay might also be the result of divergent selection. Indeed, even if Tajima's D test failed to reveal selection, the selection might occur at a contemporary scale. Moreover, the nine populations studied here are impacted by contrasting environmental factors since the characteristics of the water masses in this area are especially influenced by the Loire and Gironde estuaries. The Loire estuary is known to be greatly affected by Polycyclic Aromatic Hydrocarbon (PAH) and polychlorobiphenyls (PCB, RNO, 2004b), while the Gironde estuary (directly connected with the Charente estuary and Bay of Marennes Oleron, Boutier et al., 2000) is heavily impacted by trace metals (e.g. Cd, Zn, Cu, Grousset et al., 1999; RNO, 2004b).

This contrasting pattern obtained with these different types of markers might be a consequence of a genetic barrier to gene flow like those observed in transition zones (Lemaire et al., 2005). During the Pleistocene, Pacific populations of *M. balthica* colonized the Atlantic Ocean down to the Iberic coasts after the opening of the Bering Strait (Väinölä, 2003). Throughout the Last Glacial Maximum (LGM, 18,000 years ago), all the northern European coasts were covered by ice except for the Bay of Biscay and the Spanish region, which is known to have been one of the four major temperate refugia (Gómez and Lunt, 2007). After the LGM, natural populations of *M. balthica* in this area became the source for the recolonization of northward sites (Austin et al., 2004; Howes et al., 2006). Meanwhile, after the complete disappearance of the ice sheets from northern Europe, migration began again from North to South. Thus, the post-glacial recolonization of the northern European coasts occurred from two isolated *M. balthica* stocks via two distinct paths: (i) a South to North route from an Iberian refugium and (ii) a North to South route from the Pacific Ocean; this migration led to a secondary contact between differentiated populations. The existence of genetic signatures typical of a hybrid zone (Barton and Hewitt, 1985) might be the consequence

of the admixture of divergent *M. balthica* lineages in the Bay of Biscay. First, the incongruence between mitochondrial and nuclear data is typical of transition zones (Lemaire et al., 2005). A hybrid zone can emerge when two divergent lineages come into secondary contact (Barton and Hewitt, 1985), which has previously been shown for *M. balthica* (Väinölä, 2003; Luttikhuisen et al., 2003). Hybridization between distinct mitochondrial lineages (*M. balthica balthica* and *M. balthica rubra*) has indeed been described in the Baltic (Nikula et al., 2007). In addition, biogeographic conditions among French sampling locations is conducive to the establishment of contact zones as semi-permeable hydrographic barriers may exist (e.g. Loire and Finistère), and a population density drop was observed around Finistère (pers. obs.). Finally, two closely located hybrid zones have been well documented in the Bay of Biscay between *Mytilus edulis* and *My. galloprovincialis* (Bierne et al., 2002, 2003b).

4.2 Genetic diversity within peripheral populations

In the standard abundant-center model, species are expected to be at lower abundance at the edge of the range than at the geographical centre, with populations becoming progressively smaller, more fragmented and sparsely distributed (Lawton, 1993; Vucetich and Waite, 2003). Genetic organization is thus affected in terms of diversity and population structure. As an example, several studies showed that peripheral populations exhibit low genetic diversity, greater genetic differentiation (structured metapopulations), small effective population sizes and geographic isolation as compared to more central populations (El Mousadik and Petit, 1996; Durka, 1999; Bouzat and Johnson, 2004).

Contrary to these theoretical expectations (and to previous results based on isoenzymes only, Hummel et al., 1997b) but according to expectations of a glacial refugium (Maggs

et al., 2008), our study revealed a significantly higher genetic diversity in populations from the Bay of Biscay than in the Core population set. Haplotype number, haplotype and nucleotide diversities, observed and expected heterozygosities and allelic richness all support this tendency (Table 2, Fig. 6, Fig. 7). Besides, despite a high overall heterozygote deficiency ($F_{IS} = 0.4305$) which was also observed for many marine molluscs (Zouros and Foltz, 1984), the Bay of Biscay populations do not seem to be affected by inbreeding, as confirmed by the high levels of genetic diversity in these populations. This peculiar genetic pattern is thus more characteristic of a population coming from a refuge zone (Gómez and Lunt, 2007; Maggs et al., 2008) than of a population located at the range limit of a species. Hewitt (1999) clearly established that a feature of temperate species (*e.g.* alder, beech, grasshoppers and newts) in southern Europe was the presence of several distinct “geographical genomes” with a variety of alleles. This pattern is present in southern populations of *M. balthica*, which exhibit two of the four major haplotypes (H1 and H2) found exclusively within the Bay of Biscay. On the other hand, the haplotypes H3 and H4 were rarely found in the Bay of Biscay populations but were the most frequent in every population from the Core set (except for the strictly monomorphic population of Murman, Figs. 2 and 3). Likewise, the higher nuclear diversity observed in the Bay of Biscay populations is mainly due to the high number of private alleles (*i.e.* alleles sampled only once) found in these populations. Regarding the loci *mac40*, *mac10* and *mac84*, 44 %, 47 % and 69 % of these alleles were found only in the southernmost populations. These populations exhibit a total of 42 rare and private alleles across 8 loci while Core-populations exhibit only 19.

These results are consistent with a rapid northward expansion after the Last Glacial Maximum from a refuge zone located either in Spain or in the Bay of Biscay, and might be a reflection of cryptic populations.

476

477 4.3 Implications for conservation

478 During the last 50 years the natural range *M. balthica* has undergone a strong northward
479 contraction, as observed in many other species (Koutsikopoulos et al., 1998; Parmesan
480 and Yohe, 2003). The populations currently located near the southern range limit of the
481 species (i.e. in the Bay of Biscay) present higher genetic diversity than populations
482 located in the North. However, the Bay of Biscay populations might be at risk because
483 of the strong barrier to gene flow around Brittany, isolating these populations from the
484 center of the natural range.

485 The potential disappearance of *M. balthica* along the French Atlantic coasts may have
486 negative cascading effects on littoral ecosystems. As the favourite prey for many
487 shorebirds, fishes and macro-invertebrates (Piersma and Beukema, 1993; Edjung and
488 Bonsdorff, 1992; Mattila and Bonsdorff, 1998), *M. balthica* is a key species for
489 intertidal communities. The Pertuis Charentais area and southern Brittany (where
490 populations 1 to 9 were sampled) are known to be a major wintering area for the Black
491 Tailed Godwit *Limosa limosa* (Gill et al., 2007), a species that feeds almost exclusively
492 on *M. balthica* stocks on intertidal mudflats (Bocher P, Robin F, personal
493 communication). Indeed, Zwarts and Blomert (1992) highlighted the energetic
494 importance of *M. balthica* intake among a 5 species mix of bivalves in the diet of
495 another shorebird, *Calidris canutus*. Its disappearance could thus have a dramatic and
496 lasting impact on the whole ecosystem. As an illustration, the decreasing bivalve
497 recruitment observed in the Wadden Sea (confirmed by a positive and significant
498 Tajima's D value) led to a massive emigration, starvation and mortality of bivalve-
499 eating birds (Camphuysen et al., 2002).

500

501 In addition to the warming of surface waters in the North East Atlantic, many

anthropogenic pressures might have accelerated the shift northward of range of *M. balthica*. Pollution and habitat fragmentation induced by littoral industrialization are two possible factors. Nevertheless, many studies have been conducted on the performance of *M. balthica* in highly polluted sites (Szefer and Skwarzec, 1988; Pempkowiak and Szefer, 1992; Pempkowiak et al., 1999). These showed that *M. balthica* seemed to remain a major component of the macrozoobenthic communities in heavily polluted environments when other species were declining or had disappeared (Szaniawska et al., 1996).

In contrast, an increase in temperature is known to greatly affect the physiological capacities of individuals, especially for organisms living at the low latitude margin of their natural range (Hugues, 2000). A recent study on the metabolic and respiratory rates of *M. balthica* (Jansen et al., 2007) revealed a zero plasticity to temperature for populations close to the southern range limit. The author translocated a Gironde population to the Bidasoa Bay (where *M. balthica* was present 50 years ago) and observed rapid mortality. These results reinforced those published by Hummel et al. (1998) showing limited ecophysiological performances for *M. balthica* populations at the edge of its natural range.

The increase of surface waters temperature due to climate change is expected to affect adult survival, dispersal abilities and pelagic larval duration (O'Connor et al., 2007, Munday et al., 2008) and would potentially limit population connectivity (Cowen and Sponaugle, 2009). Indeed, temperature will affect larval stage in two ways. First, it will affect basal metabolism, growth, development and energetic costs for larvae, thus modifying the pelagic larval duration (Shanks et al., 2003). Second, the increase in temperature of water bodies encountered by pelagic larvae might be responsible for current displacement (as well as eddies or up-wellings), potentially altering larval transport, growth and survival (Meekan et al., 2003). This connectivity between sub-

populations would potentially impact the population dynamics and structure and would then have a major role in terms of conservation and species management (Cowen et al., 2007).

CONCLUSION

We showed that *M. balthica* populations located near the species southern range limit exhibited high genetic diversity, probably due to the secondary contact of two isolated gene pools after the last glacial maximum. However, a high diversity at neutral loci is not enough to guarantee the survival of a population, as suggested by the disappearance of the southern French and Spanish populations during the last 50 years. The strong barrier to gene flow isolating the Bay of Biscay populations from the central area of the species range makes them even more vulnerable to environmental changes. A fine scale study of sub-population connectivity is now needed in this zone as well as an evaluation of the hybrid index of these populations. Combining the use of a large number of SNP markers and an experimental genomic approach with genetically-divergent populations (e.g. MO and North Brittany populations) would provide some insight into the tolerance to elevated temperatures of *M. balthica* in the context of global warming.

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897

Sampling Site	Code	Latitude (N)	Longitude (W)	N _{mito}	N _{nuc}
(1) Bonne Anse	BON	45.5523	-0.9294	16	13
(2) Charente	CHA	45.9583	-1.0501	15	25
(3) Fouras	F	45.984	-1.0925	8	36
(4) Yves	Y	46.0116	-1.0561	0	39
(5) Aytres	AY	46.126	-1.1306	13	50
(6) Aiguillon	AIG	46.1617	-1.1298	31	16
(7) Noirmoutiers	NM	46.9035	-2.1671	20	40
(8) Loire	LOI	47.2675	-2.1715	24	47
(9) Pont-Mahe	PT-MH	47.4442	-2.4608	16	23
(10) Mont Saint Michel	MSM	48.438	-1.5153	30	30
(11) Seine	SEI	49.4029	0.1205	32	29
(12) Somme	SOM	50.2146	1.6227	29	28
(13) Westerschelde	WES	51.3792	3.6272	31	27
(14) Balgzand	WAD	52.9301	4.7953	31	28
(15) Wilhemshaven	WIL	53.4802	8.0641	32	28
(16) Sylt	SYL	54.7789	8.2954	40	40
(17) Ireland	IRE	53.257	-9.1201	36	31
(18) Murman	MUR	69.3073	33.556	20	15
Total				424	545

Table 1: Sampling sites, locality codes, GPS coordinates (WGS84) and number of individuals analysed with mitochondrial (N_{mito}) and microsatellites nuclear markers (N_{nuc}) for the eighteen European sampling sites of *Macoma balthica*.

Mitochondrial data							Nuclear data					
Bay of Biscay	N	S	H	Hd	π	D	N	Nall	Rall	Ho	Hs	F_{IS}
(1)	16	4	4	0.442	0.00269	- 0.792	13	6.625	4.3806	0.304	0.577	0.5027
(2)	15	3	5	0.476	0.00188	- 0.513	25	8.526	4.5149	0.368	0.630	0.4325
(3)	-	-	-	-	-	-	39	11	4.7357	0.375	0.661	0.4437
(4)	8	2	2	0.536	0.05357	1.448	36	1.75	4.6560	0.368	0.640	0.4342
(5)	13	7	5	0.628	0.07179	- 1.378	50	11.25	4.7440	0.434	0.659	0.3339
(6)	31	4	5	0.434	0.00219	- 0.686	16	9.625	4.7716	0.408	0.670	0.4207
(7)	20	2	3	0.468	0.04184	1.136	37	9.125	4.4706	0.430	0.676	0.3764
(8)	24	5	5	0.493	0.03913	- 1.204	47	10.25	4.5025	0.381	0.674	0.4444
(9)	16	2	3	0.508	0.03917	0.767	23	7.375	4.4446	0.423	0.654	0.3794
Total	143	11	11	0.464	0.0411	-	286	18.25	4.5800	0.395	0.668	0.4305
Mean	17.9	3.63	4	0.498	0.0315	-	31.78	8.392	4.5801	0.388	0.649	0.4186
(S.D.)	7.08	1.768	1.195	0.062	0.0265	-	13.16	2.932	0.1475	0.041	0.031	0.0491
Core populations	N	S	H	Hd	π	D	N	Nall	Rall	Ho	Hs	F_{IS}
(10)	30	4	2	0.129	0.00154	- 1.258	30	8.375	4.2451	0.376	0.618	0.4087
(11)	32	4	3	0.123	0.00127	- 1.443	29	8.125	4.0295	0.333	0.599	0.4598
(12)	29	4	3	0.490	0.00441	1.156	28	8.375	4.2860	0.329	0.602	0.4681
(13)	31	5	4	0.385	0.00290	- 0.613	27	8.125	4.3306	0.303	0.626	0.5298
(14)	31	3	2	0.512	0.00460	2.436*	28	8.750	4.3304	0.357	0.639	0.4570
(15)	32	6	5	0.565	0.00491	0.281	28	8.125	4.2112	0.382	0.607	0.3864
(16)	40	4	3	0.497	0.00448	1.414	40	9.500	4.2814	0.343	0.622	0.4613
(17)	36	4	2	0.056	0.00067	- 1.88	31	7.625	4.1177	0.379	0.558	0.3354
(18)	20	0	1	0	0	-	15	7.750	4.0522	0.293	0.673	0.3681
Total	281	11	10	0.523	0.07684	-	256	15.50	4.2110	0.358	0.628	0.4434
Mean	31.2	3.78	2.78	0.306	0.00275	-	28.44	8.306	4.2093	0.344	0.616	0.4305
(S.D.)	5.40	1.64	1.20	0.225	0.00192	-	6.386	0.559	0.1157	0.033	0.031	0.0604
All populations	N	S	H	Hd	π	D	N	Nall	Rall	Ho	Hs	F_{IS}
Total	424	18	19	0.720	0.08984	-	542	20.875	-	0.3740	0.6500	0.4426
Mean	24.9	3.71	3.35	0.396	0.01630	-	30.11	8.305	4.395	0.3660	0.6325	0.4246
(S.D.)	9.15	1.65	1.32	0.192	0.02300	-	10.18	0.165	0.230	0.0425	0.0347	0.0537

Table 2: Mitochondrial and nuclear diversity for each population and each set of populations (Bay of Biscay and Core populations). N: number of samples; S: segregating site; H: haplotype number; Hd: haplotype diversity; π : nucleotide diversity; D: Tajima's D ($p > 0.1$, significant value with an asterisk $p < 0.05$) Nall: allele number; Rall: allelic richness; Ho: observed heterozygosity; Hs: gene diversity and F_{IS} : heterozygote deficiency.

	Bay of Biscay populations								Core populations								
Site	(1)	(2)	(3)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)	(17)	(18)
(1)	0.000																
(2)	-0.046	0.000															
(3)	-0.017	0.041	0.000														
(5)	-0.047	-0.035	-0.018	0.000													
(6)	-0.037	-0.037	0.002	-0.032	0.000												
(7)	-0.029	0.006	-0.075	-0.029	-0.015	0.000											
(8)	-0.036	-0.009	-0.060	-0.040	-0.026	-0.054	0.000										
(9)	-0.035	-0.036	0.053	-0.030	-0.016	0.023	0.011	0.000									
(10)	0.492*	0.601*	0.374*	0.368*	0.520*	0.444*	0.431*	0.547*	0.000								
(11)	0.507*	0.622*	0.386*	0.379*	0.538*	0.460*	0.447*	0.564*	-0.021	0.000							
(12)	0.509*	0.627*	0.387*	0.382*	0.541*	0.462*	0.450*	0.567*	-0.024	-0.032	0.000						
(13)	0.442*	0.528*	0.342*	0.325*	0.466*	0.398*	0.385*	0.490*	0.012*	0.014	0.023	0.000					
(14)	0.509*	0.569*	0.446*	0.395*	0.525*	0.485*	0.480*	0.540*	0.288*	0.302*	0.318*	0.146*	0.000				
(15)	0.456*	0.517*	0.383*	0.344*	0.471*	0.425*	0.418*	0.491*	0.193*	0.204*	0.218*	0.072	-0.019	0.000			
(16)	0.461*	0.526*	0.388*	0.347*	0.477*	0.431*	0.426*	0.497*	0.180*	0.190*	0.203*	0.059	-0.007	-0.026	0.000		
(17)	0.862*	0.909*	0.836*	0.782*	0.880*	0.864*	0.868*	0.878*	0.869*	0.884*	0.891*	0.767*	0.472*	0.529*	0.571*	0.000	
(18)	0.891*	0.938*	0.866*	0.813*	0.909*	0.894*	0.899*	0.906*	0.910*	0.925*	0.931*	0.812*	0.533*	0.584*	0.626*	0.000	0.000

Table 3: Pairwise differentiation (mtDNA sequences).
Significant values are indicated with an asterisk (exact test, $p < 0.05$).

Fig. 1: Sample sites and haplotype distribution for *Macoma balthica* populations. The colors used in pie charts are the same as in Fig. 2.

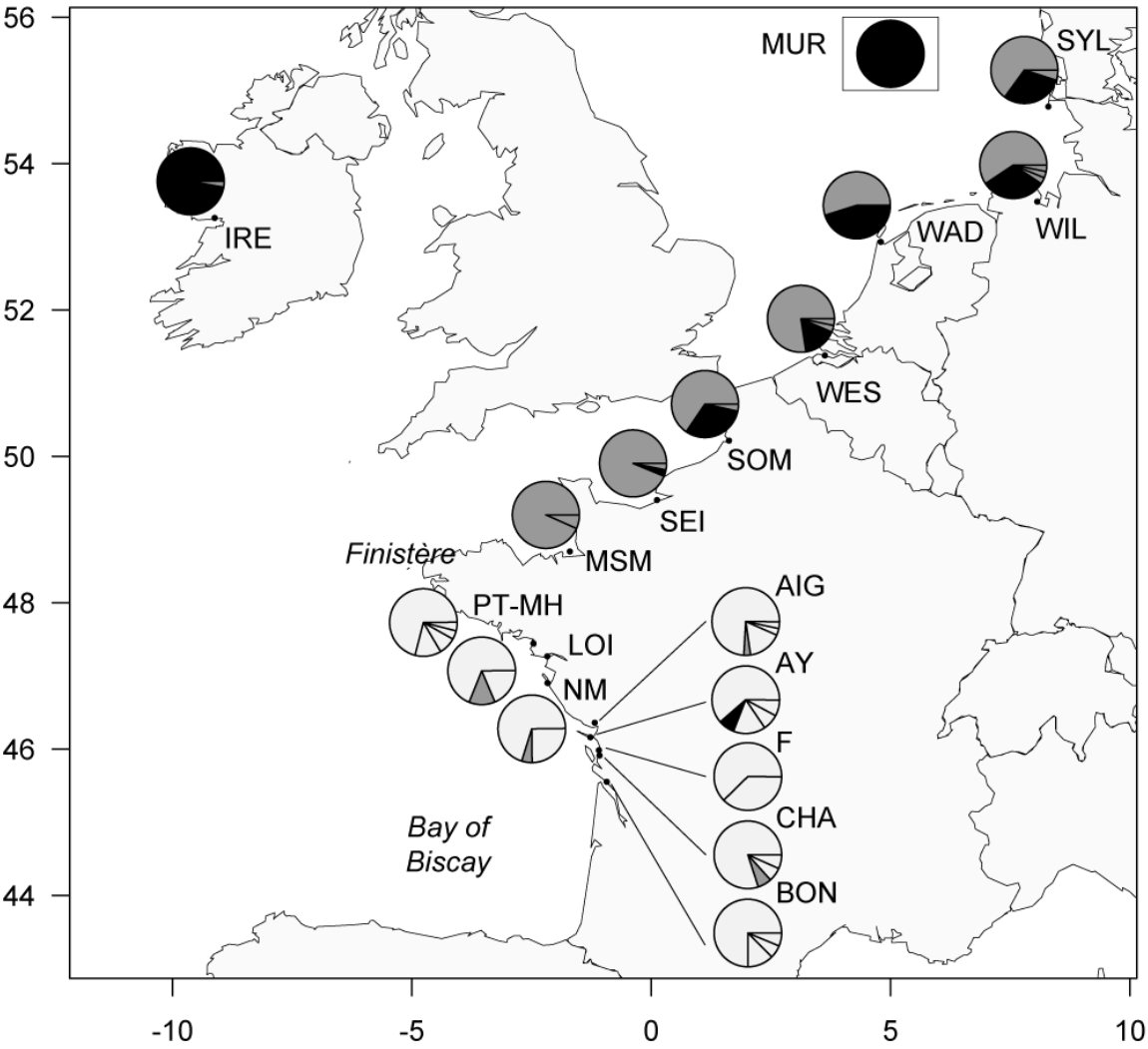


Fig. 2: Minimum spanning network displaying mitochondrial variation along 313 bp of the COI gene. Each circle represents a haplotype. Circle size is proportional to haplotype frequency. Each segment represents a single mutational event. H1 and H2 are haplotypes typical of the Bay of Biscay, and H3 and H4 are characteristic of the Core populations.

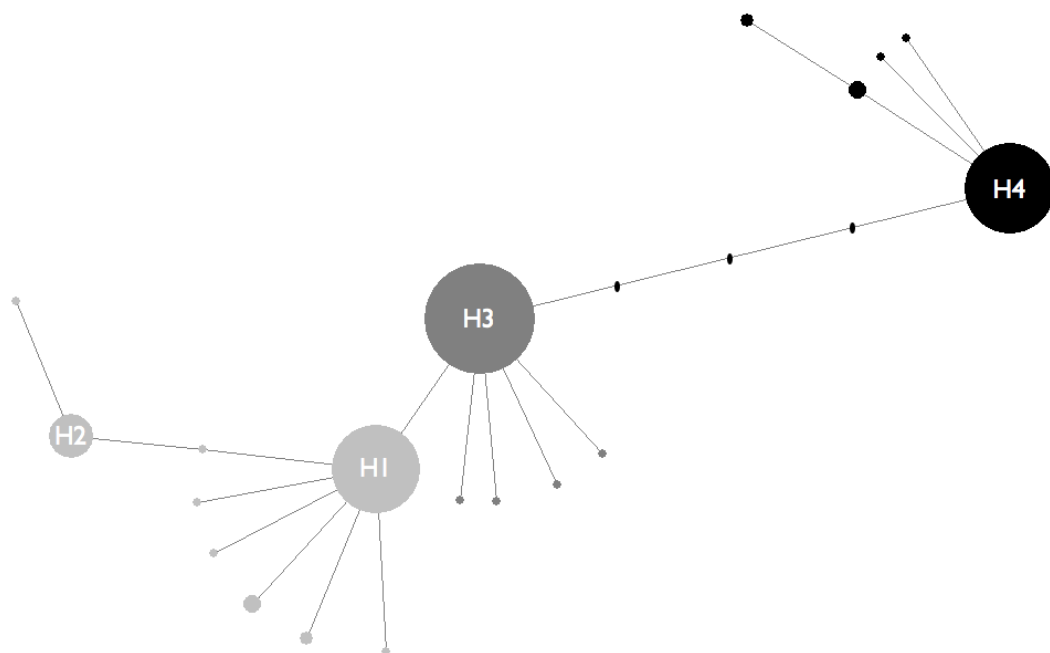


Fig. 3: Frequencies of the four most common haplotypes (H1-H4) identified along the European coast. Except for Ireland, sampling sites are ordered by increasing latitude along the x-axis.

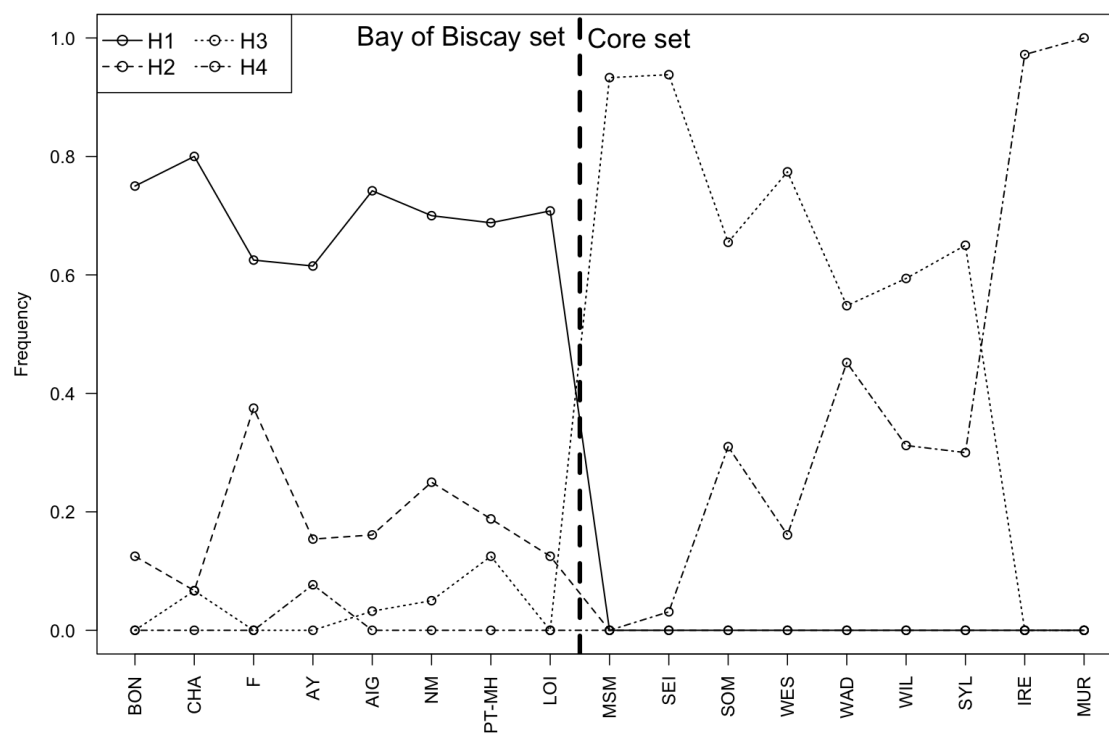


Fig. 4: Assignment proportions inferred by GeneClass. For each population presented in a column, the proportion of individuals assigned to populations on the right is represented by a square (scale: bottom left). For instance, most individuals sampled from Aytré were assigned to that same population (i.e., strong proportion of auto-assignment) and a few individuals only were assigned to the populations Y, CHA, LOI and MSM.

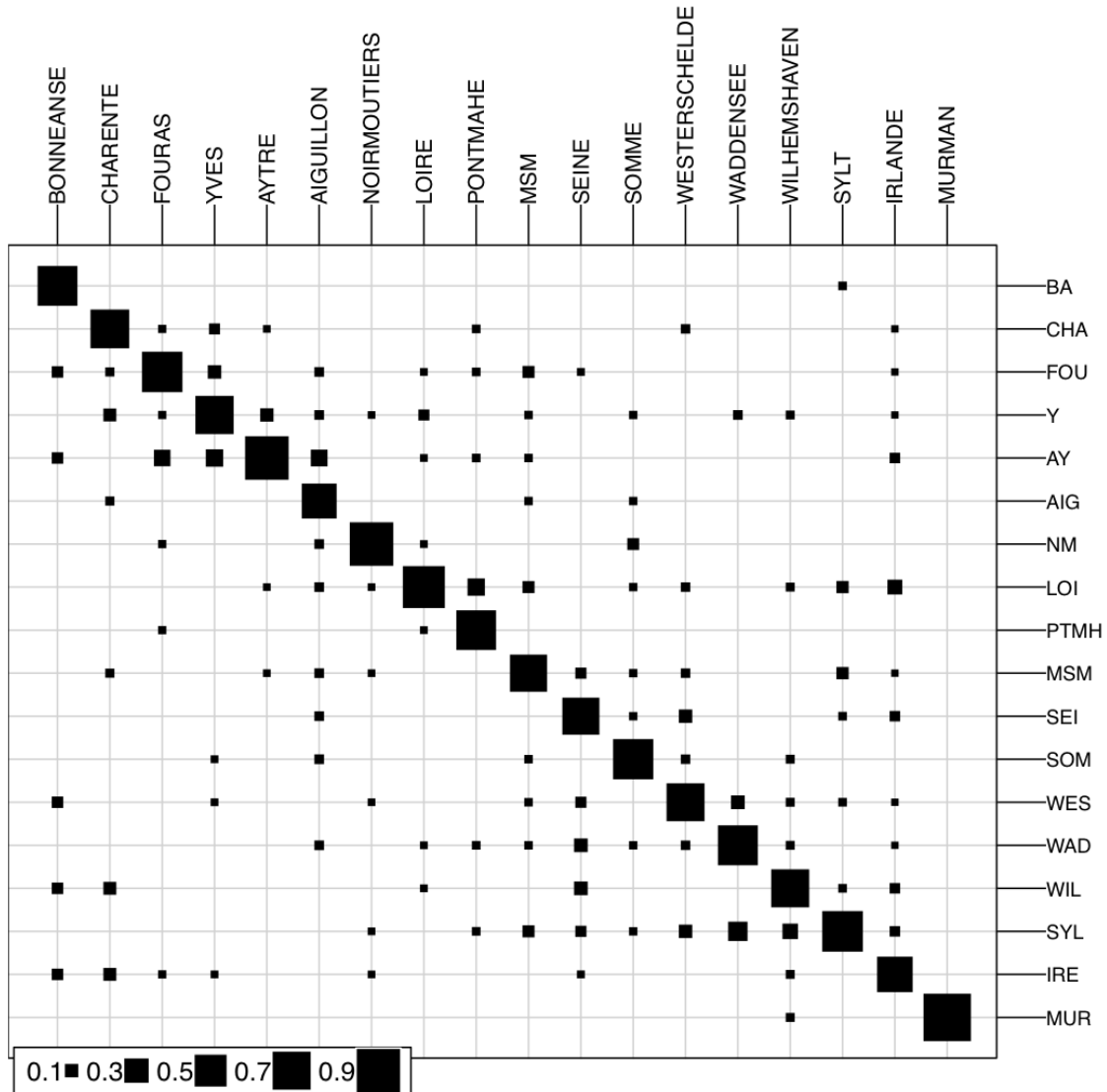


Fig. 5: Summary of spatial population clustering, based on results from Geneland, TESS and Structure analyses using mitochondrial and microsatellite dataset. At each sampling location, results from each program are given as a symbol, along a segment (see key). Filled circles represent nuclear data, while diamonds represent mitochondrial data. Symbol color represents cluster identity. Abbreviations: mG, Geneland, mitochondrial data; G, Geneland, nuclear data; T, TESS, nuclear data; S, Structure, nuclear data.

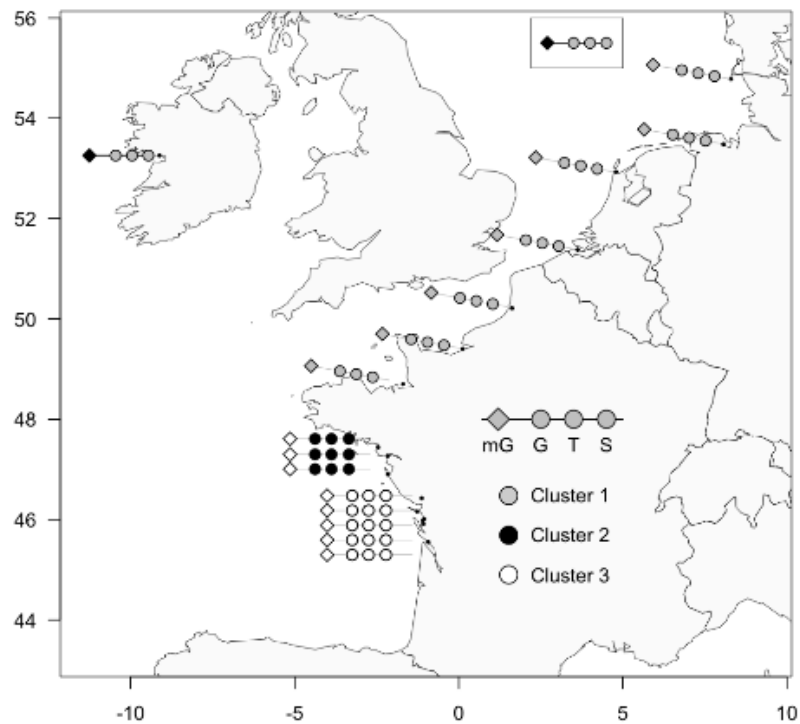


Fig. 6: Box-and-whisker plots of allelic richness in the Bay of Biscay and Core population sets. For each population set, the R_{all} values presented are (in order of decreasing values): maximum, third quartile, median, first quartile and minimum.

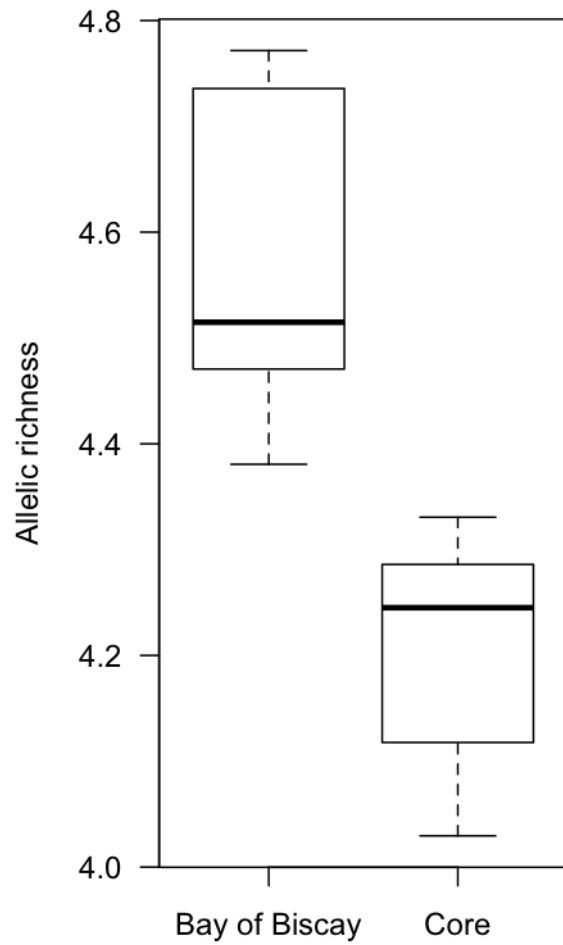


Fig. 7: Box-and-whisker plots of observed (white) and expected (grey) heterozygosity in the Bay of Biscay and Core populations sets.

