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Mitochondrial and Nuclear DNA Analysis of Genetic Heterogeneity Among Recruitment Cohorts of the European Flat Oyster *Ostrea edulis*

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Abstract:

Marine species with high fecundity and high early mortality may also have high variance in reproductive success among individuals due to stochastic factors, making successful reproduction a "sweepstakes." In some cases, the impact is sufficient to reduce the effective number of breeders in wild populations. We tested two predictions of the sweepstakes reproductive success hypothesis in a French Atlantic population of the European flat oyster, *Ostrea edulis*, by evaluating (1) whether individuals belonging to temporally discrete recruitment cohorts within a single reproductive season displayed reduced genetic variation relative to the entire adult population, and (2) whether these temporal cohorts of recruits were genetically differentiated from each other. We assayed genetic variation at four nuclear microsatellites and a 12S mitochondrial fragment in four recruitment cohorts. Nuclear markers provided no evidence for differentiation between recruitment cohorts and adults or between temporal cohorts. However, mitochondrial data indicate that the first temporal cohort showed significant differentiation with the last (Fst = 0.052, P < 0.05) and with the adult sample (Fst = 0.058, P < 0.05). These differences are most likely due to the smaller effective size of the mitochondrial genome—and hence its increased sensitivity to drift compared to the nuclear genome. This slight mitochondrial signal indicates a certain limitation in the number of contributing female parents in this species. The "sweepstakes" phenomenon was therefore limited in our case. Hypothetically, this phenomenon may occur or not, with a high variance as a result of the interaction between the oyster reproductive biology and different environmental conditions.

Introduction

Marine species with high fecundity and high early mortality are prone to high variance in reproductive success, in some cases sufficient to limit the effective number of breeders. As a result, the effective size of a population (N_e) in such species is almost always markedly smaller than the census number (N). This was demonstrated in a naturalized population of the Pacific oyster, *Crassostrea gigas*, by Hedgecock and co-workers on the basis of long-term temporal changes in allelic frequencies in Dabob Bay (Washington, USA) (1994) and confirmed by Li and Hedgecock (1998) using larval samples from the same population. Both studies support the hypothesis that larvae are produced by "relatively few parents" and if such differential reproductive success among potential parents is stochastic, then reproduction in free-spawning species may be viewed as a lottery or sweepstakes (Hedgecock, 1994; Hedrick, 2005). Depending upon the duration of the reproductive season and synchronicity of spawning, it is also possible that temporal cohorts during a single reproductive period experience similar levels of genetic drift. If so, one would predict that temporal cohorts of recruits are genetically distinct because they are produced by different, small random samples of the breeding population. It might be specially the case for non-native species when introduced in areas where environmental conditions can be limiting for their reproduction, as for the Pacific oyster in western North America, where natural recruitment is limited to a few locations due to low seawater temperature (Ruesink *et al.*, 2005).

Recent studies have found evidence for significant genetic differentiation among temporal pulses of recruitment (Selkoe *et al*., 2006; Lee and Boulding, 2007). However, although evidence has accumulated to support the sweepstakes hypothesis marine fish and invertebrates (Hedgecock, 1994; Hauser *et al.,* 2002; Turner *et al.,* 2002; Árnason, 2004; Hoarau *et al.,* 2005; see Hedgecock *et al*., 2007, for review), numerous studies of temporal genetic variation

in recruitment find no or very limited evidence of differentiation among cohorts. For example, in a study of kinship relationships among larvae, Avise and Shapiro (1986) did not detect any subdivision among cohorts of the serranid reef fish *Anthias squamipinnis*, which represented a random sample of progeny from many matings, and neither did Herbinger *et al.* (1997) in a study of kinship among larvae of the cod *Gadus morhua*. Tseng *et al.* (2003) found no significant differentiation among temporal cohorts of the Japanese eel, although they did find that effective population size was declining, and interpreted this as implicating large-scale events such as oceanographic changes. Flowers *et al.* (2002) found no evidence of differentiation between four annual cohorts spanning 7 years of the sea urchin *Strongylocentrotus purpuratus,* although computer simulations indicated that their sampling strategy had sufficient statistical power to detect large variances in reproductive success. However, in another sea urchin species, *Strongylocentrus franciscanus*, Moberg and Burton (2000) showed that samples of recruits differed significantly from adult samples collected at the same locale, and that among-year variation was substantial. Lenfant and Planes (2002) detected significant divergence between temporal cohorts of the marine fish *Diplodus sargus*, despite very low values of *Fst*, and proposed that these cohorts were mixtures of families assembled during the pelagic stage or later during recruitment. Laurent and Planes (2007) observed that populations of sardines, *Sardina pilchardus*, have weak but significant genetic differences between year-classes. Rhodes *et al.* (2003) also detected significant differences in allele frequency between successive years in a population of camouflage groupers, *Epinephelus polyphekadion*, in the western central Pacific, possibly related to genetic variation among cohorts or between local spawning groups. Arnaud-Haond *et al.* (2008) studied the genetic structure of the pearl oyster *Pinctada margaritifera* in French Polynesia at different spatial scales in relation to the collection of seed for aquaculture production (Arnaud *et al.,*

2003). They showed that pearl oyster populations may exhibit patterns of chaotic genetic patchiness at local scale.

Variance in individual reproductive success among parents has also been documented under experimental conditions using controlled crossing (*e.g.,* Hedgecock and Sly, 1990; Hedgecock *et al.*, 1992, and references therein; Petersen *et al.*, 2008). The most direct evidence comes from studies of the Pacific oyster, *Crassostrea gigas*, in which changes in family representation in progenies resulting from factorial crosses were investigated using microsatellites markers for parentage analyses (Boudry *et al.*, 2002; Taris *et al.*, 2006). Their results showed large variance in parental contributions at several developmental stages, leading to a strong reduction of experiment-wide effective population size that could be attributed to three main factors: gamete quality, sperm-egg interaction, and sperm competition and differential viability among families.

The European flat oyster (*Ostrea edulis* L.) is a marine bivalve whose natural geographical distribution ranges along the European Atlantic coast from Norway to Morocco as well as in the Mediterranean and Black Seas. Studies of allozymes, microsatellites, and mitochondrial differentiation over the whole range (Saavedra *et al.*, 1995; Launey *et al.*, 2002; Diaz-Almela *et al.*, 2004) concluded that, although overall differentiation was small, Mediterranean and Atlantic populations exhibit significant genetic divergence and a pattern of isolation-by-distance. In Northern European populations, Sobolewska and Beaumont (2005) observed low genetic subdivision using microsatellites markers and suggested that human-mediated transports of oysters played a significant role in the observed pattern. However, the average mitochondrial haplotype diversity was highly variable among populations, as a consequence of smaller effective population sizes in some locations (Diaz-Almela *et al.*, 2004). In addition, the same study found a 10-fold quantitative difference in *Fst* between the

mitochondrial and the nuclear genomes that could be due to an unbalanced sex ratio, sex-biased variance in reproductive success, or both. Moreover, individuals that settled on a collector in the Mediterranean during a 2-week period in late summer 1993 (Hedgecock *et al.*, 2007) harbored significantly less genetic variation than the local adult population; those individuals had a mean number of alleles per locus of 13.7 compared to 23 in the adults. Estimated from this reduction in the number of alleles, the number of parents that contributed to this cohort was less than 50.

In this context, we wanted to test two components of the sweepstakes reproductive success hypothesis using a larger dataset: (1) would recruitment cohorts of flat oysters show reduced genetic variation relative to the adult population in the Atlantic portion of their range, and (2) would successive cohorts of recruits be genetically differentiated. To this end, we collected four temporally separate recruitment cohorts over the reproductive period in a single French Atlantic population and assayed their levels of genetic variation and differentiation using four nuclear microsatellites and a 12S mitochondrial fragment.

Materials and Methods

Juvenile and adult sampling

We deployed collectors throughout the summer of 2001 to collect cohorts of juveniles in Baie de Quiberon, Brittany, on the Atlantic coast of France (Fig. 1)[figc1]. We use the term "cohort" to refer to a group of newly settled juveniles collected during a given period of time. Three sets of collectors were deployed every 2 or 3 weeks, with the first set (cohort 1) from 18 June to 3 July, the second from 3 July to 16 July (cohort 2), and the third from 16 July to 13 August (cohort 3). Another set of collectors was left during the whole recruitment period and constituted the seasonal cohort (Fig. 1). When removed from the field site, all collectors were

transferred to raceways at the Ifremer research station in La Tremblade until the animals were large enough to be sampled (about a 4-week period). Raceways were provided with sand-filtered seawater to prevent contamination of the collectors by local larvae. Mortality was recorded in the raceways and remained at a very low level that was not different between the cohorts and might not account for potential differences in marker frequencies. In total, we sampled 672 animals: 167 in cohort 1, 198 in cohort 2, 115 in cohort 3, and 192 in the seasonal cohort. We also collected 68 adults in the same area from 26 June 2001 to 8 August 2001 and preserved gill tissue in alcohol for DNA extraction.

Microsatellite amplification

We used four previously published microsatellite loci: *Oedu* T5, *Oedu* J12, *Oedu* H15, and *Oedu* U2 described in Launey *et al.* (2002). Primers were synthesized by MWG Biotech with each forward primer labeled with IRD-700 or IRD-800, and PCR reactions were performed in 10-µl reactions containing 5 µl template DNA (phenol-chloroform extracted), 50 mmol l^{-1} $MgCl_2$, 2 mmol l^{-1} dNTPs, 1 pmol l^{-1} each primer, 1 unit of Goldstar Licensed Polymerase (Eurogentec), and 1× polymerase buffer (supplied by the manufacturer). Amplifications were performed on a MJ Research PTC-200 DNA Engine thermal cycler as follows: pre-denaturation (95 °C, 5 min) followed by 30 cycles of denaturation-annealing-polymerization (95 °C, 20 s; T_a, 20 s; 72 °C, 20 s) and a final elongation step (72 °C, 3 min). Fragment sizes were determined by electrophoresis on 6.5% polyacrylamide gels and run at 2000 V, 35 mA, at 50 °C on a LICOR DNA sequencer (model 4200, Lincoln, NE). Genotypes were determined by comparison with samples of known fragment length using the Gene Profiler 4.0 software.

Mitochondrial analysis

We amplified a 313-bp fragment from the mitochondrial 12S rRNA gene for subsequent sequencing using previously developed primers12SOeduF and 12SOeduR (Diaz Almela *et al.,* 2004). Amplification was performed with 2.5 mmol l^{-1} $MgCl_2$, 200 μmol l^{-1} of each dNTP, 1 μmol l^{-1} of each primer, 0.7 U of Silverstar Taq polymerase (Eurogentec, Seraing, Belgium), and 10× PCR reaction buffer (Eurogentec, Seraing, Belgium). PCR was carried out with an initial denaturation step at 95 °C (5 min), followed by 30 cycles of amplification (denaturation at 95 °C for 1 min, annealing at 54 °C for 1 min, and elongation at 72 °C for 1 min) and a final elongation step at 72 °C for 7 min. The PCR products were purified with a High Pure PCR Product purification kit (Boehringer-Mannheim, Germany). Sequencing reactions consisted of an initial denaturation step (2 min, 92 °C) followed by 30 cycles (30 s, 95 °C, denaturating; 30 s, 50 °C, annealing; 1 min, 72 °C, elongation), performed with the Sequitherm EXCEL II DNA sequencing kit-LC (Epicentre Technologies). The fragments were separated on a Li-Cor 4200 automated DNA sequencer. All the novel sequences were submitted to the EMBL nucleotide sequence database.

Data analysis

Nuclear genetic polymorphism within each cohort was measured as the observed (H_o) and unbiased expected (He_{nb}) heterozygosities at the four microsatellite loci. To compare the genetic variability between the cohorts, we used a Bayesian procedure developed by Belkhir *et al.* (2006) that compares the gene diversity of two samples, incorporating the sampling theory of Ewens (1972). In this procedure, the allele-frequency spectrum of a spawning population is reconstructed from the allele-frequency spectrum of the sampled cohort produced by this

population. For each cohort, pseudosamples are simulated from which posterior distributions and credibility intervals for expected heterozygosity can be generated and can be compared statistically using a Student's *t*-test under the approximation of normality. We also estimated locus-specific allelic richness (*Rs*) for each cohort, using rarefaction to correct for unequal sizes (El Mousadik and Petit, 1996) with the program FSTAT ver. 2.9.3 (Goudet, 1995), and tested for significant differences using a Friedman test (Proc Freq, SAS/STAT Software, SAS Institute Inc., 1999). In addition, we calculated Wright's *F*-statistics (1951) according to Weir and Cockerham (1984), tested for significant deviations from Hardy-Weinberg expectations in each cohort (F_{is}), and tested for significant overall and pairwise genetic differentiation (*Fst)*, using permutation tests (1000 permutations) implemented in the package Genetix ver. 4.1 (Belkhir *et al.*, 1996–2004).

We also estimated the locus-specific frequency of null alleles using three methods, based on heterozygote deficiency (Chakraborty *et al.*, 1992), the frequency of non-amplified individuals (*i.e.,* homozygotes for the null allele; Brookfield, 1996), and a maximum likelihood approach developed by Kalinowski and Taper (2006), all of which are implemented in ML-Relate (Kalinowski *et al.*, 2006).

To investigate the family structure, we assigned all possible pairs of individuals to relatedness categories (*i.e.,* unrelated, half sibs, full sibs, parent/offspring) using the maximum likelihood approach implemented in ML-Relate (Kalinowski *et al.*, 2006). We tested for difference in the frequencies of relatedness categories between groups using contingency table analysis (PROC FREQ, SAS/STAT Software, SAS Institute Inc., 1999).

We identified mitochondrial 12S rRNA haplotypes in our 12S rRNA sequences by aligning them using ClustalW (Thompson *et al.*, 1994). We then estimated Nei's unbiased haplotype diversity H_{nb} (Nei, 1987) and tested for differentiation between populations using

Wright's fixation index *Fst* (Wright, 1951) estimated as θ (Weir and Cockerham, 1984) and computed with the package Genetix ver. 4.1 (Belkhir *et al.*, 1996–2004).

Variance effective population size was calculated according to Waples (1989) from temporal changes in allele frequencies at microsatellite and mitochondrial loci using NeEstimator 1.3 (Peel *et al.*, 2004).

Results

As previously reported for microsatellite markers in oysters, diversity parameters were very high (Table 1)[tabc1]. Allelic richness per locus ranged from 20 (adults) to 22 (cohort 2), with the exception of locus H15, which always exhibited allelic richness below 16. We found no significant differences in allelic richness between cohorts ($\chi^2 = 0.89$, $P = 0.99$). Expected heterozygosity H_{nb} varied from 0.909 to 0.944 for loci T5, J12, and U2, and from 0.816 to 0.886 for locus H15. Using the rarefaction procedure of Belkhir *et al.* (2006), we found no significant difference in heterozygosity between the adult sample recruitment cohorts. We did, however, find significant deviations from Hardy-Weinberg expectations (*Fis*) in all cohorts when we used a multilocus analysis ($P < 0.001$ after Bonferroni correction, Table 1). Single locus tests revealed, however, that only H15 exhibited significant *P* values for all groups. Table 3 presents the frequencies of null alleles for each locus. In loci T5, J12, and U2, the values of "r" ranged between 0% and 7% according to the method of Chakraborty *et al.* (1992) and between 5% and 13% according to the method of Brookfield (1996). The estimates of null allele frequencies in locus H15 were higher, with an average of 11% according to Chakraborty and 17% according to Brookfield. The maximum likelihood resulted in estimates of null allele frequencies close to those obtained with Chakraborty's method. Our analysis of pairwise

kinship categories between all pairs of individuals revealed a similar family structure between cohorts (χ^2= 0.96, P = 0.99) (Table 5), with most pairs categorized as unrelated (from 83.17% in cohort 1 to 85.93% in adults).

The number of mitochondrial 12S RNA fragments sequenced for each group ranged between 37 and 42. Sequencing of these fragments identified 10 haplotypes: 5 had been previously observed and were named A, B, C, F, and I (Diaz-Almela *et al.*, 2004), and 5 were new haplotypes that we named Z1 through Z5. These new haplotypes differ from the B haplotype by one (Z1) or two (Z2, Z3, Z4, and Z5) base pairs. In all cohorts, the "A" haplotype was most common, as previously found in the Atlantic Ocean by Diaz-Almela *et al.* (2004). Average mitochondrial haplotype diversity (H_{nb} = 0.26 ± 0.05) was much lower than microsatellite diversity (H_{nb} = 0.909 ± 0.003). Based on nuclear markers, pairwise comparisons of *Fst* among cohorts indicated no differentiation between cohorts or between adults and any cohorts. In contrast, *Fst* values for mitochondrial data found significant differentiation between cohort 1 and both the seasonal cohort and the adult sample (Table 4).

According to the method of Waples (1989) using NeEstimator, nuclear microsatellite markers estimated *Ne* for the adults to cohort 1 interval as 231 [68 - +∞]), for the adults to cohort 2 interval as 304 [88 - +∞]), for the adults to cohort 3 interval as 601 [102 - +∞]), and the adults to seasonal cohort interval as 458 [97 - +∞]). For the mitochondrial data, *Ne* estimates for the adults to seasonal cohort interval was 29 [3 - +∞]).

Discussion

Although there are a wealth of published studies on molecular population genetics in marine organisms, most represent a single "snapshot" in time, with individuals often of mixed

ages sampled once. Most do not include historic samples, and thus they implicitly assume temporal genetic stability (Waples, 1998). However, recent studies have found evidence for significant genetic differentiation among temporal pulses of recruitment, for example in *Paralabrax clathratus,* the kelp bass (Selkoe *et al*., 2006), or *Littorina keenae,* the eroded periwinkle (Lee and Boulding, 2007). Hence, the lack of temporal stability in genetic diversity may indicate that subpopulations experience severe fluctuations in effective population size (*e.g.,* Nielsen *et al.*, 1997). Some studies indicate that variance in reproductive success is large enough to limit effective population numbers to a small fraction of the actual abundance. Hedgecock (1994) described this phenomenon as a "sweepstakes" (or lottery) for recruitment success in highly fecund marine organisms such as broadcast spawners, in which chance environmental events lead to high variance in offspring survival and a reduction in effective population size that has been termed the Hedgecock Effect. This high variance in recruitment success may be explained by spatial and temporal heterogeneities in the distribution of larvae that originate from distinct reproductive events involving a limited number of parents with variable allelic compositions. The European flat oyster, *Ostrea edulis,* fits well with this model since, like most of the flat oyster species, it is a brooding species, releasing the larvae after about 10 days in the palleal cavity of the female. In addition, spermatozoids have been observed to be tightly grouped in spermatozeugmata (*i.e.,* "balls" of sperm) in male gonads (Ó Foighil, 1989). Moreover, they are highly fecund, with larvae likely to be transported by oceanic currents into bays and estuaries. Those biological characteristics may be responsible for "pulses" of larvae or gametes with a relatively low intra-pulse genetic diversity and conversely high inter-pulse variability; as demonstrated by Hedgecock *et al.* (2007), these conditions can lead to genetic variation among cohorts of recruits and decreased genetic variation within cohorts relative to the adult population. However, we did not find such a

pattern in our study. On the basis of data from nuclear microsatellite markers, genetic variation within temporal cohorts (allelic richness and heterozygosity) was not significantly different from that in the adult population. In addition, pairwise *Fst* estimates between recruitment cohorts was not significant, and our estimate of the number of effective breeders (*Ne*) from the adult population that contribute to the next generation (represented by the seasonal cohort) was large (*Ne* = 458). However, the confidence intervals that we obtained for *Ne* estimates show that they are not reliable.

Turning to the mitochondrial data, we found two significant pairwise values of *Fst*—between cohort 1 and the seasonal cohort, and between cohort 1 and the adults. One possible explanation is that maternal transmission makes mitochondrial polymorphisms more sensitive to genetic drift (Diaz Almela *et al.*, 2004), but it is also possible that this result reflects higher variance in reproductive success for females than males. This is partly responsible for the 16-fold lower estimate of *Ne* for the mitochondrial data (*Ne* = 29) than the nuclear data (*Ne* = 458). In addition to the intrinsic specificities of cytoplasmic markers (haploid nature), life-history elements could reduce female effective population size and thus mitochondrial *Ne*. Le Dantec and Marteil (1976) showed that female gonad development is slower. This may reflect an important energy cost in the development of ovocytes and could induce a lower probability of becoming female. Indeed, the sex ratio within a reproductive season is male-biased (up to a 3:1 ratio). Moreover, *O. edulis* is a protandrous species. The action of the parasite *Bonamia ostreae,* which is known to induce high mortality within 2- and 3-year-old adults (Culloty and Mulcahy, 1996), could influence the proportion of individuals that achieve to reproduce as females in a population. From a methodological standpoint, these

results warrant the use of mtDNA in future studies, due to its greater power to detect limitations in the number of spawning females.

Unlike the case of sweepstakes reproductive success Hedgecock *et al.* (2007) reported for flat oyster juveniles in the Mediterranean, the present study showed no evidence of such a strong signal . We did, however, detect significant differentiation between the seasonal cohort and cohort 1 and between cohort 1 and the adult population, thus showing that the number of contributing parents, especially females, is limited in this species. Additional empirical examples are required to document the phenomenon, then generate larger database to draw broad-scale conclusions. The mtDNA differences between the seasonal cohort and cohort 1 might reflect haplotypic differences in spawning time. This could be tested with multi-year data. Indeed, more temporal sampling is needed to rule out temporal assortative mating, in which genetically differentiated populations spawning at different times during the spawning season might account for the observations (Hendry and Day, 2005).

Overall, our study suggests that the stochastic "sweepstake" phenomenon might be itself a lottery, occurring or not with a high variance as a result of the interaction between the oyster's reproduction biology and different environmental conditions.

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Table 1 Population genetics parameters estimated in the different samples studied

Microsatellite locus	Parameters	Adults *n* = 68	Cohort 1 *n* = 167	Cohort 2 *n* = 198	Cohort 3 *n* = 115	Seasonal cohort *n* = 192
OeduT5	N_i	64	162	192	108	174
	A	21.84	25.09	21.34	22.66	21.52
	Ho	0.859	0.864	0.859	0.889	0.885
	He_{nb}	0.908	0.925	0.906	0.921	0.902
		[0.871–0.922]	[0.892–0.937]	[0.878–0.917]	[0.887–0.932]	[0.860–0.920]
	F_{is}	0.062 ns	0.066^{*}	0.056^{**}	0.037 ns	0.026 ns
OeduJ12	N_i	62	164	191	107	176
	A	21.00	21.13	24.04	23.01	23.56
	Ho	0.807	0.878	0.848	0.897	0.869
	He_{nb}	0.911	0.907	0.924	0.923	0.924
		[0.869–0.927]	[0.871–0.929]	[0.893–0.937]	[0.888–0.934]	[0.882–0.939]
	F_{is}	0.123^{**}	0.036 ns	0.086^{**}	0.033ns	0.066^{**}
OeduH15	N_i	64	155	184	106	168
	A	13.91	15.13	15.97	14.74	15.4
	Ho	0.641	0.677	0.728	0.698	0.696
	He_{nb}	0.811	0.851	0.883	0.87	0.865
		[0.721–0.871]	[0.786–0.873]	[0.848–0.900]	[0.824–0.892]	[0.807–0.889]
	F_{is}	0.216^{**}	0.211^{**}	$0,178^{**}$	0.196^{**}	0.192^{**}
OeduU2	N_i	67	158	190	109	179
	A	24.7	23.22	26.29	24.7	25.61
	Ho	0.836	0.892	0.926	0.908	0.844
	He_{nb}	0.928	0.932	0.931	0.937	0.939
		[0.879–0.945]	[0.901–0.939]	[0.892–0.940]	[0.910–0.941]	[0.915–0.943]
	F_{is}	0.109^{**}	0.043^{*}	0.008 ns	0.033 ns	0.106^{**}
Multilocus	A	20.36	21.14	21.91	21.28	21.52

Ho	0.786	0.828	0.841	0.848	0.824
He_{nb}	0.884	0.895	0.904	0.904	0.899
	[0.863–0.904]	[0.877–0.908]	[0.890–0.915]	[0.891–0.915]	[0.887–0.913]
F_{is}	0.125**	0.087**	0.081**	0.073**	0.096**

Sample sizes (*n*) for adults and temporal cohorts, number of amplified samples (N_i), allelic richness (A), observed (H_o) and expected (He_{nb}) heterozygosity (followed by the confidence interval), and F_{is} estimates according to Weir and Cockerham (1984). Comparison between Fis estimates and 0 allow testing for the deviation from Hardy-Weinberg expectations in each cohort. F_{is} values are followed by a significance test based on 1000 permutations; (ns) corresponds to nonsignificant values of *P*,

* of $P < 0.05$ and

** $P < 0.001$ after Bonferroni correction on locus.

Table 2 Frequencies of null alleles estimated by three methods for each locus

Locus	Sample	N	Method reference*		
			rC	rB	rM
T5	Adults	0.06	0.03	0.09	0.03
	Cohort 1	0.03	0.03	0.06	0.03
	Cohort 2	0.03	0.03	0.06	0.02
	Cohort 3	0.06	0.02	0.08	0.03
	Seasonal cohort	0.09	0.01	0.10	0.00
J12	Adults	0.09	0.07	0.13	0.06
	Cohort 1	0.02	0.02	0.05	0.01
	Cohort 2	0.04	0.05	0.08	0.04
	Cohort 3	0.07	0.02	0.08	0.01
	Seasonal cohort	0.08	0.03	0.10	0.04
H15	Adults	0.06	0.12	0.16	0.12
	Cohort 1	0.07	0.12	0.16	0.12
	Cohort 2	0.07	0.10	0.15	0.10
	Cohort 3	0.08	0.11	0.16	0.10
	Seasonal cohort	0.13	0.11	0.19	0.11
U2	Adults	0.02	0.06	0.07	0.05
	Cohort 1	0.05	0.02	0.07	0.02
	Cohort 2	0.04	0.00	0.05	0.01
	Cohort 3	0.05	0.02	0.07	0.01
	Seasonal cohort	0.07	0.06	0.10	0.06

* rC, Chakraborty *et al.* (1992); rB, Brookfield (1996); rM, Kalinowski and Taper (2006).

N, percentage of non-amplified samples.

Table 3 Percentage of four common relationship categories for each group

Relationship	Adults	Cohort 1	Cohort 2	Cohort 3	Seasonal cohort
Unrelated	85.93	83.17	83.33	83.52	83.73
Half Sibs	11.22	14.18	14.35	13.68	12.97
Full Sibs	1.49	1.54	1.65	1.54	1.67
Parent/Offspring	1.40	1.36	1.11	0.68	1.26

For each group, a list of relationship between two individuals has been estimated from the microsatellite data. Only the relationship with the highest likehood has been considered.

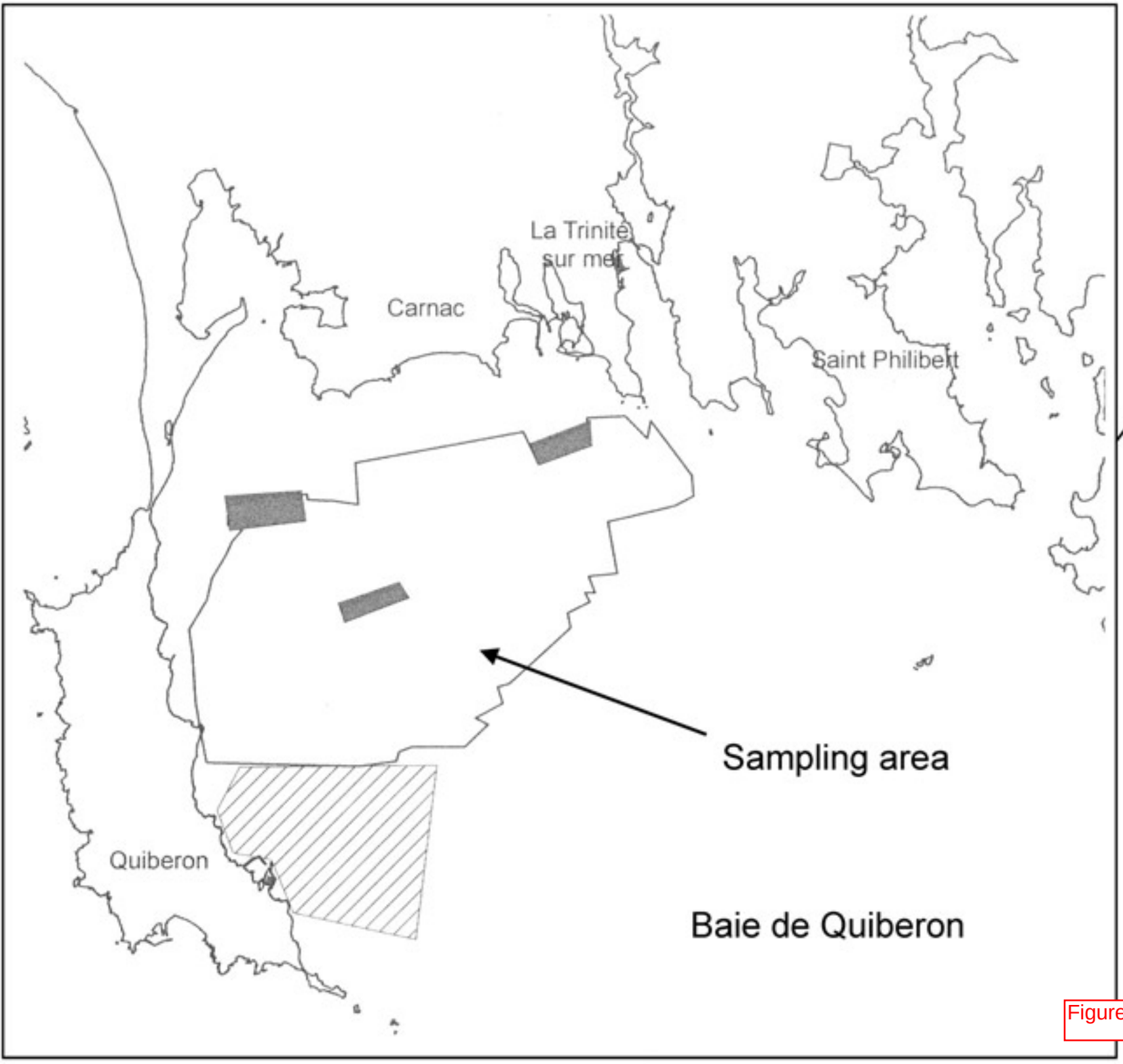
Table 4 Number of the 12S haplotypes obtained in each cohort and the adults with related pair base position and expected (H_{nb}) haplotypic diversity

						Pair base position										
Haplotypes	Adults 42	Cohort 1 43	Cohort 2 37	Cohort 3 39	Seasonal cohort 40	76	121	137	139	144	146	153	158	160	164	262
B	7	1	2	3	7	G	G	C	T	G	C	T	C	A	G	T
A	31	39	34	35	31	A	–	–	–	–	–	–	–	–	–	–
C	1	1	1	1	0	A	A	–	C	–	–	C	–	C	–	C
F	1	0	0	0	0	–	–	–	–	A	–	–	–	–	A	–
I	1	0	0	0	0	A	A	–	C	–	–	C	–	G	–	C
Z1	1	0	0	0	0	–	A	–	–	–	–	–	–	–	–	–
Z2	0	0	0	0	1	A	–	–	–	–	–	–	–	–	–	C
Z3	0	0	0	0	1	A	–	T	–	–	–	–	–	–	–	–
Z4	0	1	0	0	0	A	–	–	–	–	–	–	T	–	–	–
Z5	0	1	0	0	0	A	–	–	–	–	T	–	–	–	–	–
H_{nb}	0.43	0.18	0.15	0.19	0.37											

Table 5 *Fst* values for pairwise comparison based on mitochondrial 12S rRNA and microsatellite markers (in italic)

	Cohort 2	Cohort 3	Seasonal cohort	Adults
Cohort 1	–0.019	–0.014	**0.052***	**0.058***
	0.000	*0.001*	*0.000*	*0.001*
Cohort 2		–0.024	0.041	0.049
		–0.001	*0.002*	*0.002*
Cohort 3			0.021	0.029
			0.002	*0.002*
Seasonal cohort				–0.019
				0.000

Significance was tested with 1000 permutations indicated with an asterisk. Significant values are in bold.



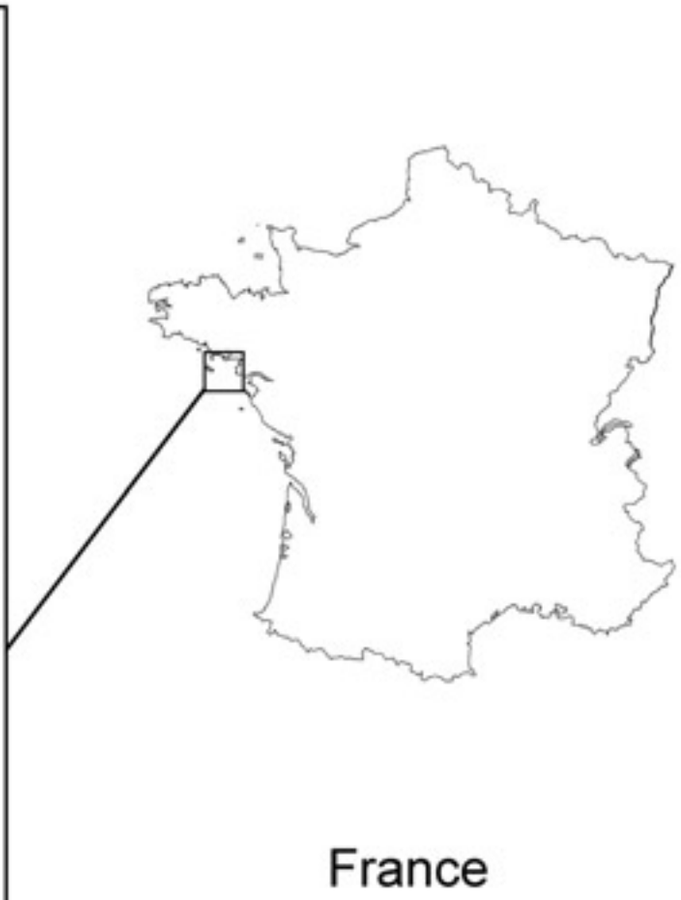


Figure 1. Sampling area in the Baie de Quiberon.