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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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18 Abstract: The bivalve *Macoma balthica* (L.) is a key species of intertidal mudflats in  
19 France and Europe. Its natural range has experienced a contraction along the European  
20 coastline towards the northeast during the past five decades. This southern boundary  
21 shift seems to be correlated with the increased sea surface temperature in the Bay of  
22 Biscay (France), a major glacial refugium during the LGM (Last Glacial Maximum,  
23 18000 years ago). In this study, we used one mitochondrial marker (COI) and eight  
24 nuclear microsatellite markers to reveal patterns consistent with populations that are  
25 close to a glacial refugium. Meridional populations exhibit high genetic diversity,  
26 contrary to what is expected from populations at the edge of a species range. In  
27 addition, we highlight a barrier to gene flow in the Bay of Biscay populations, near  
28 Brittany. So considering (i) the previously-reported sensitivity of *M. balthica* to  
29 elevated temperatures, (ii) the genetic isolation of the southernmost populations, and  
30 (iii) the importance of this species in the trophic web, the population ecology and  
31 genetic structure of this species should be monitored in the context of global warming.

32

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

## 35 1. INTRODUCTION

36 Populations located near the boundary of their distribution area theoretically  
37 present structural and genetic characteristics that may limit their adaptive capacity  
38 (Eckert et al., 2008). Indeed, near the species range limit, populations are not at their  
39 optimum fitness level and most of the time exhibit a reduced growth rate (Brown et al.,  
40 1995; Thomas and Kunin, 1999). As a consequence they are often small, fragmented  
41 populations, vulnerable to genetic drift and exposed to the Allee effect and a reduced  
42 mutation rate (Courchamp et al., 1999; Alleaume-Benharira et al., 2006). Moreover,  
43 asymmetric gene flow from central populations toward those on the periphery restricts  
44 opportunities to develop local adaptations. This process, known as migration-selection  
45 balance, results in chronic maladaptation (Ronce and Kirkpatrick, 2001; Bolnick et al.,  
46 2008). Furthermore, environmental changes associated with supposedly low genetic  
47 diversity in populations close to the species range limit might increase the risk of  
48 extinction for such populations (Lawton, 1993; Vucetich and Wayne, 2003). The  
49 dynamic equilibrium between extinction and recolonization in these areas is more  
50 fragile, and renewal of populations more difficult (Lande et al., 2003). Under these  
51 conditions, the rate of environmental change is of direct importance because it is the  
52 main limitation to the development of an adaptive process.

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

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

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

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

89           Several evolutionary studies with contradictory results have already been  
90 performed on *M. balthica*. Väinölä (2003) used allozymes to show the existence of two  
91 groups of populations, one large homogeneous NE Atlantic population (including the  
92 Norwegian Sea, the North Sea and the coasts of the British Isles), and one structured  
93 meta-population with a high genetic diversity and a large number of alleles in the  
94 northern part of the natural range of the species (the Pacific ocean, the Baltic Sea and  
95 the White Sea). These two groups are supposedly two subspecies coexisting in Europe:  
96 the Pacific lineage *M. balthica balthica* and the Atlantic lineage *M. balthica rubra*  
97 (Väinölä, 2003). Mitochondrial markers confirmed these results (Luttikhuisen et al.,  
98 2003; Nikula et al., 2008) but also revealed a very specific mitochondrial pattern in the  
99 southernmost population of *M. balthica* (i.e. Gironde estuary, France), with haplotypes  
100 that are quite divergent from the other European and Pacific populations. In contrast,  
101 Hummel et al. (1995, 1997b) and Strelkov et al. (2007) did not find any isolation of  
102 French Atlantic populations (including the Gironde and the Loire estuary) from other  
103 populations using allozymes. The French populations were indeed similar to the  
104 Norwegian Sea and western Barents Sea populations.

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

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

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

128

## 129 2. MATERIALS AND METHODS

### 130 2.1 Sampling

131         Adult specimens of *M. balthica* were collected between 2003 and 2007 at  
132 18 locations ranging from the Gironde estuary (known southern limit) to the Barents  
133 Sea (Murman population, see Fig. 1 and Table 1 for precise locations and sample sizes).  
134 Specimens were stored at -20 °C or in 95 % ethanol.

### 135 2.2 Mitochondrial COI sequencing of PCR products

136         Total DNA was extracted from less than 15 mg of muscle using the  
137 Dneasy<sup>TM</sup> Tissue Kit (Qiagen, Germany). Amplifications of a COI gene fragment

138 (Cytochrome Oxidase Subunit 1) were performed using the set of primers described in  
139 Luttikhuisen et al. (2003). Amplifications were carried out in a total volume of 50  $\mu$ L  
140 consisting of 1X PCR buffer, 1.85 mM MgCl<sub>2</sub>, 125  $\mu$ M dNTPs, 0.25  $\mu$ M of each  
141 primer, 1.6 U of Taq DNA polymerase (Red Hot <sup>®</sup> TAQ DNA Polymerase, Abgène)  
142 and about 10 ng of template DNA. A MJResearch PTC 100 Thermal Cycler was used  
143 with the following cycling profile: 3 min of initial denaturation at 95 °C, followed by 35  
144 cycles of denaturation at 95 °C for 45 s, annealing at 60 °C for 60 s and extension at 72  
145 °C for 90 s. A final extension step was carried out for 5 min at 72 °C. Double-stranded  
146 PCR products were cleaned using MultiScreen-PCR MANU03010 plates (Millipore).  
147 The sequencing was performed by the Genoscreen corporation (Campus Pasteur - 1 rue  
148 du Professeur Calmette - 59000 Lille - France) using an ABI PRISM<sup>®</sup> 3730 XL  
149 automated DNA Sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA).  
150 Sequence data were aligned using Clustal W (Thompson et al., 1994) and ambiguities  
151 were manually checked, comparing each sequence with its complementary fragment  
152 using BioEdit (Hall, 1999).

### 153 2.3 Microsatellite amplification and genotyping

154 PCR amplifications of 8 microsatellite loci were conducted using the  
155 primers described in Becquet et al. (2009). The protocol for DNA amplification  
156 consisted in a touch-down PCR procedure: 3 min of an initial denaturation step at 95 °C  
157 followed by 10 cycles of denaturation at 95 °C for 45 s, annealing at temperatures  
158 decreasing by 1 °C per cycle from T<sub>initial</sub> to T<sub>annealing</sub> (T<sub>initial</sub> varied between 68 °C and 62  
159 °C depending on primers, Becquet et al., 2009) for 45 s and extension at 72 °C for 45 s,  
160 followed by 30 cycles at 95 °C for 45 s, annealing at a final T<sub>annealing</sub> for 45 s and  
161 extension at 72 °C for 45 s. A final extension step was carried out for 7 min at 72 °C.  
162 The PCR products were screened on 6.5 % polyacrylamide gels using a Li-Cor NEN  
163 Global IR2 DNA sequencer. Allele sizes were determined using a known DNA



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

## 166 2.4 COI sequence analyses

167           The haplotype number  $H$ , haplotype diversity  $H_d$  (Nei, 1987), the number of  
168 polymorphic sites  $S$ , and nucleotide diversity  $\pi$  (i.e. average number of nucleotide  
169 differences between pairs of sequences) were calculated for each population using the  
170 DnaSP v.5 software (Librado and Rozas, 2009). In order to test the assumption that  
171 populations at the range limit are less diversified than core populations, we defined two  
172 sets of populations separated by Finistère (Brittany, Fig. 1): the 9 southernmost  
173 locations were labelled as the ‘Bay of Biscay’ set (populations 1 to 9, Table 1) and the  
174 remaining 9 sampling sites were labelled as the ‘Core populations’ set (populations 10  
175 to 18, Table 1). We examined the genetic structure of the overall and regional  
176 population by performing an analysis of molecular variance (AMOVA, Excoffier et al.,  
177 1992) as implemented in ARLEQUIN v.3.11. (Excoffier et al., 2005). Fixation indices  
178 (Wright, 1951) analogous to the  $F_{ST}$ ,  $F_{CT}$  and  $F_{SC}$  parameters, were calculated to  
179 analyze genetic differentiation between populations over the whole study area ( $\Phi_{ST}$ ),  
180 among groups of populations within the species range ( $\Phi_{CT}$ ) and among populations  
181 within groups ( $\Phi_{SC}$ ) as defined in Excoffier et al. (1992).  $\Phi$ -statistics take into account  
182 both haplotype frequencies and the molecular distances between haplotypes. Statistical  
183 significance of the  $\Phi$  indices was tested using a non-parametric permutation procedure  
184 implemented in ARLEQUIN v.3.11. Moreover, one-sided tests to compare mean  
185 haplotype numbers of the two sets and mean nucleotide diversities were performed in R  
186 v. 2.11.1 (R Development Core Team, 2010) using either the Student  $t$ -test or the  
187 Wilcoxon test when parametric assumptions were not met.

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

190 on the median-joining algorithm (Cassens et al., 2003). This process combines both  
191 minimum spanning trees and maximum parsimony approaches to simplify the complex  
192 branching pattern and represent all the most parsimonious intra-specific phylogenies.  
193 Such networks describe the evolutionary relationships (based on mutational events)  
194 between the different haplotypes observed over the whole dataset. Finally, for each  
195 population we calculated the D parameter defined by Tajima (1989) and estimated the  
196 significance of deviations from zero in DNAsp v.5 (Librado and Rozas, 2009).  
197 Geneland v. 4.0 (Guillot et al., 2005a, 2005b, 2012) was used to infer spatial population  
198 structure. Geneland incorporates geographical information as a weak prior, with the  
199 assumption that most populations exhibit some degree of spatial structuring, and that  
200 the joint probability of any two individuals belonging to the same population decreases  
201 with the geographical distance between them. For each run, the number of groups (K) at  
202 Hardy-Weinberg equilibrium is assessed using maximum-likelihood estimations. Then,  
203 K is fixed at its most likely value and the posterior probability of belonging to each  
204 class is computed for each individual of the dataset. Finally, maps of posterior  
205 probabilities are generated. A total of 30 independent runs were computed in order to  
206 check for the convergence of MCMC computations with the following parameters:  
207 length of MCMC = 150,000 steps, thinning = 10, maximum rate of Poisson process =  
208 550, maximum seed number for the Poisson-Voronoi tessellation = 1,650.

209

## 210 2.5 Microsatellite analyses

### 211 2.5.1 Genetic diversity within localities

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

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

#### 224 2.5.2 Genetic differentiation and relationships among localities

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

228 In addition to Geneland, two other Bayesian clustering programs, Structure and TESS,  
229 were used to infer structure among populations. Structure explores the number of  
230 populations in a dataset by optimizing Hardy-Weinberg equilibrium within putative  
231 groups, without taking geographical information into account. As Geneland, TESS uses  
232 geographical information in membership assignment. The three programs have different  
233 sensitivities to population structure and admixture levels (Chen et al., 2007; Guillot  
234 2009; François and Durand 2010), and their comparative use allows weighing of the  
235 relative importance of geography.

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

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

251

## 252 3. RESULTS

### 253 3.1 Spatial population structuring

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

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

271 The mitochondrial pairwise  $F_{ST}$  estimates (Table 3) were found to be elevated and  
272 highly significant when comparing populations from the Bay of Biscay set (populations  
273 1 to 9) with populations from the Core set (populations 10 to 18,  $0.325 < F_{ST} < 0.938$ ,  $p$   
274  $< 0.001$ ). This clustering was also significant according to the AMOVA results: the  
275 overall high genetic structure ( $\Phi_{ST} = 0.613$ ,  $p < 0.0001$ ) was mostly due to a large  
276 genetic differentiation between these 2 groups ( $\Phi_{CT} = 0.587$ ,  $p < 0.0001$ ) while the  
277 genetic differentiation among populations within these groups appeared to be low ( $\Phi_{SC}$   
278  $= 0.0613$ ,  $p < 0.0001$ ).

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

283 Spatial population clustering inferred from Structure, TESS and Geneland analyses led  
284 to contrasting mitochondrial and nuclear patterns. Mitochondrial data, analysed using  
285 Geneland, revealed significant clustering among (1) Bay of Biscay populations, (2) core  
286 populations from Brittany to Germany, and (3) Ireland and Murman populations (Fig. 5,  
287 Fig. S1). Microsatellite data, analysed with Geneland, Structure and TESS, could be  
288 split into three clusters. Bay of Biscay populations were separated by the Loire estuary  
289 and formed two groups (1<sup>st</sup> group: populations 1-6; 2<sup>nd</sup> group: populations 7-9;  $F_{ST} =$   
290  $0.044$ , Table S1). A second genetic break was detected at the tip of Finistère ( $F_{ST}$  ranges  
291 between  $0.021$  and  $0.035$ , Table S1). In addition, in a four-cluster scenario (data not  
292 shown), TESS further split core populations into two strongly-admixed groups: (1)

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

294

### 295 3.2 Molecular diversity

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

305 The multilocus genotype (8 microsatellite markers) was obtained for 545 individuals  
306 collected at 18 sampling sites (Table 1). No linkage disequilibrium was detected  
307 between loci (pairwise exact tests,  $p > 0.2$ ). Overall, the mean number of alleles ( $N_{\text{all}}$ )  
308 ranged from 6.62 (Bonne Anse) to 11.25 (Aytré) with a total of 20.87 over the whole  
309 dataset (Table 2). Most of the differences observed across populations were due to  
310 varying sample sizes since all allelic richness values ( $R_{\text{all}}$ ) were similar, ranging from  
311 4.0295 (Seine) to 4.7716 (Aiguillon) (overall population mean and standard deviation =  
312  $4.39 \pm 0.23$ ). However, a highly significant difference was found when comparing  
313 mean  $R_{\text{all}}$  values for both population sets (one-sided two-sample Student  $t$ -test,  $t =$   
314  $5.933$ ,  $df = 16$ ,  $p = 1.051e^{-5}$ ): a greater mean  $R_{\text{all}}$  value was found within the Bay of  
315 Biscay set ( $R_{\text{all}} = 4.58 \pm 0.15$ ) than within the Core set ( $R_{\text{all}} = 4.21 \pm 0.12$ , see also Fig.  
316 6).

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

319 dataset (the mean and standard deviation across the 18 populations was  $0.366 \pm 0.0425$   
320 and  $0.6325 \pm 0.0347$  for  $H_o$  and  $H_s$  respectively, Table 2), both indices were found to be  
321 significantly greater in the Bay of Biscay set than in the Core set ( $t = 2.513$ ,  $df = 16$ ,  $p =$   
322  $0.012$  for  $H_o$  and  $t = 2.246$ ,  $df = 16$ ,  $p = 0.020$  for  $H_s$ ). Heterozygote deficiency was high  
323 for all populations (mean  $F_{IS}$  value and standard deviation of  $0.424 \pm 0.054$ ). The same  
324 levels of heterozygote deficiency were found in both sets (mean  $F_{IS}$  and standard  
325 deviation =  $0.4187 \pm 0.0491$  and  $0.4305 \pm 0.0603$  for the Bay of Biscay set and for the  
326 Core set, respectively) and a two-sided Student  $t$  test showed no difference between the  
327 set means ( $t = 0.457$ ,  $df = 16$ ,  $p = 0.654$ ).

328

329

## 330 4. DISCUSSION

331

### 332 4.1 Genetic structure

#### 333 4.1.1 High gene flow at macro-scale

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

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

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

368

#### 369 4.1.2 Discordance between mitochondrial and nuclear genetic structure.

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



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

385 Local hydrodynamics during the larval stage might explain this genetic barrier within  
386 the Bay of Biscay. Indeed, a postzygotic isolation might exist within the semi-closed  
387 Bay of Marennes-Oléron and, on a wider scale, within the Pertuis Charentais area (i.e.  
388 populations 1 to 6) where a 1300 km<sup>2</sup> semi-closed area is delineated by two 30 km-long  
389 islands only a few kilometres apart. This assumption is supported by the high self-  
390 recruitment detected in this study (Fig. 4). Larval retention might be reinforced for  
391 populations 1 to 6 as waters from the Gironde and Loire estuaries rarely mix, leading to  
392 the formation of eddies and up-wellings that favor larval retention (Lazure and Jegou,  
393 1998). The incongruence between mitochondrial and nuclear data, however, would  
394 suggest that this barrier is too recent to be detected with mitochondrial markers, which  
395 tend to evolve more slowly than microsatellite ones (e.g. Brown et al., 1979; Estoup and  
396 Angers, 1998). This hypothesis is unlikely, as the Loire estuary was already present in  
397 the Pleistocene, when profound structuration of mitochondrial lineages occurred.

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

408 This contrasting pattern obtained with these different types of markers might be a  
409 consequence of a genetic barrier to gene flow like those observed in transition zones  
410 (Lemaire et al., 2005). During the Pleistocene, Pacific populations of *M. balthica*  
411 colonized the Atlantic Ocean down to the Iberic coasts after the opening of the Bering  
412 Strait (Väinölä, 2003). Throughout the Last Glacial Maximum (LGM, 18,000 years  
413 ago), all the northern European coasts were covered by ice except for the Bay of Biscay  
414 and the Spanish region, which is known to have been one of the four major temperate  
415 refugia (Gómez and Lunt, 2007). After the LGM, natural populations of *M. balthica* in  
416 this area became the source for the recolonization of northward sites (Austin et al.,  
417 2004; Howes et al., 2006). Meanwhile, after the complete disappearance of the ice  
418 sheets from northern Europe, migration began again from North to South. Thus, the  
419 post-glacial recolonization of the northern European coasts occurred from two isolated  
420 *M. balthica* stocks via two distinct paths: (i) a South to North route from an Iberian  
421 refugium and (ii) a North to South route from the Pacific Ocean; this migration led to a  
422 secondary contact between differentiated populations. The existence of genetic  
423 signatures typical of a hybrid zone (Barton and Hewitt, 1985) might be the consequence

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

436

437

#### 438 4.2 Genetic diversity within peripheral populations

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

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

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

473 These results are consistent with a rapid northward expansion after the Last Glacial  
474 Maximum from a refuge zone located either in Spain or in the Bay of Biscay, and might  
475 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

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

510 In contrast, an increase in temperature is known to greatly affect the physiological  
511 capacities of individuals, especially for organisms living at the low latitude margin of  
512 their natural range (Hugues, 2000). A recent study on the metabolic and respiratory  
513 rates of *M. balthica* (Jansen et al., 2007) revealed a zero plasticity to temperature for  
514 populations close to the southern range limit. The author translocated a Gironde  
515 population to the Bidasoa Bay (where *M. balthica* was present 50 years ago) and  
516 observed rapid mortality. These results reinforced those published by Hummel et al.  
517 (1998) showing limited ecophysiological performances for *M. balthica* populations at  
518 the edge of its natural range.

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

528 populations would potentially impact the population dynamics and structure and would  
529 then have a major role in terms of conservation and species management (Cowen et al.,  
530 2007).

#### 531 CONCLUSION

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

544

545

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557



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Sampling Site	Code	Latitude (N)	Longitude (W)	N <sub>mito</sub>	N <sub>nuc</sub>
(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) Willemshaven	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<sub>mito</sub>) and microsatellites nuclear markers (N<sub>nuc</sub>) for the eighteen European sampling sites of *Macoma balthica*.

Mitochondrial data							Nuclear data					
Bay of Biscay	N	S	H	Hd	$\pi$	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
<b>Total</b>	143	11	11	0.464	0.0411	-	286	18.25	4.5800	0.395	0.668	0.4305
<b>Mean</b>	17.9	3.63	4	0.498	0.0315	-	31.78	8.392	4.5801	0.388	0.649	0.4186
<b>(S.D.)</b>	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	$\pi$	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
<b>Total</b>	281	11	10	0.523	0.07684	-	256	15.50	4.2110	0.358	0.628	0.4434
<b>Mean</b>	31.2	3.78	2.78	0.306	0.00275	-	28.44	8.306	4.2093	0.344	0.616	0.4305
<b>(S.D.)</b>	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	$\pi$	D	N	Nall	Rall	Ho	Hs	$F_{IS}$
<b>Total</b>	424	18	19	0.720	0.08984	-	542	20.875	-	0.3740	0.6500	0.4426
<b>Mean</b>	24.9	3.71	3.35	0.396	0.01630	-	30.11	8.305	4.395	0.3660	0.6325	0.4246
<b>(S.D.)</b>	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;  $\pi$ : 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.

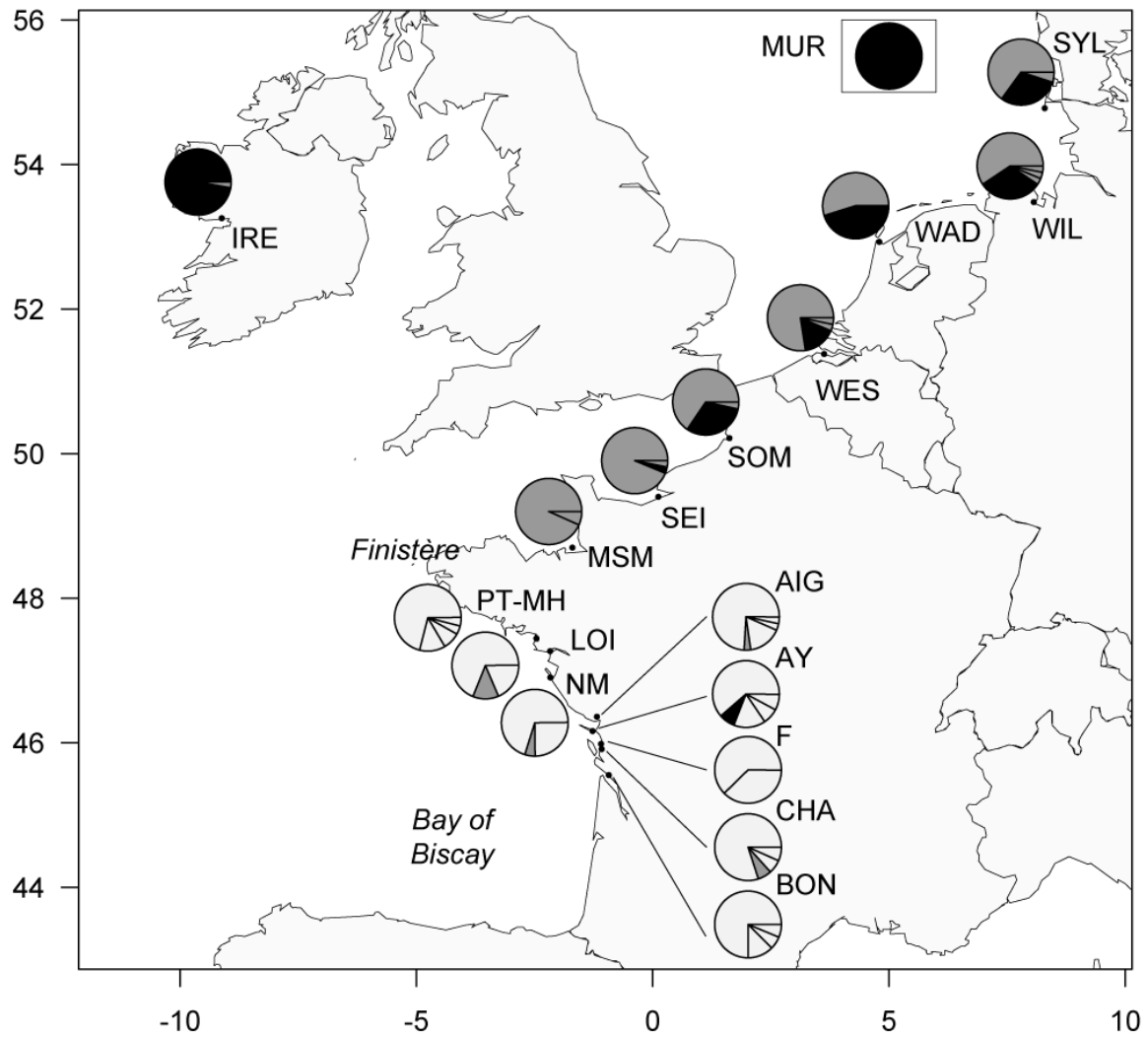


Fig. 2: Minimum spanning network displaying mitochondrial variation along 313 bp of the COI gene. Each circle represents a haplotype. Circle size is proportional to haplotype frequency. Each segment represents a single mutational event. H1 and H2 are haplotypes typical of the Bay of Biscay, and H3 and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

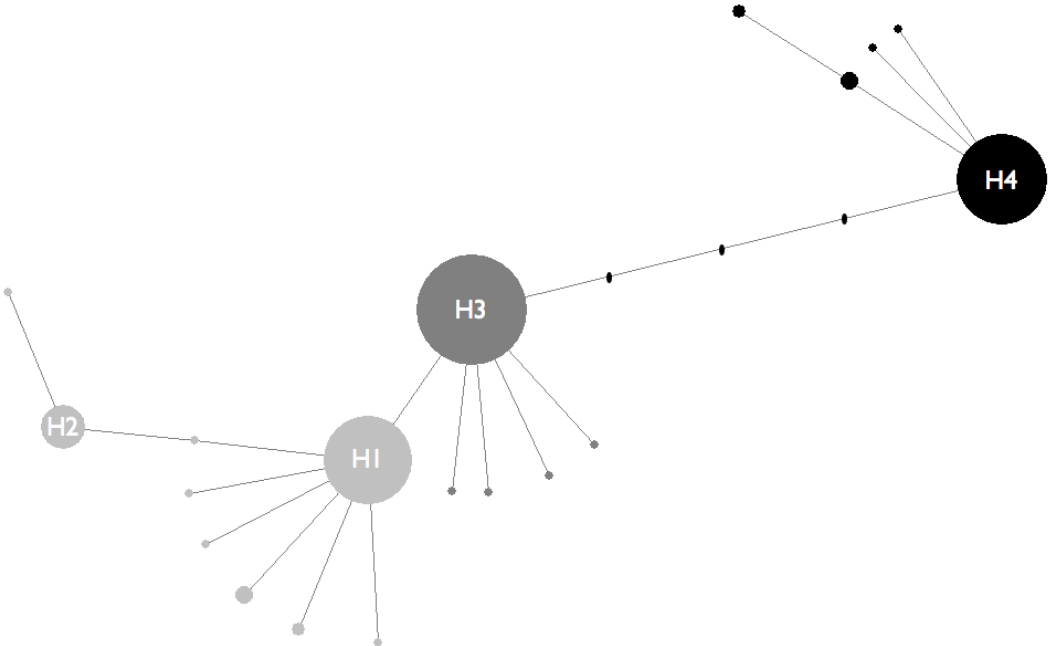


Fig. 3: Frequencies of the four most common haplotypes (H1-H4) identified along the European coast. Except for Ireland, sampling sites are ordered by increasing latitude along the x-axis.

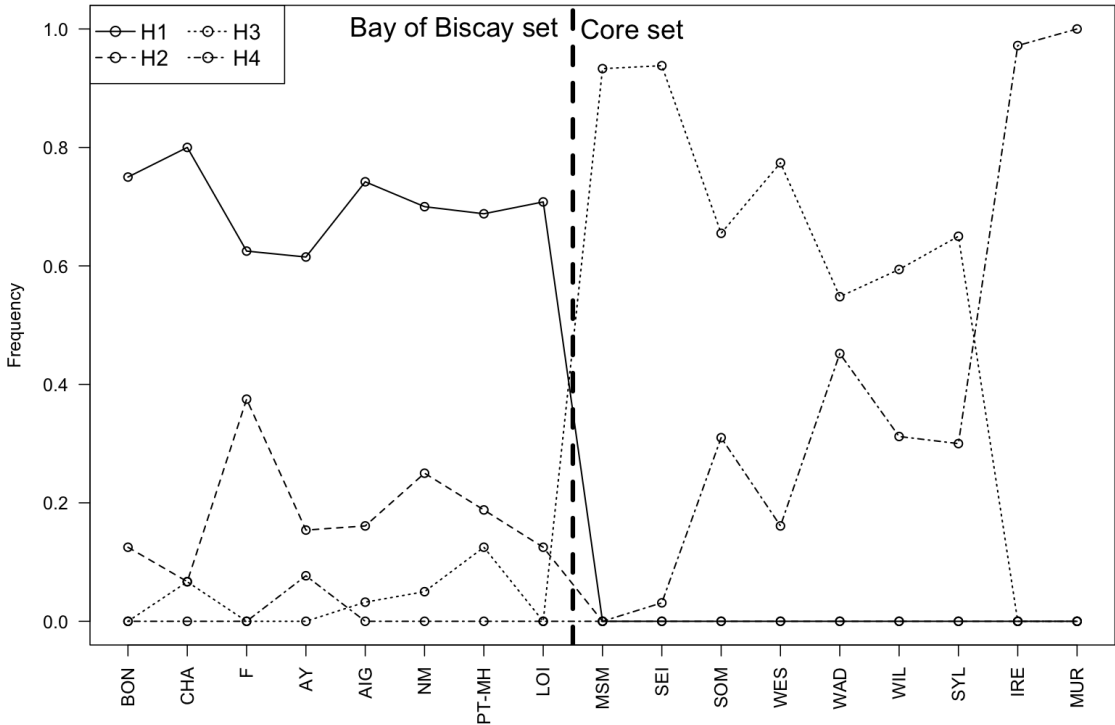




Fig. 5: Summary of spatial population clustering, based on results from Geneland, TESS and Structure analyses using mitochondrial and microsatellite dataset. At each sampling location, results from each program are given as a symbol, along a segment (see key). Filled circles represent nuclear data, while 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.

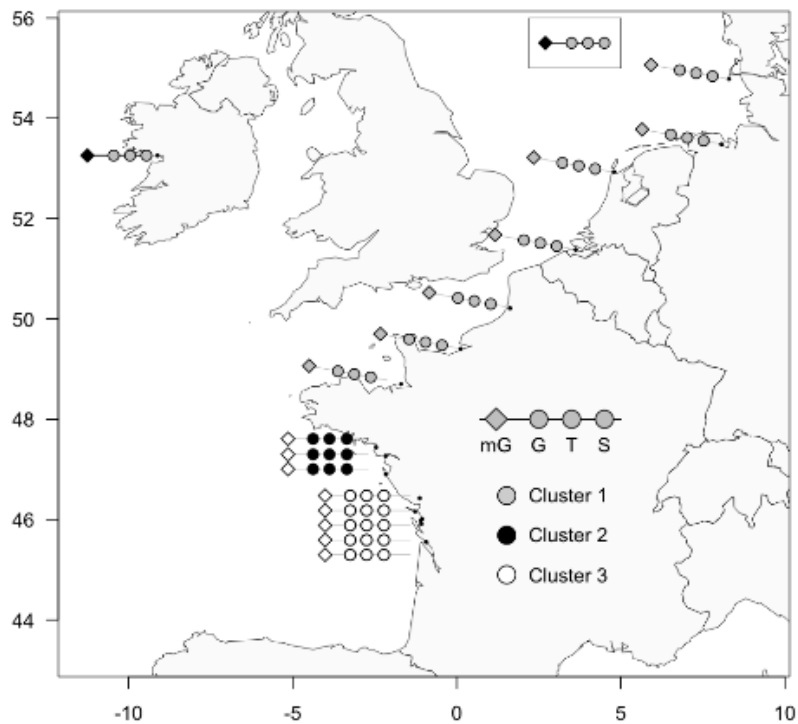


Fig. 6: Box-and-whisker plots of allelic richness in the Bay of Biscay and Core population sets. For each population set, the  $R_{all}$  values presented are (in order of decreasing values): maximum, third quartile, median, first quartile and minimum.

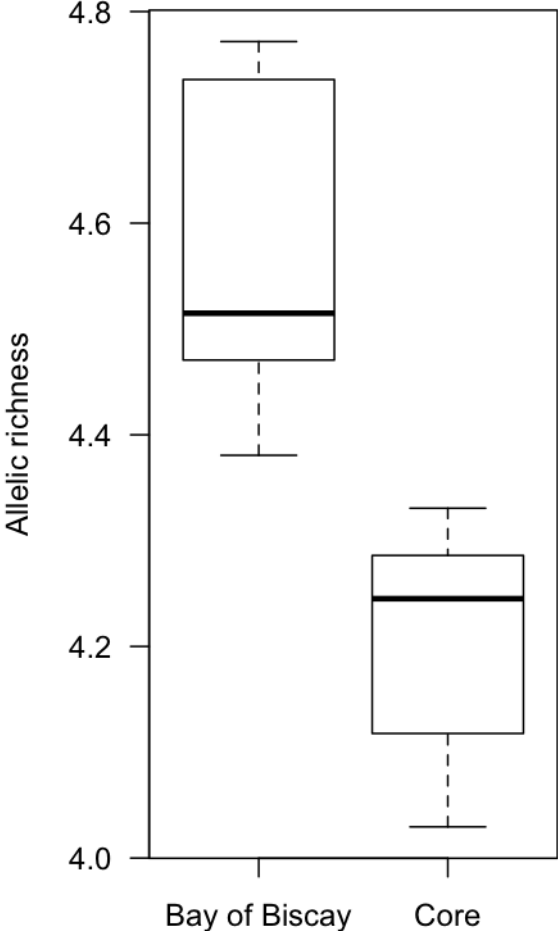


Fig. 7: Box-and-whisker plots of observed (white) and expected (grey) heterozygotes in the Bay of Biscay and Core populations sets.

