Evaluating the genetic effects of the invasive *Ocenebra inornata* on the native oyster drill *Ocenebra erinacea*

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Abstract

Studies focusing on the effect of invasive species on the genetic diversity of native marine invertebrates remain scant. Here we report diversity among French populations of the intertidal gastropod *Ocenebra erinacea* (Linnaeus, 1758) sampled in the presence and absence of the invasive *Ocenebra inornata* (Recluz, 1851). Between 1999 and 2004 a total of 352 individuals of *O. erinacea* were collected from 15 sites, five of which in the presence of the invasive, and genotyped at the mitochondrial locus *cox1*. No statistical difference was observed between polymorphism levels recorded within native populations exposed to the invasive, compared to populations sampled in the absence of *O. inornata*. No sign of native population decline was detected in response to the invader. While significant shifts in native *O. erinacea* population sizes were previously reported in the literature, genetic effects may take longer to accumulate, or may be undetectable without a larger panel of genetic markers. In contrast, large genetic distances and significant population differentiation were recorded between Atlantic and Mediterranean *O. erinacea* samples, suggesting that these populations have distinct evolutionary histories. Comparison of genetic divergence within the closely-related genus *Nucella* suggests that the Atlantic populations and the Mediterranean *O. erinacea* populations from Thau Lagoon may belong to different species or subspecies.

Introduction

Natural movements of species ranges on the surface of the world (expansions, regressions, displacements) play a considerable role in the evolution of species. Most of the time, these phenomena are progressive and marked by the tempo of geological processes (e.g. Hewitt 1996). However, during the last few decades a growing number of species has undergone changes in their natural range due to both changes at a global scale (e.g. Parmesan & Yohe 2003) and artificial transfers due to human activities (e.g. Carlton 1989; Seebens *et al.* 2013).

The number of biological invasions has strongly increased during the last decades (e.g. Mack *et al.* 2000; Mooney & Cleland, 2001; Ruiz *et al.* 2000). In the marine environment, these phenomena are mainly due to aquaculture and especially to shellfish farming, which represents a major cause of introduction, intentional or not, of exogenous species (Elton 1958; Carlton 1992).

Apart from potentially important economic consequences, the arrival of these introduced species can also cause serious ecological impacts on local fauna. Introduced species are likely to decrease the abundance of indigenous species, excluding them from part of their distributional area or even causing their extinction by modifying invaded habitats, hybridizing with native species, exchanging pathogens, preying on them, or competing with them (e.g. Lockwood *et al.* 2007 and references therein). Moreover, when they exert strong selective pressures, introduced species can also reduce the genetic diversity of native populations (Kim *et al.* 2003; Wittmann *et al.* 2013).

Such impacts, although poorly known (e.g. Strauss *et al.* 2006), may have heavy ecological consequences since adaptive potential depends on the genetic diversity of a

population. Reductions in genetic diversity are generally considered detrimental (e.g. Frankham 1995; Lande 1995; Strauss *et al.* 2006) and might contribute to extinction (Wittmann *et al.* 2013). Thus, various authors showed a link between the fitness of a species and its genetic diversity, particularly in mollusks (Mitton & Grant 1984; Garton & Haag 1991; Zouros 1993; Launey & Hedgecock 2001; Hedgecock *et al*. 2007). In addition, the reduced genetic variability of an indigenous population could promote the expansion process of other species that are phylogenetically close. However, very few studies address changes in genetic diversity of an indigenous species under the competitive pressure generated by a biological invader.

The oyster drills *Ocenebra erinacea* (Linnaeus, 1758) and *Ocenebra inornata* (Recluz, 1851) constitute a noteworthy model to study the genetic effects of indigenous-invasive interactions on indigenous populations. A native of the northwestern Pacific (Choe & Park 1997; Amano & Vermeij 1998), *O. inornata* (previously known as *Ocinebrellus inornatus*; see Houart and Sirenko 2003; Bouchet and Houart 2014) has recently invaded European coasts, probably following massive oyster imports (Pigeot 2000; De Montaudouin & Sauriau 2000; review of Lützen *et al.* 2012). Genetic data suggest that French populations may come from Asia and the United States (Martel *et al*. 2004a). The introduction of *O. inornata* may have important economical consequences, as it is a predator of cultivated mollusks (e.g. oysters, blue mussels, Goulletquer *et al.* 2002). It coexists at several French sites with an indigenous muricid, *O. erinacea* (Linnaeus, 1758), which ranges from the straits of Gibraltar to the Netherlands, and inhabits all British and Mediterranean coasts (Graham 1988). Although *O. erinacea* and *O. inornata* differ in some life history traits (Martel *et al*. 2004c), these muricid gastropods fill similar ecological niches, and may compete for habitat (both species live on hard substrates and drill the shell of bivalves to feed on them; e.g. Lutzen et al 2012). Pigeot *et al*. (2000) recorded a decrease in population density of *O. erinacea*, in

parallel to an in increase in numbers of *O. inornata* in Marennes-Oléron (Charente-Maritime, France), between 1997 and 1999 (two years after the invasive was first detected). While the introduction and expansion patterns of *O. inornata* have been investigated in previous studies (Martel *et al.* 2004a; Martel *et al.* 2004b), its ecological impacts on the native *O. erinacea* are poorly known.

In previous studies (Martel 2003), seven allozyme loci were analyzed on populations of *Ocenebra erinacea* and *Ocenebra inornata* collected in 7 sites of the French Atlantic coast where the two species live in sympatry. These markers revealed genetic diversity indices systematically weaker within the native *O. erinacea* than within the invasive *O. inornata*. This result was counterintuitive, as (i) the founder effect linked to an introduction event should lead to a low genetic diversity within the populations of the introduced species, (ii) this phenomenon should be all the more marked as the invasion is recent (review of Sakai *et al.* 2001). Consequently, a lower genetic diversity within the populations of the exogenous species compared to the populations of the indigenous species was expected. It is thus of importance to test whether the genetic diversity of the indigenous species *O. erinacea* is correlated with the presence of the introduced species *O. inornata* in zones of sympatry. Indeed, *O. inornata* could induce a selective pressure on *O. erinacea* leading to decrease of polymorphism in this local species.

Here, we test this hypothesis by sampling *Ocenebra erinacea* from the Atlantic and Mediterranean French coasts, in the presence and absence of *Ocenebra inornata*, and by measuring genetic diversity of the native species using the mitochondrial marker *cox1*. While investigating the genetic effects that the presence of *O. inornata* may have on sympatric populations of *O. erinacea*, we came across a very strong genetic break between Atlantic and Mediterranean populations. This break is detailed and potential biogeographic causes are discussed.

Materials and methods

Sampling

A total of 352 adult specimens of *Ocenebra erinacea* were collected between 1999 and 2004 at 15 sites on the French coast, along line transects (<200 m in length). At each site, specimens from different rocks were collected to reduce sampling bias in favor of a particular lineage. The sites were located both within oyster farming zones and unexploited areas (Table 1). In order to show a possible impact of the presence of *Ocenebra inornata* on genetic diversity of *O. erinacea*, five locations where the two species live in sympatry and 10 sites free of *O. inornata* have been sampled. The presence of *O. inornata* was assessed by direct observation. After collection, specimens were stored in 95% ethanol before DNA extraction.

DNA extraction, amplification and sequencing

Total DNA was extracted from <15 mg of foot muscle using Dneasy™ Tissue Kit according to the manufacturer's protocol (Qiagen, Germany). Part of the mitochondrial *cox1* gene was PCR-amplified with the HCO2198/LCO1490 primers (Folmer *et al.* 1994), which have proved useful for neogastropod studies (e.g. Zou et al 2011, 2012). Polymorphism at *cox1* is high in *Ocenebra inornata*, a phylogenetically close species (Martel *et al.* 2004a).

PCRs were done in 50 µL, with 1X PCR buffer, 1.85 mM $MgCl_2$, 125 µM dNTPs, 0.25 µM of each primer, 1.6 U of Red Hot® DNA Polymerase (ABgene) and about 10 ng of DNA template. The following cycling profile was performed using a MJResearch PTC 100 Thermal Cycler: initial 5-min denaturation step at 94°C followed by 40 cycles of 30 sec at

94°C, 30 sec at 50°C and 1 min at 72°C, and by a final 5-min extension period at 72°C. PCR products were purified using MultiScreen-PCR MANU03010 plates (Millipore).

Sequencing was done by GenoScreen (Lille - France) using an ABI PRISM® 3730 XL Automated DNA Sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA). Sequences were aligned using ClustalX (Thompson *et al.* 1994).

Data analyses.

Genetic analyses aimed at (i) quantifying and comparing genetic diversity among populations, (ii) analyzing the spatial distribution of polymorphism and genetic exchanges among populations, and (iii) studying the evolutionary relationships among populations. Haplotype number H, number of polymorphic sites S, haplotype diversity H_e and average per site nucleotide diversity π (Nei 1987) were calculated for each population using the software DnaSP 5.10.1 (Librado & Rozas 2009).

We tested the null hypothesis of the standard neutral model in Arlequin v.3.5 (Excoffier & Lischer 2010), by calculating the D and Fs statistics, as defined by Tajima (1989) and Fu (1997). When these statistics are significantly different from zero, populations may have undergone purifying selection, a selective sweep and/or expansion (<0), or balancing selection and/or a population decline (>0). Statistical significance was tested by generating 10,000 random samples under the hypothesis of selective neutrality and population equilibrium. These tests were performed for each sampling site separately, and also for pooled sites in presence or absence of the invasive.

The differentiation index Φ_{ST} (Excoffier *et al.* 1992), an estimator of F_{ST} (Wright 1951) calculated from frequency values and distances between haplotypes, was computed with Arlequin v.3.5. The Kimura 2 Parameter (K2P) model of nucleotide substitution was used to

estimate genetic distances, and 10,000 permutations were used to test statistical significance under the null hypothesis of no difference between populations (Excoffier *et al.* 1992).

Finally, a haplotype network was built using the median-joining algorithm implemented in Network 4.6.1.1 (fluxus-engineering.com, Bandelt 1999). This method is one of the most accurate for inferring intra-specific networks in the absence of recombination (Woolley *et al.* 2008).

To help interpret the large genetic divergence observed between Atlantic and Mediterranean specimens of *Ocenebra erinacea*, we looked for mitochondrial *cox1* data from the BOLD database (Ratnasingham & Hebert 2007). However, besides three other BOLD *cox1* sequences from Spanish specimens of *O. erinacea*, we produced the only available mitochondrial sequences for the genus *Ocenebra*. We therefore used *cox1* sequences from six species of the closely-related genus *Nucella* Röding, 1798 (e.g. Pascal 2004) to measure intra-specific and inter-specific genetic distances. *Nucella* and *Ocenebra* are both characterized by a non-planktonic larval development and lay egg capsules on hard substrates (Martel *et al.* 2004c; review by Krug 2011). We used the K2P model of nucleotide substitution (Kimura 1980), widely used in DNA barcoding (Hebert *et al.* 2003; Barrett & Hebert 2005), to measure genetic distances among *cox1* haplotypes.

Results

A 550 bp fragment of *cox1* was sequenced for 352 individuals, and 29 haplotypes were identified (GenBank accession numbers AY995771-99; Popset 63109090). Sequences include 37 polymorphic sites, 20 of which are parsimony informative, and one of which has three character states. No indels were observed (Table 2).

Population-level genetic diversity and demographic stability

Genetic diversity is comparable among Atlantic populations of *Ocenebra erinacea*, but values for the different diversity indices are low compared to other recently-studied marine mollusks. The number of polymorphic sites between two different sequences varies between 0 and 6 among Atlantic populations. Two of these populations (Morbihan and St Quay) are each characterized by a single haplotype, and the 12 other Atlantic sites have no more than five haplotypes (sample sizes provided in Table 1). Consequently, haplotype diversity H_e is low, except for Oléron (H_e = 0.564; Table 2). In Fouras, where *Ocenebra inornata* and *O. erinacea* are sympatric, the haplotype and nucleotide diversities are respectively four- and six-fold higher in the invasive drill (H_e = 0.348 and π x10^{-3} = 0.83; data from Martel 2003) than in the local one (H_e = 0.074 and π x10^{-3} = 0.14).

A single Mediterranean population (Thau) was sampled. The haplotype diversity (H_e = 0.342) and the nucleotide diversity (π x10^{-3} = 2.52) are respectively two- and five-fold higher than on the whole of Atlantic populations (Table 2). Moreover, 22 polymorphic sites were found among 37 individuals sampled in Thau, a value considerably higher than the 24 polymorphic sites observed among 315 Atlantic individuals.

Last, haplotype and nucleotide diversities of *Ocenebra erinacea* populations co-occurring with *Ocenebra inornata* were lower (H_e = 0.247 ± 0.0535 and π x10^{-3}= 0.57 ± 0.65) than for populations located in zones where *O. inornata* was not detected (H_e = 0.399 ± 0.3976, π x10^{-3}= 10.04 ± 5.52) (Table 2). However, this pattern is entirely due to the higher diversity encountered at Thau; when removing this site from the group of populations that were not found in contact with *O. inornata*, diversity values dropped significantly (H_e = 0.179 ± 0.0368, π x10^{-3}= 0. 38 ± 0. 51). Comparing molecular diversity at the site level revealed the same pattern (Welch Two Sample t-test, including Thau, for H_e: t = -0.83, df = 6.22, p = 0.44;

for π: t = -0.16, df = 12.81, p = 0.88. In both cases, results were also non-significant when removing the population of Thau).

Except for one case, the *D* and *Fs* statistics were never positive (Table 2). Furthermore, the only slightly positive *D* value (deviation from zero non significant) was observed when sites where the invasive was absent were pooled, including Thau, and this result was therefore likely influenced by the underlying population structure (see "Genetic differentiation among populations" Results section). There is therefore no supporting evidence that *Ocenebra erinacea* populations exposed to *O. inornata* suffered from a population decline. Some sites exhibited significant negative values of *D* and *Fs*, which can be interpreted as signs of purifying selection, selective sweep and/or population expansion. Particularly, the pooled Atlantic sites showed significantly negative values for both tests, regardless of whether the invasive was present or not. These molecular signatures must, however, be interpreted with care, as they might reflect older demographic events.

Genealogical relationships and spatial distribution of haplotypes

Two haplogroups, separated by 18 mutational steps, were observed using the median-joining network (Fig. 1). The first haplogroup (23 haplotypes) was mainly composed of Atlantic specimens, and included one specimen from Thau, characterized by haplotype H26 (separated from other Mediterranean haplotypes by 20 to 22 mutational steps). Haplotypes from this group diverged by ≤ 2 mutations. Haplotype H1 was common (represented in 89% of Atlantic individuals) and central to the Atlantic haplogroup, while the other 22 haplotypes were rare (7 individuals for H3, 3 for H13, 2 for H6, H9, H15, H19 and a single individual for the others) and peripheral to H1. The second haplogroup was strictly composed of Mediterranean individuals. Of six haplotypes from this group, one was common (H21,

represented in 82% of Thau individuals), and four were rare (3 individuals for H22 and a single individual for H23-25). Haplotypes from this group diverged by one mutation. The Atlantic samples share no haplotype with the Mediterranean sample (Fig. 2). Except for H1 (present at all sites except Thau), H3 (shared between Loix and Aytré) and H13 (shared between Loix, Aytré and Trébeurden), all haplotypes are private (observed only within one population).

Genetic differentiation among populations

Genetic differentiation among pairs of populations was measured using Φ_{ST}. The population from Thau was significantly differentiated from all other populations. Pairwise Φ_{ST} values ranged between 0.94 and 0.96, corresponding to a substantial genetic differentiation between the Mediterranean and Atlantic populations (Table 3). Inside the Atlantic group, no significant differentiation was observed after sequential Bonferroni correction (lowest corrected alpha level for Atlantic populations: 0.00055).

Levels of intra-specific divergence in *Ocenebra* compared to *Nucella*

Given the differences in haplotype composition and divergence between Mediterranean and Atlantic sites, we investigated whether the genetic distances correspond to intra- or inter-specific divergence by comparing *Ocenebra* to its close relative *Nucella*. The pairwise K2P distance between *Ocenebra erinacea* haplotypes ranged between 0.18 and 4.54% (maximum observed between haplotypes 20 from Loire and 25 from Thau Lagoon). Within *Nucella*, K2P was calculated for 532 sequences and 117 haplotypes distributed among six species, along a 434 bp stretch of *cox1*. Intra-specific distances ranged from 0 to 3.32%, while inter-specific distance ranged from 4.81 to 12.2%, for specimens distributed over 1000 to

2000 km (East and West coasts of North America, respectively; BOLD database). For comparison, Zou et al (2011), analyzing 108 neogastropod *cox1* sequences (same gene region as analysed here; not including *Ocenebra* or *Nucella*) found maximum intra-specific K2P distances of 2.2% and minimum inter-specific distances of 2.1%.

Discussion

Relation between *Ocenebra erinacea* and the invasive species *Ocenebra inornata*

While a decrease in genetic diversity in response to invaders was reported in the past (Kim *et al.* 2003), we did not detect such a pattern among *Ocenebra erinacea* exposed to the invasive species *Ocenebra inornata*. The relatively low polymorphism levels recorded may have hampered our ability to detect genetic effects of the invasive on the native species, and the use of additional molecular markers such as nuclear microsatellites or SNPs might further help detect possible demographic events associated with the presence *O. inornata*. However, this remains to be tested, as even genome-wide scans can fail at detecting recent demographic events and selective pressures. Riquet *et al.* (2013), for instance, used AFLPs to compare native and invasive populations of the marine mollusk *Crepidula fornicata*. They reported little genetic differentiation among these populations, and detected no F_{ST} outliers out of 344 tested loci. An alternative hypothesis explaining the apparent absence of genetic effects of the invasive on the native is that the competitive and selective pressures inflicted on *O. erinacea* are too low to have genetic effects (e.g. Wittmann *et al.* 2013). Finally, deviations from neutrality observed for pooled Atlantic sites suggest selection could have shaped the current genetic diversity and could have blurred signatures of demographic processes.

Ocenebra inornata was first documented on the French Atlantic coast in 1995 (De Montaudouin & Sauriau 2000, Pigeot *et al.* 2000), and the specimens of *Ocenebra erinacea*

used in this study were collected between 1999 and 2004. The introduction of *O. inornata* may have been too recent at the time of sampling for genetic consequences on the native species to be detectable. About ten years later, the distributional landscape of *O. inornata* on Atlantic coasts have significantly changed, and the invasive is now found as far north as the entrance of the Baltic Sea (Lützen *et al.* 2012). A new survey of the genetic diversity of *O. erinacea* may today unfold the genetic consequences of the invasion by *O. inornata*, and this study therefore provides a snapshot in time that may help better understand the temporal dynamics of loss of genetic diversity. In addition to sampling in the field, we searched for *Ocenebra* specimens in the collections of the Museum national d'Histoire naturelle in Paris (France), in order to look for genetic diversity in *O. erinacea* specimens collected prior to, or soon after the invasion by *O. inornata* (MNHN voucher numbers IM-2008-7101, IM-2008-7102, IM-2008-7103). Unfortunately, we were not able to amplify the *cox1* marker from these specimens.

Remarkably low genetic diversity of *Ocenebra erinacea* populations

Mitochondrial genetic diversity, as measured using part of *cox1*, was low relative to what was observed in other marine mollusks. Overall, *Ocenebra erinacea* haplotype and nucleotide diversities were (disregarding the sample from Thau, see below) H_e = 0.18-0.25 and π x10^{-3}= 0.38-0.57 (Table 2). Comparatively, H_e = 0.684 and π x10^{-3}= 2.25 for *Ocenebra inornata* in its natural range (data from Martel *et al.* 2004a), H_e = 0.734 and π x10^{-3}= 14.78 in the gastropod *Cyclope neritea* (Simon-Bouhet *et al.* 2006), and H_e = 0.720 and π x10^{-3}= 89.84 in the bivalve *Macoma balthica* (Becquet *et al.* 2012).

The low genetic diversity observed at *cox1* was consistent with the low diversity observed using allozymes: Martel (2003) reported that the number of alleles N_{all} and the

observed heterozygosity H_o characterizing the Atlantic populations of *Ocenebra erinacea* are respectively two to four times, and twenty to thirty times lower (N_{all} = 1.1 ± 0.1; H_o = 0.01 ± 0.01; mean ± SD) than in other marine gastropods sampled in their native range, such as *Bedeva hanleyi* (N_{all} = 2.2 ± 0.1, H_o = 0.30 ± 0.02; Hoskin 2000), *Drupella sp.* (N_{all} = 2.3 ± 1.0, H_o = 0.25; Johnson & Cumming 1995), and *Littorina striata* (N_{all} = 4.2 ± 1.0, H_o = 0.18 ± 0.17; De Wolf *et al.* 2000). Congruent patterns across mitochondrial and allozyme markers thus suggest a low genetic diversity in these populations rather than an absence of variability at *cox1*. Still, our sampling remains restricted compared to the native range of the species, and additional monitoring may reveal new patterns of genetic diversity.

Genetic diversity and connectivity among populations

The genetic diversity of the Mediterranean population was among the highest (Table 2). The oyster farmers of the Thau lagoon, one of the main shell farming areas of the French Mediterranean coasts, carry out commercial exchanges with distant production sites, and *Ocenebra erinacea* is likely to be transferred during these exchanges. In fact, the morphological survey done by Berrou *et al.* (2004) evidenced exchanges from Oléron Island to Thau Lagoon (this is congruent with the observation of the Atlantic haplotype H26 being observed at Thau; Figs. 1-2). In our study, the high genetic diversity observed in Thau Lagoon could be the result of the introduction of Mediterranean specimens imported from other production sites such as Oléron. As no Mediterranean site was sampled other than Thau Lagoon, the artificial mixing induced by shellfish exchanges cannot be further evaluated here.

Alternatively, the difference in genetic diversity observed between the Atlantic and the Mediterranean population of Thau lagoon could be explained by historical and biogeographical factors, and/or selection. *Ocenebra erinacea* is not well adapted to cold water

and Belgium currently constitutes the northern limit of its natural range (Graham 1988). Consequently, the species may have found, as other marine species (e.g. Nikula & Väinölä 2003, Ladhar-Chaabouni *et al.* 2010), a refugium on the Iberian coast or in the Mediterranean basin during past glaciations. *O. erinacea* may have disappeared from the French Atlantic coasts during the Würm Glacial period (115,000 to 10,000 years BP) but survived on the coasts of the Iberian peninsula, which is known as one of the major Pleistocene refugia (Gómez & Lunz 2007). At the end of this climatic crisis, a reduced number of individuals from southern refuges may have reached northern coasts. Maggs *et al.* (2008), reviewing molecular signatures of glacial refugia on marine species, made predictions of low genetic and haplotype diversity in northern regions previously covered by ice sheets, and comparatively high diversity in refugial southern regions (and see Hewitt 1996). These predictions are generally met for *O. erinacea*, but additional sampling from the Iberian peninsula and the Mediterranean Sea would be necessary to further characterize the historical biogeography of this species. Given *O. erinacea*'s maladaptation to cold-water, another possibility is that selection (either purifying selection, or selective sweeps) linked to differences in water temperature between Thau and the Atlantic sites produced the observed patterns of genetic diversity. The negative Tajima's *D* and Fu's *Fs* observed for the Atlantic population (in absence and presence of the invasive) would support this scenario.

One potential consequence of biogeographic divergence between Atlantic and Mediterranean populations is the emergence of new species (Hewitt 1996, 2004). Recently, Salicini *et al.* (2013) have shown that in the bat *Myotis naterreri*, a complex of four cryptic species exist in the Western Palearctic region (central and southern Europe, northwestern Maghreb), each species coinciding with a glacial refugium. In *Ocenebra erinacea*, inter-clade divergence overlaps with the inter-specific distances observed in the closely-related genus *Nucella* (even though the geographical distances separating *Nucella* specimens were greater

than the distances separating *O. erinacea* specimens; see Bergsten *et al*. 2012). In addition, *O. erinacea* specimens from the Atlantic and Mediterranean can readily be distinguished using morphology, and the morphological distance between Atlantic and Mediterranean *O. erinacea* is comparable to what is observed between *O. erinacea* and *Ocenebra brevirobusta* Houart 2000 (Berrou *et al.* 2004). It is therefore possible that the Atlantic and Mediterranean clades sampled for this study belong to groups in incipient stages of speciation, or even undescribed species.

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Table 1. *Ocenebra erinacea* collection sites (listed from North to South, following the coastline), site name abbreviations (Ab., as used in Figure 2), number of *O. erinacea* specimens collected and sequenced (n), geographical coordinates, year of collection, and characteristics of the locations: presence (+) or absence (-) of oyster farms and of *O. inornata*.

Location	Ab.	n	Latitude	Longitude	Year of collection	Shellfish area	*Presence of O. inornata*
Blainville	Bl	31	49°03' N	1°36' W	2003	+	-
Chaussey	Ch	25	48°52' N	1°48' W	2003	+	-
Saint Malo	SM	22	48°39' N	2°01' W	2004	-	-
Saint Quay	SQ	23	48°39' N	2°50' W	2004	-	-
Trébeurden	Tr	24	48°48' N	3°35' W	2004	-	-
Crozon	Cr	23	48°17' N	4°27' W	2004	-	-
Le Croisic	LC	22	47°18' N	2°31' W	2004	-	-
Morbihan	Mo	12	47°33' N	2°51' W	2004	-	-
Bourgneuf	Bo	24	47°01' N	2°01' W	2003	+	+
Loix	Lo	28	46°13' N	1°24' W	2003	+	+
Aytré	Ay	20	46°06' N	1°07' W	2003	+	+
Fouras	Fo	27	46°00' N	1°07' W	2004	+	+
Oléron	Ol	13	45°53' N	1°10' W	2004	+	+
Arcachon	Ar	21	44°40' N	1°12' W	1999	+	-
Thau	Th	37	43°24' N	3°35' W	1999	+	-

Table 2. Molecular diversity of populations and results of the neutrality tests. The number of segregating sites (*S*), the number of haplotypes (*H*), the haplotype diversity (H_e ± one standard deviation), and the nucleotide diversity (π ± one standard deviation) are given for each sampling site, site groups in presence and in absence of the invasive (with and without the Mediterranean population of Thau), and for the entire data set. Sites where *Ocenebra inornata* was present are indicated by an asterisk. For neutrality tests of Tajima and Fu, statistical significance after sequential Bonferroni correction is indicated with an asterisk.

Sampling sites	S	H	*He* ± SD	π ± SD (x10^{-3})	Tajima's *D*	Fu's *Fs*
Blainville	1	2	0.065 ± 0.059	0.12 ± 0.28	-1.14	-1.24*
Chaussey	3	4	0.230 ± 0.110	0.44 ± 0.58	-1.73	-3.08*
Saint Malo	1	2	0.091 ± 0.081	0.17 ± 0.34	-1.16	-0.96
Saint Quay	0	1	0	0	0	0
Trébeurden	5	5	0.377 ± 0.122	0.90 ± 0.89	-1.83	-2.80*
Crozon	2	3	0.316 ± 0.118	0.60 ± 0.70	-0.86	-0.87
Le Croisic	4	4	0.333 ± 0.124	0.81 ± 0.84	-1.67	-1.74
Morbihan	0	1	0	0	0	0
Bourgneuf *	2	2	0.083 ± 0.075	0.30 ± 0.47	-1.51	-0.19
Loix *	5	5	0.270 ± 0.109	0.65 ± 0.72	-2.01*	-3.57*
Aytré *	4	4	0.363 ± 0.131	0.89 ± 0.89	-1.64	-1.61
Fouras *	1	2	0.074 ± 0.067	0.14 ± 0.30	-1.15	-1.12
Oléron *	2	3	0.564 ± 0.112	1.12 ± 1.06	-0.13	-0.17
Arcachon	1	2	0.095 ± 0.084	0.17 ± 0.35	-1.16	-0.92
Thau	22	6	0.342 ± 0.098	2.52 ± 1.76	-2.50*	-0.42
Sites in presence of invasive	**11**	**9**	**0.247 ± 0.0535**	**0.57 ± 0.65**	**-2.10***	**-9.42***
Sites in absence of invasive	**31**	**22**	**0.399 ± 0.3976**	**10.04 ± 5.52**	**0.22**	**-0.06**
Sites in absence of invasive (without Thau)	**16**	**16**	**0.179 ± 0.0368**	**0. 38 ± 0. 51**	**-2.38***	**< -10***
All populations	**37**	**29**	**0.356 ± 0.0325**	**7.63 ± 4.20**	**-0.81**	**-4.97**

Table 3. Pairwise Φ_{ST} values calculated using the Kimura 2 Parameter model of nucleotide substitution. Only pairwise comparisons involving the population of Thau are statistically significant after sequential Bonferroni correction.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. Blainville	0														
2. Chausey	0.0048	0													
3. Saint Malo	0.00242	0.00244	0												
4 . Saint Quay	-0.01001	-0.00356	0.00207	0											
5. Trebeurden	0.02194	0.01045	0.00921	0.01207	0										
6. Crozon	0.05037	0.02772	0.03452	0.04529	0.02682	0									
7. Le Croisic	0.03001	0.01442	0.01577	0.02118	0.01654	0.01135	0								
8. Morbihan	-0.03708	-0.03371	-0.03067	0	-0.02156	-0.00476	-0.01543	0							
9. Bourgneuf	0.00458	-0.00021	0.00102	0.00181	0.01083	0.0316	0.01598	0.03274	0						
10. Loix	0.00242	-0.0008	-0.00559	-0.00747	0.00258	0.02217	0.012	-0.0358	-0.01337	0					
11. Aytré	0.03811	0.01838	0.02156	0.0293	0.00956	0.03281	0.02045	0.01049	0.00555	0.01096	0				
12. Oleron	0.28743	0.18725	0.23275	0.28709	0.1342	0.16138	0.13706	0.18898	0.15611	0.12163	0.04004	0			
13. Fouras	0.0004	0.00162	0.00096	-0.00611	0.01656	0.04351	0.02394	-0.03502	0.00196	-0.00096	0.03104	0.26425	0		
14. Arcachon	0.0032	-0.00323	0.00014	0.00443	0.00763	0.03266	0.01404	0.02941	-0.00153	0.00658	0.01955	0.22606	0.00145	0	
15. Thau	0.96208	0.95559	0.95617	0.95856	0.94988	0.95236	0.95001	0.94928	0.95614	0.9547	0.9484	0.94167	0.95959	0.95541	0

Fig. 1. Median-joining haplotype network. Each circle represents a haplotype, which frequency is proportional to circle diameter (legend: bottom right). Distances between haplotypes are proportional to the number of mutation events (see text). The Atlantic haplogroup contains one Mediterranean population, represented by haplotype H26 (marked with **).

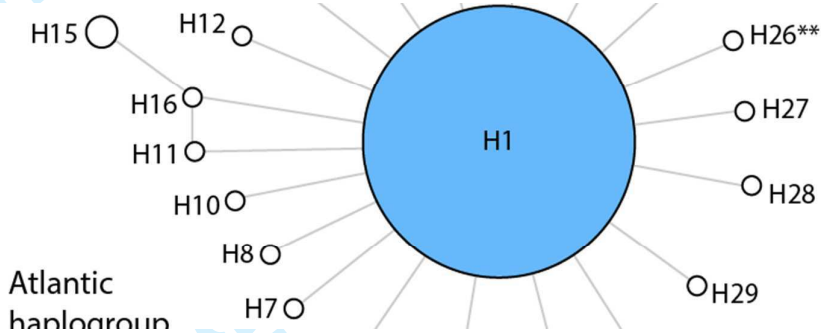
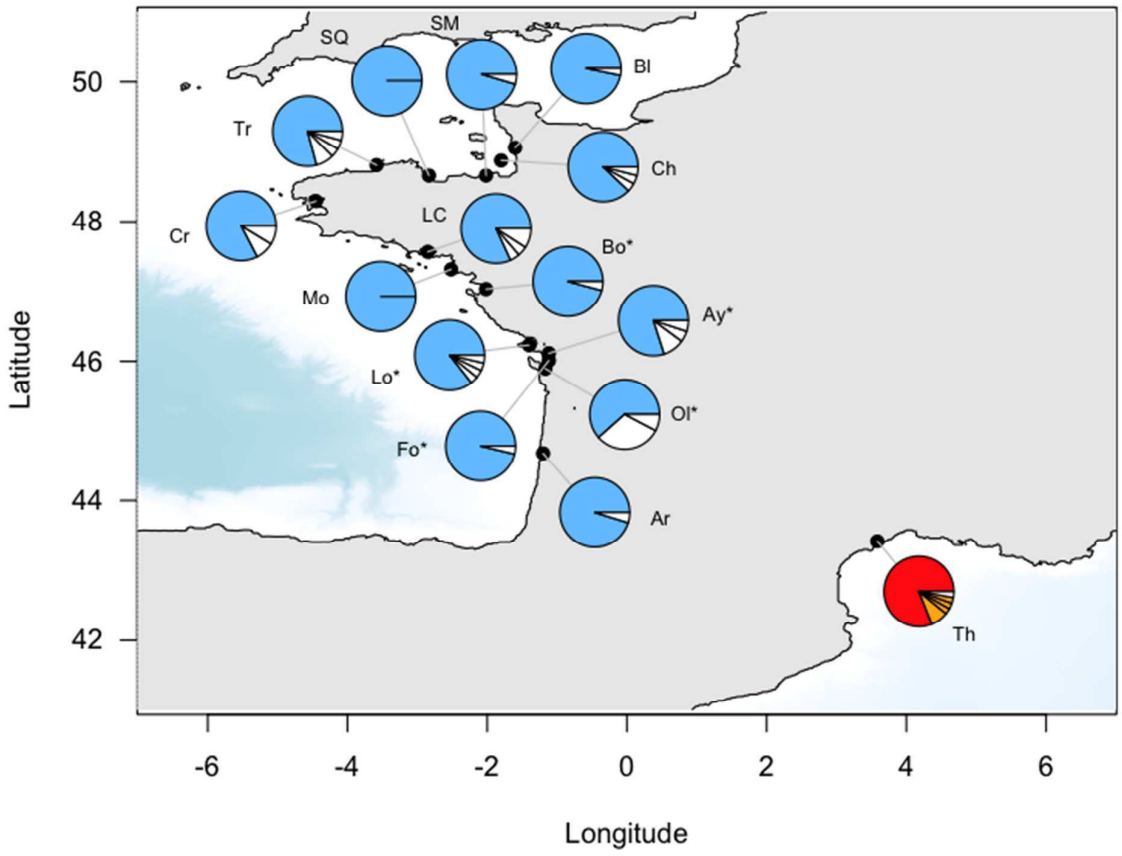


Fig. 2. Distribution of haplotype frequencies along the French coast. Abbreviations for site names are detailed in Table 1. Haplotype colors correspond to the colors used in Figure 1. The stations where both *Ocenebra erinacea* and *Ocenebra inornata* were observed are marked with an asterisk. Map constructed with R package marmap (Pante & Simon-Bouhet 2013).



Evaluating the genetic effects of the invasive *O~~cinebrellus~~cenebra inornat~~aus~~* on the native oyster drill *Ocenebra erinacea*

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Keyword: *Ocenebra erinaceus*; *Ocinebrellus inornatus*; haplotype diversity; evolutionary response; evolutionary ecology; invasive species; glacial refugium; cryptic species

Abstract

Studies focusing on the effect of invasive species on the genetic diversity of native marine invertebrates remain scant. Here we report diversity among French populations of the intertidal gastropod *Ocenebra erinacea* (Linnaeus, 1758) sampled in the presence and absence of the invasive ~~*Ocinebrellus inornatus*~~*Ocenebra inornata* (Recluz, 1851). Between 1999 and 2004 a total of 352 individuals of *O. erinacea* were collected from 15 sites, five of which in the presence of the invasive, and genotyped at the mitochondrial locus *cox1*. No statistical difference was observed between polymorphism levels recorded within native populations exposed to the invasive, compared to populations sampled in the absence of ~~*O. inornatus*~~*O. inornata*. No sign of native population ~~bottleneck~~ decline was detected in response to the invader. While significant shifts in native *O. erinacea* population sizes were previously reported in the literature, genetic effects may take longer to accumulate, or may be undetectable without a larger panel of genetic markers~~or may be detectable using a larger panel of genetic markers~~. In contrast, large genetic distances and significant population differentiation were recorded between Atlantic and Mediterranean *O. erinacea* samples, suggesting that these populations have distinct evolutionary histories. Comparison of genetic divergence within the closely-related genus *Nucella* suggests that the Atlantic populations and the Mediterranean *O. erinacea* populations from Thau Lagoon may belong to different species or subspecies.

Introduction

Natural movements of species ranges on the surface of the world (expansions, regressions, displacements) play a considerable role in the evolution of species. Most of the time, these phenomena are progressive and marked by the tempo of geological processes (e.g. Hewitt 1996). However, ~~for a~~ during the last few decades a growing number of species has undergone changes in their natural range due to both ~~global~~ changes at ~~the planet~~a global scale (e.g. Parmesan & Yohe 2003) and artificial transfers due to human activities (e.g. Carlton 1989; Seebens *et al.* 2013).

The number of biological invasions has strongly increased during the last decades (e.g. Mack *et al.* 2000; Mooney & Cleland, 2001; Ruiz *et al.* 2000). In the marine environment, these phenomena are mainly due to aquaculture and especially to shellfish farming, which represents a major cause of introduction, intentional or not, of exogenous species (Elton 1958; Carlton 1992).

Apart from potentially important economic consequences, the arrival of these introduced species can also cause serious ecological impacts on local fauna. Introduced species are likely to decrease the abundance of indigenous species, excluding them from part of their distributional area or even causing their extinction by modifying invaded habitats, hybridizing with native species, exchanging pathogens, preying on them, or competing with them (e.g. Lockwood *et al.* 2007 and references therein). Moreover, when they exert strong selective pressures, introduced species can also reduce the genetic diversity of native populations (Kim *et al.* 2003; Wittmann *et al.* 2013).

Such impacts, although poorly known (e.g. Strauss *et al.* 2006), may have heavy ecological consequences since adaptive potential depends on the genetic diversity of a

population. Reductions in genetic diversity are generally considered detrimental (e.g. Frankham 1995; Lande 1995; Strauss *et al.* 2006) and m~~ay lead~~ight contribute to extinction (Wittmann *et al.* 2013). Thus, various authors showed a link between the fitness of a species and its genetic diversity, particularly in mollusks (Mitton & Grant 1984; Garton & Haag 1991; Zouros 1993; Launey & Hedgecock 2001; Hedgecock *et al.* 2007). ~~Conversely~~In addition, the reduced genetic variability of an indigenous population could promote the expansion process of other species that are phylogenetically close. However, very few studies address changes in ~~the evolution of~~ genetic diversity of an indigenous species under the competitive ~~selection~~ pressure generated by a biological invader.

The oyster drills *Ocenebra erinacea* (Linnaeus, 1758) and ~~*Ocinebrellus inornatus*~~*Ocenebra inornata* (Recluz, 1851) constitute a noteworthy model to study the genetic effects of indigenous-invasive interactions on indigenous populations. A native of the northwestern Pacific (Choe & Park 1997; Amano & Vermeij 1998), ~~*Ocinebrellus inornatus*~~*O. inornata* (previously known as *Ocinebrellus inornatus*; see Houart and Sirenko 2003; Bouchet and Houart 2014) ~~(Recluz, 1851)~~ has recently invaded European coasts, probably following massive oyster imports (Pigeot 2000; De Montaudouin & Sauriau 2000; review of Lützen *et al.* 2012). Genetic data suggest that French populations may come from Asia and the United States (Martel *et al.* 2004a). The introduction of ~~*O. inornatus*~~*O. inornata* may ha~~s~~ves important economical consequences, as it is a predator of cultivated mollusks (e.g. oysters, blue mussels, Goulletquer *et al.* 2002). It coexists ~~on~~ at several French sites with an indigenous muricid, *O.* ~~*cenebra*~~ *erinacea* (Linnaeus, 1758), which ranges from the straits of Gibraltar to the Netherlands, and inhabits all British and Mediterranean coasts (Graham 1988). Although *O. erinacea* and ~~*O. inornatus*~~*O. inornata* differ in some life history traits (Martel *et al.* 2004c), ~~they~~ these muricid gastropods fill similar ecological niches, and may compete for habitat (both species live on hard substrates and drill the shell of bivalves to feed on them; e.g.

Lutzen et al 2012). Pigeot *et al*. (2000) recorded a decrease in population density of *O. erinacea*, in parallel to an in increase in numbers of *O. inornata* in Marennes-Oléron (Charente-Maritime, France), between 1997 and 1999 (two years after the invasive was first detected). While the introduction and expansion patterns of ~~*O. inornatus*~~*O. inornata* have been investigated in previous studies (Martel *et al.* 2004a; Martel *et al.* 2004b), its ecological impacts ~~and the influence of~~on the ~~local~~ native *O. erinacea* ~~species on its capacity of integration~~ are poorly known.

In previous studies (Martel 2003), seven allozyme loci were analyzed on populations of *Ocenebra*~~*O.*~~ *erinacea* and ~~*O. inornatus*~~*Ocenebra inornata* collected in 7 sites of the French Atlantic coast where the two species live in sympatry. These markers revealed genetic diversity indices systematically weaker within the native *O. erinacea* than within the invasive ~~*O. inornatus*~~*O. inornata*. This result was counterintuitive, as (i) the founder effect linked to an introduction event should lead to a low genetic diversity within the populations of the introduced species, (ii) this phenomenon should be all the more marked as the invasion is recent (review of Sakai *et al.* 2001). Consequently, a lower genetic diversity within the populations of the exogenous species compared to the populations of the indigenous species was expected. It is thus of importance to test whether the genetic diversity of the indigenous species *O. erinacea* is correlated with the presence of the introduced species ~~*O. inornatus*~~*O. inornata* in zones of sympatry. Indeed, ~~*O. inornatus*~~*O. inornata* could induce a selective pressure on *O. erinacea* leading to decrease of polymorphism in this local species.

Here, we test this hypothesis by sampling *O.*~~*cenebra*~~ *erinacea* from the Atlantic and Mediterranean French coasts, in the presence and absence of ~~*O. inornatus*~~*Ocenebra inornata*, and by measuring genetic diversity of the native species using the mitochondrial marker *cox1*. While investigating the genetic effects that the presence of ~~*O. inornatus*~~*O. inornata* may have on sympatric populations of *O. erinacea*, we came across a very strong genetic break between

Atlantic and Mediterranean populations. This break is detailed and potential biogeographic causes are discussed.

Materials and methods

Sampling

A total of 352 adult specimens of *Ocenebra erinacea* were collected between 1999 and 2004 at 15 sites on the French coast, along line transects (<200 m in length). At each site, specimens from different rocks were collected to reduce sampling bias in favor of a particular lineage. The sites were located both within oyster farming zones and unexploited areas (Table 1). In order to show a possible impact of the presence of ~~*O. inornatus*~~*Ocenebra inornata* on genetic diversity of *O. erinacea*, five locations where the two species live in sympatry and 10 sites free of ~~*O. inornatus*~~*O. inornata* have been sampled. The presence of *O. inornata* was assessed by direct observation. After collection, specimens were stored in 95% ethanol before DNA extraction.

DNA extraction, amplification and sequencing

Total DNA was extracted from <15 mg of foot muscle using Dneasy™ Tissue Kit according to the manufacturer's protocol (Qiagen, Germany). Part of the mitochondrial *cox1* gene was PCR-amplified with the HCO2198/LCO1490 primers (Folmer *et al.* 1994), which ~~are~~ have ~~particularly~~ proved useful for neogastropod studies (e.g. ~~Harasewych *et al.* 1997~~Zou et al 2011, 2012). Polymorphism at *cox1* is high in ~~*O. inornatus*~~*Ocenebra inornata*, a phylogenetically close species (Martel *et al.* 2004a).

PCRs were done in 50 µL, with 1X PCR buffer, 1.85 mM $MgCl_2$, 125 µM dNTPs, 0.25 µM of each primer, 1.6 U of Red Hot® DNA Polymerase (ABgene) and about 10 ng of DNA template. The following cycling profile was performed using a MJResearch PTC 100 Thermal Cycler: initial 5-min denaturation step at 94°C followed by 40 cycles of 30 sec at 94°C, 30 sec at 50°C and 1 min at 72°C, and by a final 5-min extension period at 72°C. PCR products were purified using MultiScreen-PCR MANU03010 plates (Millipore).

Sequencing was done by GenoScreen (Lille - France) using an ABI PRISM® 3730 XL Automated DNA Sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA). Sequences were aligned using ClustalX (Thompson *et al.* 1994).

Data analyses.

Genetic analyses aimed at (i) quantifying and comparing genetic diversity among populations, (ii) analyzing the spatial distribution of polymorphism and genetic exchanges among populations, and (iii) studying the evolutionary relationships among populations. Haplotype number *H*, number of polymorphic sites *S*, haplotype diversity H_e ~~*He*~~ and average per site nucleotide diversity π (Nei 1987) were calculated for each population using the software DnaSP 5.10.1 (Librado & Rozas 2009).

We tested the null hypothesis of the standard neutral model in Arlequin v.3.5 (Excoffier & Lischer 2010), by calculating the *D* and *Fs* statistics, as defined by Tajima (1989) and Fu (1997). ~~In each location, we checked the demographic equilibrium of the populations in Arlequin v.3.5, by calculating the *D* and *Fs* statistics, as defined by Tajima (1989) and Fu (1996).~~ When these statistics are significantly different from zero, populations may have undergone purifying selection, a selective sweep and/or expansion (<0), or balancing selection and/or a population decline ~~or overdominant selection and/or a bottleneck~~ (>0). Statistical

significance was tested by generating 10,000 random samples under the hypothesis of selective neutrality and population equilibrium. These tests were performed for each sampling site separately, and also for pooled sites in presence or absence of the invasive.

The differentiation index Φ_{ST} (Excoffier *et al.* 1992), an estimator of F_{ST} (Wright 1951) calculated from frequency values and distances between haplotypes, was computed with Arlequin v.3.5 ~~(Excoffier & Lischer 2010)~~. The Kimura 2 Parameter (K2P) model of nucleotide substitution was used to estimate genetic distances, and 10,000 permutations were used to test statistical significance under the null hypothesis of no difference between populations (Excoffier *et al.* 1992).

Finally, a haplotype network was built using the median-joining algorithm implemented in Network 4.6.1.1 (fluxus-engineering.com, Bandelt 1999). This method is one of the most accurate for inferring intra-specific networks in the absence of recombination (Woolley *et al.* 2008).

To help interpret the large genetic divergence observed between Atlantic and Mediterranean specimens of *O.* ~~*cenebra*~~ *erinacea*, we looked for mitochondrial *cox1* data from the BOLD database (Ratnasingham & Hebert 2007). However, besides three other BOLD *cox1* sequences from Spanish specimens of *O. erinacea*, we produced the only available mitochondrial sequences for the genus *Ocenebra*. We therefore used *cox1* sequences from ~~seven~~ six species of the closely-related genus *Nucella* Röding, 1798 (e.g. Pascal 2004) to measure intra-specific and inter-specific genetic distances. *Nucella* and *Ocenebra* are both characterized by a non-planktonic larval development and lay egg capsules on hard substrates (Martel *et al.* 2004c; review by Krug 2011). We used the K2P model of nucleotide substitution (Kimura 1980), ~~a~~ widely used ~~metric~~ in DNA barcoding (Hebert *et al.* 2003; Barrett & Hebert 2005), to measure genetic distances among *cox1* haplotypes.

Results

A 550 bp fragment of *cox1* was sequenced for 352 individuals, and 29 haplotypes were identified (GenBank accession numbers AY995771-99; Popset 63109090). Sequences include 37 polymorphic sites, 20 of which are parsimony informative, and one of which has three character states. No indels were observed (Table 2).

Population-level genetic diversity and demographic stability

Genetic diversity is comparable among Atlantic populations of *Ocenebra~~.~~ erinacea*, but values for the different diversity indices are low compared to other recently-studied marine mollusks. The number of polymorphic sites between two different sequences varies between 0 and 6 among Atlantic populations. Two of these populations (Morbihan and St Quay) are each characterized by a single haplotype, and the 12 other Atlantic sites have no more than five haplotypes (sample sizes provided in Table 1). Consequently, haplotype diversity H_e is low, except for Oléron (H_e = 0.564; Table 2). In Fouras, where ~~*O. inornatus*~~*Ocenebra inornata* and *O. erinacea* are sympatric, the haplotype and nucleotide diversities are respectively four- and six-fold higher in the invasive drill (H_e = 0.348 and π x10^{-3} = 0.83~~78~~; data from Martel 2003) than in the local one (H_e = 0.074 and π x10^{-3} = 0.14).

A single Mediterranean population (Thau) was sampled. The haplotype diversity (H_e = 0.342) and the nucleotide diversity (π x10^{-3} = 2.52) are respectively two- and five-fold higher than on the whole of Atlantic populations (Table 2). Moreover, 22 polymorphic sites were found among 37 individuals sampled in Thau, a value considerably higher than the 24 polymorphic sites observed among 315 Atlantic individuals.

Last, haplotype and nucleotide diversities of *O. cenebra erinacea* populations co-occurring with ~~*O. inornatus*~~*Ocenebra inornata* were lower (H_e = 0.247 ± 0.0535 and π x10^{-3}= 0.57 ± 0.65) than for populations located in zones where ~~*O. inornatus*~~*O. inornata* was not detected (H_e = 0.399 ± 0.3976, π x10^{-3}= 10.04 ± 5.52) (Table 2). However, this pattern is entirely due to the higher diversity encountered at Thau; when removing this site from the group of populations that were not found in contact with ~~*O. inornatus*~~*O. inornata*, diversity values dropped significantly (H_e = 0.179 ± 0.0368, π x10^{-3}= 0. 38 ± 0. 51). Comparing molecular diversity at the site level revealed the same pattern (Welch Two Sample t-test, including Thau,~~;~~ for H_e: t = -0.83, df = 6.22, p = 0.44; for π: t = -0.16, df = 12.81, p = 0.88. In both cases, results were also non-significant when removing the population of Thau).

Except for one case, the *D* and *Fs* statistics were never positive (Table 2). Furthermore, the only slightly positive *D* value (deviation from zero non significant) was observed when sites where the invasive was absent were pooled, including Thau, and this result was therefore likely influenced by the underlying population structure (see "Genetic differentiation among populations" Results section). ~~Except for one case (all sites without *O. inornatus* pooled, deviation from zero non significant), the *D* and *Fs* statistics were never positive.~~ There is therefore no supporting evidence that *O. cenebra erinacea* populations exposed to ~~*O. inornatus*~~*O. inornata* suffered from a population decline~~bottleneck~~. Some sites ~~populations~~ exhibited significant negative values of *D* and *Fs*, which can be interpreted as signs of purifying selection, selective sweep and~~-~~/-or population expansion. Particularly, the pooled Atlantic sites showed significantly negative values for both tests, regardless of whether the invasive was present or not. These molecular signatures must, however, be interpreted with care, as they might reflect older demographic events.

~~Phylogenetic~~ Genealogical relationships and spatial distribution of haplotypes

Two haplogroups, separated by 18 mutational steps, were observed using the median-joining network (Fig. 1). The first haplogroup (23 haplotypes) was mainly composed of Atlantic specimens, and included one specimen from Thau, characterized by haplotype H26 (separated from other Mediterranean haplotypes by 20 to 22 mutational steps). Haplotypes from this group diverged by ≤ 2 mutations. Haplotype H1 was common (represented in 89% of Atlantic individuals) and central to the Atlantic haplogroup, while the other 22 haplotypes were rare (7 individuals for H3, 3 for H13, 2 for H6, H9, H15, H19 and a single individual for the others) and peripheral to H1. The second haplogroup was strictly composed of Mediterranean individuals. Of six haplotypes from this group, one was common (H21, represented in 82% of Thau individuals), and four were rare (3 individuals for H22 and a single individual for H23-25). Haplotypes from this group diverged by one mutation. The Atlantic samples share no haplotype with the Mediterranean sample (Fig. 2). Except for H1 (present at all sites except Thau), H3 (shared between Loix and Aytré) and H13 (shared between Loix, Aytré and Trébeurden), all haplotypes are private (observed only within one population).

Genetic differentiation among populations

Genetic differentiation among pairs of populations was measured using Φ_{ST}. The population from Thau was significantly differentiated from all other populations. Pairwise Φ_{ST} values ranged between 0.94 and 0.96, corresponding to a~~n important~~ substantial genetic differentiation between the Mediterranean and Atlantic populations (Table 3). Inside the Atlantic group, no significant differentiation was observed after sequential Bonferroni correction (lowest corrected alpha level for Atlantic populations: 0.00055).

Levels of intra-specific divergence in *Ocenebra* compared to *Nucella*

Given the differences in haplotype composition and divergence between Mediterranean and Atlantic sites, we investigated whether the genetic distances correspond to intra- or inter-specific divergence by comparing *Ocenebra* to its close relative *Nucella*. The pairwise K2P distance between *Ocenebra ~~O.~~ erinacea* haplotypes ranged between 0.18 and 4.54% (maximum observed between haplotypes 20 from Loire and 25 from Thau Lagoon). Within *Nucella*, K2P was calculated for 532 sequences and 117 haplotypes distributed among six species, along a 434 bp stretch of *cox1*. Intra-specific distances ranged from 0 to 3.32%, while inter-specific distance ranged from 4.81 to 12.2%, for specimens distributed over 1000 to 2000 km (East and West coasts of North America, respectively; BOLD database). For comparison, Zou et al (2011), analyzing 108 neogastropod *cox1* sequences (same gene region as analysed here; not including *Ocenebra* or *Nucella*) found maximum intra-specific K2P distances of 2.2% and minimum inter-specific distances of 2.1%.

Discussion

Relation between *O~~.~~cenebra erinacea* and the invasive species *~~O. inornatus~~Ocenebra inornata*

While a decrease in genetic diversity in response to invaders was reported in the past (Kim *et al.* 2003), we did not detect such a pattern among *Ocenebra~~O.~~ erinacea* exposed to the invasive species *~~O. inornatus~~Ocenebra inornata*. The relatively low polymorphism levels recorded may have hampered our ability to detect genetic effects of the invasive on the native species, and the use of ~~more sensitive~~additional molecular markers such as nuclear microsatellites or SNPs might further help detect possible demographic events associated with

the presence ~~*O. inornatus*~~*O. inornata*. However, this remains to be tested, as even genome-wide scans can fail at detecting recent demographic events and selective pressures. Riquet *et al.* (2013), for instance, used AFLPs to compare native and invasive populations of the marine mollusk *Crepidula fornica~~l~~ta*. They reported little genetic differentiation among these populations, and detected no F_{ST} outliers out of 344 tested loci. An alternative hypothesis explaining the apparent absence of genetic effects of the invasive on the native is that the competitive and selective pressures inflicted on *O. erinacea* are too ~~mild~~ low to have genetic effects (e.g. Wittmann *et al.* 2013). Finally, deviations from neutrality observed for pooled Atlantic sites suggest selection could have shaped the current genetic diversity and could have blurred signatures of demographic processes.

Ocenebra ~~*O.*~~ *inornat~~us~~a* ~~invaded~~ was first documented on the French Atlantic coast in ~~the~~ 19~~9~~75~~0's~~ (De Montaudouin & Sauriau 2000, Pigeot *et al.* 2000), and the specimens of *Ocenebra~~.~~ erinacea* used in this study were collected between 1999 and 2004. The introduction of *O. inornata* may have been too recent at the time of sampling for genetic consequences on the native species to be detectable. About ten years later, the distributional landscape of ~~*O. inornatus*~~*O. inornata* on Atlantic coasts have significantly changed, and the invasive is now found as far north as the entrance of the Baltic Sea (Lützen *et al.* 2012). A new survey of the genetic diversity of *O. erinacea* may today unfold the genetic consequences of the invasion by ~~*O. inornatus*~~*O. inornata*, and this study therefore provides a snapshot in time that may help better understand the temporal dynamics of loss of genetic diversity. In addition to sampling in the field, we searched for *Ocenebra* specimens in the collections of the Museum national d'Histoire naturelle in Paris (France), in order to look for genetic diversity in *O. erinacea* specimens collected prior to, or soon after the invasion by *O. inornata* ~~*Ocinebrellus*~~ (MNHN voucher numbers IM-2008-7101, IM-2008-7102, IM-2008-7103). Unfortunately, we were not able to amplify the *cox1* marker from these specimens.

Remarkably low genetic diversity of *Ocenebra~~.~~ erinacea* populations

Mitochondrial genetic diversity, as measured using part of *cox1*, was low relative to what was observed in other marine mollusks. Overall, *Ocenebra~~O.~~ erinacea* haplotype and nucleotide diversities were (disregarding the sample from Thau, see below) H_e = 0.18-0.25 and π x10^{-3}= 0.38-0.57 (Table 2). Comparatively, H_e = 0.684 and π x10^{-3}= 2.25 for *~~O. inornatus~~Ocenebra inornata* in its natural range (data from Martel *et al.* 2004a), H_e = 0.734 and π x10^{-3}= 14.78 in the gastropod *Cyclope neritea* (Simon-Bouhet *et al.* 2006), and H_e = 0.720 and π x10^{-3}= 89.84 in the bivalve *Macoma balthica* (Becquet *et al.* 2012).

The low genetic diversity observed at *cox1* was consistent with the low diversity observed using allozymes: Martel (2003) reported that the number of alleles N_{all} and the observed heterozygosity H_o characterizing the Atlantic populations of *O~~.~~cenebra erinacea* are respectively two to four times, and twenty to thirty times lower (N_{all} = 1.1 ± 0.1; H_o = 0.01 ± 0.01; mean ± SD) than in other marine gastropods sampled in their native range, such as *Bedeva hanleyi* (N_{all} = 2.2 ± 0.1, H_o = 0.30 ± 0.02; Hoskin 2000), *Drupella sp.* (N_{all} = 2.3 ± 1.0, H_o = 0.25; Johnson & Cumming 1995), and *Littorina striata* (N_{all} = 4.2 ± 1.0, H_o = 0.18 ± 0.17; De Wolf *et al.* 2000). Congruent patterns across mitochondrial and allozyme markers thus suggest a low genetic diversity in these populations rather than an absence of variability at *cox1*. Still, our sampling remains restricted compared to the native range of the species, and additional monitoring may reveal new patterns of genetic diversity.

Genetic diversity and connectivity among populations

The genetic diversity of the Mediterranean population was among the highest~~ than that of each Atlantic population (except Oléron,~~ Table 2). The oyster farmers of the Thau

lagoon, one of the main shell farming areas of the French Mediterranean coasts, carry out commercial exchanges with distant production sites, and *O. cenebra erinacea* is likely to be transferred during these exchanges. In fact, the morphological survey done by Berrou *et al.* (2004) evidenced exchanges between from Oléron Island and to Thau Lagoon (this is congruent with the observation of the Atlantic haplotype H26 being observed at Thau; Figs. 1-2). In our study, the high genetic diversity observed in Thau Lagoon could be the result of the introduction of Mediterranean specimens imported from other production sites such as Oléron. As no Mediterranean site was sampled other than Thau Lagoon, the artificial mixing induced by shellfish exchanges cannot be further evaluated here.

Alternatively, the difference in genetic diversity observed between the Atlantic and the Mediterranean population of Thau lagoon could be explained by historical and biogeographical factors, and/or selection. *Ocenebra erinacea* is not well adapted to cold water and Belgium currently constitutes the northern limit of its natural range (Graham 1988). Consequently, the species may have found, as other marine species (e.g. Nikula & Väinölä 2003, Ladhar-Chaabouni *et al.* 2010), a refugium on the Iberian coast or in the Mediterranean basin during past glaciations. *O. erinacea* may have disappeared from the French Atlantic coasts during the Würm Glacial period (115,000 to 10,000 years BP) but survived on the coasts of the Iberian peninsula, which is known as one of the major Pleistocene refugia (Gómez & Lunz 2007). At the end of this climatic crisis, a reduced number of individuals from southern refuges may have reached northern coasts. Maggs *et al.* (2008), reviewing molecular signatures of glacial refugia on marine species, made predictions of low genetic and haplotype diversity in northern regions previously covered by ice sheets, and comparatively high diversity in refugial southern regions (and see Hewitt 1996). These predictions are generally met for *O. erinacea*: a mitochondrial genealogy matching geography, but additional sampling from the Iberian peninsula and the Mediterranean Sea would be necessary to further

characterize the historical biogeography of this species. Given *O. erinacea*'s maladaptation to cold-water, another possibility is that selection (either purifying selection, or selective sweeps) linked to differences in water temperature between Thau and the Atlantic sites produced the observed patterns of genetic diversity. The negative Tajima's *D* and Fu's *Fs* observed for the Atlantic population (in absence and presence of the invasive) would support this scenario. ~~and Hewitt's "northern purity, southern richness" genetic diversity pattern (Magg's et al. Model IIa).~~

One potential consequence of biogeographic divergence between Atlantic and Mediterranean populations is the emergence of new species (Hewitt 1996, 2004). Recently, Salicini *et al.* (2013) have shown that in the bat *Myotis naterreri*, a complex of four cryptic species exist in the Western Palearctic region (central and southern Europe, northwestern Maghreb), each species coinciding with a glacial refugium. In *Ocenebra~~-~~ erinacea*, inter-clade divergence overlaps with the inter-specific distances observed in the closely-related genus *Nucella* (even though the geographical distances separating *Nucella* specimens were greater than the distances separating *O. erinacea* specimens; see Bergsten *et al.* 2012). In addition, *O. erinacea* specimens from the Atlantic and Mediterranean can readily be distinguished using morphology, and the morphological distance between Atlantic and Mediterranean *O. erinacea* is comparable to what is observed between *O. erinacea* and *Ocenebra~~-~~ brevirobusta* Houart 2000 (Berrou *et al.* 2004). It is therefore possible that the Atlantic and Mediterranean clades sampled for this study belong to groups in incipient stages of speciation, or even undescribed species.

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Table 1. ~~*Sampling sites.*~~*Ocenebra erinacea* ~~Collection~~ collection sites (listed from North to South, following the coastline), site name abbreviations (Ab., as used in Figure 2), number of *O. erinacea* specimens collected and sequenced (n), geographical coordinates, year of collection, and characteristics of the locations: presence (+) or absence (-) of oyster farms and of ~~*O. inornatus*~~*O. inornata*.

~~Location~~	~~Ab.~~	~~*n*~~	~~Latitude~~	~~Longitude~~	~~Shellfish area~~	~~*O. inornatus*~~
~~Blainville~~	~~Bl~~	~~31~~	~~49°03' N~~	~~1°36' W~~	~~+~~	~~-~~
~~Chaussey~~	~~Ch~~	~~25~~	~~48°52' N~~	~~1°48' W~~	~~+~~	~~-~~
~~Saint Malo~~	~~SM~~	~~22~~	~~48°39' N~~	~~2°01' W~~	~~-~~	~~-~~
~~Saint Quay~~	~~SQ~~	~~23~~	~~48°39' N~~	~~2°50' W~~	~~-~~	~~-~~
~~Trébeurden~~	~~Tr~~	~~24~~	~~48°48' N~~	~~3°35' W~~	~~-~~	~~-~~
~~Crozon~~	~~Cr~~	~~23~~	~~48°17' N~~	~~4°27' W~~	~~-~~	~~-~~
~~Le Croisic~~	~~LC~~	~~22~~	~~47°18' N~~	~~2°31' W~~	~~-~~	~~-~~
~~Morbihan~~	~~Mo~~	~~12~~	~~47°33' N~~	~~2°51' W~~	~~-~~	~~-~~
~~Bourgneuf~~	~~Bo~~	~~24~~	~~47°01' N~~	~~2°01' W~~	~~+~~	~~+~~
~~Loix~~	~~Lo~~	~~28~~	~~46°13' N~~	~~1°24' W~~	~~+~~	~~+~~
~~Aytré~~	~~Ay~~	~~20~~	~~46°06' N~~	~~1°07' W~~	~~+~~	~~+~~
~~Fouras~~	~~Fo~~	~~27~~	~~46°00' N~~	~~1°07' W~~	~~+~~	~~+~~
~~Oléron~~	~~Ol~~	~~13~~	~~45°53' N~~	~~1°10' W~~	~~+~~	~~+~~
~~Arcachon~~	~~Ar~~	~~21~~	~~44°40' N~~	~~1°12' W~~	~~+~~	~~-~~
~~Thau~~	~~Th~~	~~37~~	~~43°24' N~~	~~3°35' W~~	~~+~~	~~-~~

Location	Ab.	n	Latitude	Longitude	Year of collection	Shellfish area	*Presence of O. inornata*
Blainville	Bl	31	49°03' N	1°36' W	2003	+	-
Chaussey	Ch	25	48°52' N	1°48' W	2003	+	-
Saint Malo	SM	22	48°39' N	2°01' W	2004	-	-
Saint Quay	SQ	23	48°39' N	2°50' W	2004	-	-
Trébeurden	Tr	24	48°48' N	3°35' W	2004	-	-
Crozon	Cr	23	48°17' N	4°27' W	2004	-	-
Le Croisic	LC	22	47°18' N	2°31' W	2004	-	-
Morbihan	Mo	12	47°33' N	2°51' W	2004	-	-

Bourgneuf	Bo	24	47°01’ N	2°01’ W	2003	+	+
Loix	Lo	28	46°13’ N	1°24’ W	2003	+	+
Aytré	Ay	20	46°06’ N	1°07’ W	2003	+	+
Fouras	Fo	27	46°00’ N	1°07’ W	2004	+	+
Oléron	Ol	13	45°53’ N	1°10’ W	2004	+	+
Arcachon	Ar	21	44°40’ N	1°12’ W	1999	+	-
Thau	Th	37	43°24’ N	3°35’ W	1999	+	-

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Table 2. Molecular diversity of populations and results of the neutrality tests. The number of segregating sites (*S*), the number of haplotypes (*H*), the haplotype diversity (H_e ± one standard deviation), and the nucleotide diversity (π ± one standard deviation) are given for each sampling site, site groups in presence and in absence of the invasive (with and without the Mediterranean population of Thau), and for the entire data set. Sites where ~~*O. inornatus*~~*Ocenebra inornata* was present are indicated by an asterisk. For neutrality tests of Tajima and Fu, statistical significance after sequential Bonferroni correction is indicated with an asterisk.

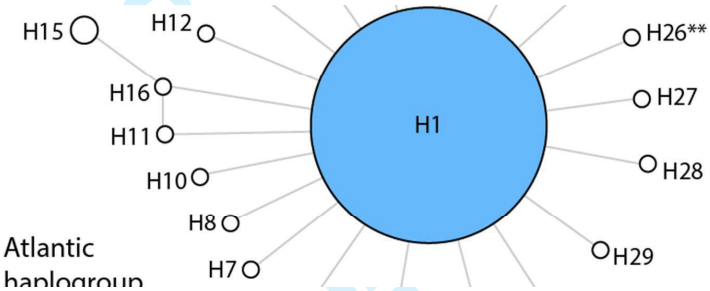
~~Sampling sites~~	~~*S*~~	~~*H*~~	~~H_e ± SD~~	~~π ± SD (x10^{-3})~~	~~Tajima's *D*~~	~~Fu's *Fs*~~
~~Blainville~~	~~1~~	~~2~~	~~0.065 ± 0.059~~	~~0.12 ± 0.28~~	~~-1.14~~	~~-1.24*~~
~~Chaussey~~	~~3~~	~~4~~	~~0.230 ± 0.110~~	~~0.44 ± 0.58~~	~~-1.73~~	~~-3.08*~~
~~Saint Malo~~	~~1~~	~~2~~	~~0.091 ± 0.081~~	~~0.17 ± 0.34~~	~~-1.16~~	~~-0.96~~
~~Saint Quay~~	~~0~~	~~1~~	~~0~~	~~0~~	~~0.00~~	~~0.00~~
~~Trébeurden~~	~~5~~	~~5~~	~~0.377 ± 0.122~~	~~0.90 ± 0.89~~	~~-1.83~~	~~-2.80*~~
~~Crozon~~	~~2~~	~~3~~	~~0.316 ± 0.118~~	~~0.60 ± 0.70~~	~~-0.86~~	~~-0.87~~
~~Le Croisic~~	~~4~~	~~4~~	~~0.333 ± 0.124~~	~~0.81 ± 0.84~~	~~-1.67~~	~~-1.74~~
~~Morbihan~~	~~0~~	~~1~~	~~0~~	~~0~~	~~0.00~~	~~0.00~~
~~Bourgneuf *~~	~~2~~	~~2~~	~~0.083 ± 0.075~~	~~0.30 ± 0.47~~	~~-1.51~~	~~-0.19~~
~~Loix *~~	~~5~~	~~5~~	~~0.270 ± 0.109~~	~~0.65 ± 0.72~~	~~-2.01*~~	~~-3.57*~~
~~Ayré *~~	~~4~~	~~4~~	~~0.363 ± 0.131~~	~~0.89 ± 0.89~~	~~-1.64~~	~~-1.61~~
~~Fouras *~~	~~1~~	~~2~~	~~0.074 ± 0.067~~	~~0.14 ± 0.30~~	~~-1.15~~	~~-1.12~~
~~Oléron *~~	~~2~~	~~3~~	~~0.564 ± 0.112~~	~~1.12 ± 1.06~~	~~-0.13~~	~~-0.17~~
~~Arcachon~~	~~1~~	~~2~~	~~0.095 ± 0.084~~	~~0.17 ± 0.35~~	~~-1.16~~	~~-0.92~~
~~Thau~~	~~22~~	~~6~~	~~0.342 ± 0.098~~	~~2.52 ± 1.76~~	~~-2.50*~~	~~-0.42~~
~~**Sites in presence of invasive**~~	~~**11**~~	~~**9**~~	~~**0.247 ± 0.0535**~~	~~**0.57 ± 0.65**~~	~~**-2.10***~~	~~**-9.42***~~
~~**Sites in absence of invasive**~~	~~**31**~~	~~**22**~~	~~**0.399 ± 0.3976**~~	~~**10.04 ± 5.52**~~	~~**0.22**~~	~~**-0.06**~~
~~**Sites in absence of invasive (without Thau)**~~	~~**16**~~	~~**16**~~	~~**0.179 ± 0.0368**~~	~~**0. 38 ± 0. 51**~~	~~**-2.38***~~	~~**< -10***~~
~~**All populations**~~	~~**37**~~	~~**29**~~	~~**0.356 ± 0.0325**~~	~~**7.63 ± 4.20**~~	~~**-0.81**~~	~~**-4.97**~~
Sampling sites	**S**	**H**	***He* ± SD**	**π ± SD (x10^{-3})**	**Tajima's *D***	**Fu's *Fs***
Blainville	1	2	0.065 ± 0.059	0.12 ± 0.28	-1.14	-1.24*
Chaussey	3	4	0.230 ± 0.110	0.44 ± 0.58	-1.73	-3.08*
Saint Malo	1	2	0.091 ± 0.081	0.17 ± 0.34	-1.16	-0.96

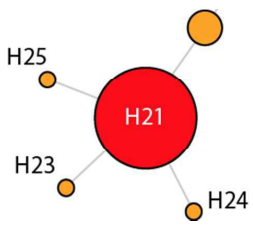
Saint Quay	0	1	0	0	0	0
Trébeurden	5	5	0.377 ± 0.122	0.90 ± 0.89	-1.83	-2.80*
Crozon	2	3	0.316 ± 0.118	0.60 ± 0.70	-0.86	-0.87
Le Croisic	4	4	0.333 ± 0.124	0.81 ± 0.84	-1.67	-1.74
Morbihan	0	1	0	0	0	0
Bourgneuf *	2	2	0.083 ± 0.075	0.30 ± 0.47	-1.51	-0.19
Loix *	5	5	0.270 ± 0.109	0.65 ± 0.72	-2.01*	-3.57*
Aytré *	4	4	0.363 ± 0.131	0.89 ± 0.89	-1.64	-1.61
Fouras *	1	2	0.074 ± 0.067	0.14 ± 0.30	-1.15	-1.12
Oléron *	2	3	0.564 ± 0.112	1.12 ± 1.06	-0.13	-0.17
Arcachon	1	2	0.095 ± 0.084	0.17 ± 0.35	-1.16	-0.92
Thau	22	6	0.342 ± 0.098	2.52 ± 1.76	-2.50*	-0.42
Sites in presence of invasive	**11**	**9**	**0.247 ± 0.0535**	**0.57 ± 0.65**	**-2.10***	**-9.42***
Sites in absence of invasive	**31**	**22**	**0.399 ± 0.3976**	**10.04 ± 5.52**	**0.22**	**-0.06**
Sites in absence of invasive (without Thau)	**16**	**16**	**0.179 ± 0.0368**	**0. 38 ± 0. 51**	**-2.38***	**< -10***
All populations	**37**	**29**	**0.356 ± 0.0325**	**7.63 ± 4.20**	**-0.81**	**-4.97**

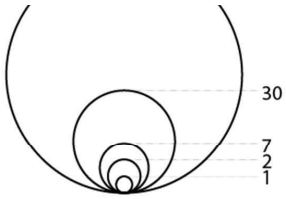
Table 3. Pairwise Φ_{ST} values calculated using the Kimura 2 Parameter model of nucleotide substitution. Only pairwise comparisons involving the population of Thau are statistically significant after sequential Bonferroni correction ~~(p=0)~~.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. Blainville	0														
2. Chausey	0.0048	0													
3. Saint Malo	0.00242	-0.00244	0												
4 . Saint Quay	-0.01001	-0.00356	-0.00207	0											
5. Trebeurden	0.02194	0.01045	0.00921	0.01207	0										
6. Crozon	0.05037	0.02772	0.03452	0.04529	0.02682	0									
7. Le Croisic	-0.03001	-0.01442	-0.01577	-0.02118	-0.01654	-0.01135	0								
8. Morbihan	0.03708	0.03371	0.03067	0	0.02156	0.00476	0.01543	0							
9. Bourgneuf	0.00458	-0.00021	-0.00102	-0.00181	0.01083	0.0316	0.01598	-0.03274	0						
10. Loix	0.00242	-0.0008	0.00559	0.00747	0.00258	0.02217	0.012	-0.0358	-0.01337	0					
11. Aytré	0.03811	0.01838	0.02156	0.0293	0.00956	0.03281	0.02045	-0.01049	-0.00555	-0.01096	0				
12. Oleron	0.28743	0.18725	0.23275	0.28709	0.1342	0.16138	0.13706	0.18898	0.15611	0.12163	0.04004	0			
13. Fouras	0.0004	-0.00162	-0.00096	-0.00611	0.01656	0.04351	0.02394	-0.03502	-0.00196	-0.00096	-0.03104	0.26425	0		
14. Arcachon	0.0032	-0.00323	0.00014	0.00443	0.00763	0.03266	0.01404	0.02941	-0.00153	-0.00658	0.01955	0.22606	0.00145	0	
15. Thau	0.96208	0.95559	0.95617	0.95856	0.94988	0.95236	0.95001	0.94928	0.95614	0.9547	0.9484	0.94167	0.95959	0.95541	0

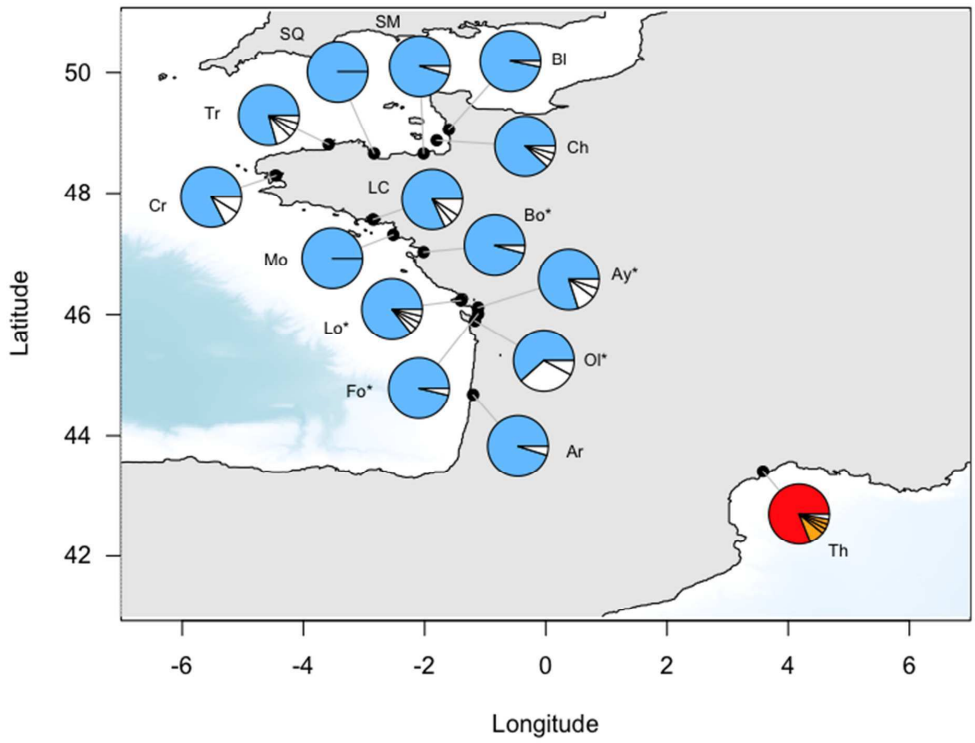
Fig~~ure~~ 1. Median-joining haplotype network. Each circle represents a haplotype, which frequency is proportional to circle diameter (legend: bottom right). Distances between haplotypes are proportional to the number of mutation events (see text). The Atlantic haplogroup contains one Mediterranean population, represented by haplotype H26 (marked with **).







Fig~~ure~~ 2. Distribution of haplotype frequencies along the French coast. Abbreviations for site names are detailed in Table 1. Haplotype colors correspond to the colors used in Figure 1. The stations where both *Ocenebra~~.~~ erinacea* and ~~*O. inornatus*~~*Ocenebra inornata* were observed are marked with an asterisk. Map constructed with R package marmap (Pante & Simon-Bouhet 2013).



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