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1 **Mitochondrial gene genealogies provide evidence for multiple**
2 **origins of recently recorded *Cyclope neritea* (Nassariidae)**
3 **populations in France**

4

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1 **ABSTRACT**

2

3 Since the 1970's, the nassariid gastropod *Cyclope neritea* has extended its

4 previously recognized natural range by colonizing the French Atlantic coasts. Since French

5 populations have gradually spread northwards at the edge of its native range, a natural spread

6 due to recent warming of the North-Eastern Atlantic was hypothesized. However, given the

7 low dispersal ability of the species and its location in the vicinity of shellfish culture sites,

8 human-mediated introductions appeared as an alternative vector. In order to address the

9 relevancy of both hypotheses (i.e. natural spread or human-mediated introductions), a

10 comprehensive study of the six recently colonized French bays along with two populations

11 located in the recognized native range of the species was carried out. Based on mitochondrial

12 gene sequences (Cytochrome Oxidase I, 262 individuals), phylogenetic reconstruction

13 revealed 22 haplotypes split into several evolutionary clades. The appearance of dense and

14 sustainable French populations appeared to be mainly due to recurrent human-mediated

15 introductions from various genetically differentiated sites in the natural range. The large

16 within-population genetic variation due to genetic admixture appears to have contributed to

17 the successful settlement of *C. neritea* in France. Nevertheless, a process of isolation by

18 distance suggested that *C. neritea* may have been present but unnoticed for a long time on the

19 French Atlantic coasts. Altogether, since the natural or introduced status for French Atlantic

20 populations is not straightforward, *C. neritea* can be classified as a cryptogenic species. Such

21 a category is of particular interest when dealing with species close to their recognized native

22 range.

INTRODUCTION

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In marine habitats and especially in coastal areas, the rate of biological invasions has significantly increased in the last few years (Williamson, 1996), inducing economical and social risks (Gouletquer, 1998; Grosholz, 2002). Moreover, introduction of non-indigenous species (NIS) is identified as the second most important cause (after habitat destruction by human activities) of the loss of biological diversity (Carlton, 1996, Coleman & Williams, 2002; Mooney & Cleland, 2001; Palumbi, 2001). However, the study of biological introductions in coastal systems has only really begun a little more than twenty years ago (Carlton, 1996; Grosholz, 2002) and the processes that underlie biological invasions in these habitats are still not precisely identified. Marine biological invasions are known to be highly related to the increase of human-mediated transport of non-native species in ship ballast waters as well as to intentional or accidental releases for aquaculture and fisheries (Carlton & Geller, 1993; Cohen & Carlton, 1998; Zibrowius, 1983). However, fast environmental changes, by altering native communities, may also favour settlement of NIS (Carlton, 2000; Dukes & Mooney, 1999). Habitat alterations or climate changes may weaken competitive ability of indigenous species, consequently facilitating the settlement of exotic species (Sax & Brown, 2000). For instance, the temperature is known to be a critical factor influencing the natural distribution of both animal and plant species: a wide array of species has been shown to have colonized western and northern Europe from the Iberian Peninsula after the ice ages (see Comes & Kadereit 1998; Taberlet *et al.*, 1998 or Gomez & Lunt, in press for a detailed review) and Stachowicz *et al.* (2002) recently showed that changes in the maximum/minimum temperatures may have promoted the establishment of two invasive ascidians at native species' expense. Species located on both sides of a biogeographic boundary are also able to

1 extent or restrict their natural range in response to changes in environmental conditions
2 (Gaylord & Gaines, 2000).

3 In this context, the Bay of Biscay (North Eastern Atlantic) is an interesting area
4 since the mean temperature of this geographical area increased by 0.6 to 0.8 °C per decade
5 during the last 30 years (IPCC, 2001). The rapid and recent warming of the Bay of Biscay has
6 already been proved to promote displacement of mollusc species within this Bay (e.g.
7 *Macoma balthica*, Hummel *et al.*, 2000). The recent changes of the distribution area of the
8 gastropod *Cyclope neritea* might also be related to this process. This nassariid gastropod is
9 native to the Mediterranean and Black Seas, and to the Atlantic coasts of Morocco, southern
10 Spain and Portugal. Up to the 1950's only discontinuous and ephemeral populations were
11 recorded in North of Spain and South of the French Basque coasts, the Basque country being
12 defined as the northern edge of the natural range of the species (for a detailed description, see
13 Sauriau, 1991). Since 1976, new populations have been recorded along the French coasts,
14 gradually from South to North: first in Arcachon Bay, then in 1983-84, in the Marennes-
15 Oléron Bay, the Isle of Ré, and the Gulf of Morbihan, and more recently, on English Channel
16 coasts in 2000s (see references in Bachelet *et al.*, 2004). On one hand, the gradual appearance
17 of *C. neritea* populations from South to North favours the hypothesis of a natural spread
18 towards North during the past thirty years in relation to environmental changes. On the other
19 hand, a natural spread over such a short period of time seems unlikely given the limited
20 dispersal ability of this species. As a direct developer, *C. neritea* has no planktonic larval
21 stage (Gomoiu, 1964) and is subjugated to a very particular substrate (i.e. sheltered spots,
22 characterized by sandy-muddy substrates) such that the numerous discontinuities (due to
23 occurrence of rocky shores) along the southern French Atlantic coasts may slow down natural
24 migrations (Sauriau, 1991). Therefore, the appearance of *C. neritea* along the French Atlantic
25 and English Channel (FAEC) coasts may be due not to a natural process but to human-

1 mediated introductions via oyster exchanges between Mediterranean and Atlantic shellfish
2 culture sites (Boulhic & Tardy, 1986a; Le Roux *et al.*, 1988; Pigeot, 1988; Sauriau, 1991).
3 Exchanges of commercial species between distant shell-farming sites have been proved to be
4 an efficient way of primary and secondary introductions of alien species (including mollusks)
5 in new biotas (Wolff & Reise, 2002) and all the newly established French populations of *C.*
6 *neritea* were found in the vicinity of oyster beds. Indeed, focusing on Arcachon Bay (Bachelet
7 *et al.*, 2004), the first recorded site of introduction of *C. neritea*, a mitochondrial sequence
8 pattern suggestive of recurrent introductions was revealed. However, the colonization history
9 of *C. neritea* along the French coasts cannot be elucidated based on a single population.

10

11 The aim of the present study was thus to examine all the currently recorded
12 populations along the FAEC coasts to determine (i) if the French populations share common
13 mechanisms for their establishment (e.g. number of sources, number of founders etc.) and (ii)
14 the likelihood of two non-exclusive hypotheses to explain the actual distribution of *C. neritea*
15 in France, i.e. a natural spread towards North and accidental human-mediated introductions.
16 To address these questions, we combined classical population genetics analyses and an intra-
17 specific phylogenetic approach by using mitochondrial markers. Mitochondrial gene
18 genealogies are indeed useful to assess the mechanisms on which rely the settlement of
19 introduced species (Kolbe *et al.*, 2004). Moreover, because population effective size at
20 mitochondrial genes is two to four-times lower than those of nuclear markers, genetic drift
21 effects are emphasized and isolation-by-distance patterns (IBD, populations in close
22 geographical proximity are expected to be genetically closer than more distant populations,
23 Slatkin & Maddison, 1990; Wright, 1943) are expected to be more easily detected (Diaz-
24 Almela *et al.*, 2004). Given the low dispersal ability of *C. neritea*, if the gradual appearance
25 of this species along the FAEC coasts was due to natural spread, IBD pattern should be

1 observed. Conversely, in the case of random human-mediated introductions, a stochastic
2 haplotype distribution should be observed. Moreover, strong losses of genetic polymorphism
3 are generally revealed when the introduction is due to rare events from a limited number of
4 sources (see for instance introductions of Argentina ants *Linepithema humile* (Tsutsui *et al.*,
5 2000), Bennett's wallabies *Macropus rufogriseus rufogriseus* (Le Page *et al.*, 2000) or
6 Mauritian macaques *Macaca fascicularis* (Lawler *et al.*, 1995)). Conversely, if several
7 genetically differentiated source populations contributed to the settlement of NIS, the
8 introduced population is expected to show a mixture of evolutionary divergent lineages
9 (admixture), each of them resulting from distinct local evolution histories in the native range
10 (Hamblin & Veuille, 1999). These expectations were analyzed, based on a comprehensive
11 sampling of *C. neritea* along the FAEC coasts including the Arcachon Bay population as well
12 as two populations from the native range (Bachelet *et al.* 2004) by analyzing the
13 mitochondrial gene Cytochrome Oxidase I (COI, 533 base pairs) over 262 individuals.

14

MATERIALS AND METHODS

Study species and sampling. *C. neritea* is a small gastropod (about 15 mm in maximum shell width) which lives in shallow waters and/or intertidal habitats, especially in sheltered areas such as estuaries and lagoons (Morton, 1960). *C. neritea* is a direct developer, depositing encapsulated eggs (a single embryo per capsule) on hard substrata such as cockle shells. Eggs take 2-5 weeks to mature and then hatching as benthic juveniles (Boulhic & Tardy, 1986b; D'Asaro, 1993; Gomoiu, 1964; Kisch, 1950).

Sampling sites (see Table 1, Fig. 1) were chosen in each bay of the French Atlantic and Channel coasts (FAEC) where *C. neritea* has been recorded since the 1970's so that, to our knowledge, this sampling fits exactly with the actual distribution of the species in its current FAEC range, beginning at the edge of its previous recognized natural range. In addition to the sample of Arcachon Bay (Arguin, Bachelet *et al.*, 2004), five populations of *C. neritea* were sampled between March 2002 and June 2003: Hossegor (French Basque Country) where *C. neritea* was unnoticed until our sampling and located at 50 km north of Socoa where the species was recorded in 1950 (Kisch, 1950); Ronce les Bains in Marennes-Oléron Bay (Sauriau, 1989), Rivedoux in Isle of Ré (Tardy *et al.*, 1985) and Séné in Gulf of Morbihan (Le Roux *et al.*, 1988), three bays where *C. neritea* was first observed in 1983-84; and Stérec in the Bay of Morlaix (English Channel), the most northern population known on French coasts since the end of the 1990s. For each population, adult specimens were collected and stored in 95% ethanol before DNA extraction. Two populations included in the study by Bachelet *et al.* (2004), Thau lagoon (French Mediterranean Sea) and Faro (South Portugal), were used as representatives of populations from the native range.

1 **DNA extraction, amplification and sequencing.** We followed the protocol as
2 detailed in Bachelet *et al.* (2004). To summarize, total DNA was extracted from less than 15
3 mg of foot muscle using Dneasy™ Tissue Kit according to the manufacturer's protocol
4 (Qiagen, Germany). Amplification of the COI gene were performed using specific primers
5 (Bachelet *et al.*, 2004) and PCR products were directly sequenced using ABI PRISM®
6 BigDye™ Terminators v3.0 Cycle Sequencing Kit following the manufacturer's protocol
7 (Applied Biosystems). Both strands were sequenced for each individual using an ABI
8 PRISM® 3100 Automated DNA Sequencer (Perkin-Elmer Applied Biosystems, Foster City,
9 CA) and sequence data were aligned using ClustalW (Thompson *et al.*, 1994).

11 **Sequence analysis.** Over the whole dataset and for each population, genetic
12 diversity was examined by computing the number of mitochondrial haplotypes N_H , the
13 number of segregating sites S , nucleotide diversity π (i.e. average number of nucleotide
14 differences between pairs of sequences) and gene diversity H_e (Nei, 1987) by using the
15 software DnaSP (Rozas & Rozas, 1999).

16 Genetic divergence between each pair of populations was investigated by calculating
17 an estimate of the pairwise F_{ST} parameter (Wright, 1951) using Arlequin v.2.0. (Schneider *et*
18 *al.*, 2001). The statistical significance of \hat{F}_{ST} values under the null hypothesis of no
19 differentiation between populations was assessed using the permutation procedure
20 implemented in Arlequin v.2.0. Isolation-by-distance model was used to test the hypothesis of
21 a step by step dispersal of *C. neritea* from South to North. In this model, a positive correlation
22 is expected between genetic distances (expressed by Rousset (1997) in term of $\frac{\hat{F}_{ST}}{1 - \hat{F}_{ST}}$) and
23 geographic distances. To picture IBD, the genetic distance between all FAEC population pairs
24 was plotted against the geographic distance in kilometres. Correlation between matrices of

1 pairwise geographic and genetic distances was estimated using Mantel tests as implemented
2 in the software IBD 1.5 (Bohonak, 2002).

3 Three complementary analyses were used to test for the hypothesis of human-
4 mediated introductions of *C. neritea* along the FAEC coasts and to determine if several
5 populations might have contributed to these introductions. First, the link between the
6 geographical distribution of the haplotypes and their genealogical relationships was analysed
7 by building a haplotypic network: compared to tree analyses, network reconstructions are
8 particularly accurate for the study of within-species phylogeny (Posada & Crandall, 2001). A
9 median-joining algorithm performed with the software Network 4.0.0.0. (Bandelt *et al.*, 1999,
10 Cassens *et al.* 2003) was used: a minimum-spanning network was constructed and a
11 maximum parsimony algorithm was applied to simplify the complex branching pattern and
12 represent all the most parsimonious intra-specific phylogenies. Second, for polymorphic
13 populations, the distribution of the observed number of pairwise nucleotide site differences
14 (i.e. mismatch distribution) was computed in DnaSP v.3.53 (Rozas & Rozas, 1999) to
15 examine the mixing of evolutionary divergent lineages at the population level. Finally, given
16 the low resolving power of Tajima's *D*-test (Galtier *et al.*, 2000), tests for departure from
17 mutation-drift equilibrium under an infinite-site model were carried out following Depaulis &
18 Veuille (1998). This test (*K*-test) is based on the comparison of the observed haplotype
19 number (N_H) with the expected number under equilibrium (N_{EH}) given the size of the sample
20 (N) and the observed number of segregating sites (S). It detects recent population admixture
21 when testing a departure from equilibrium in the direction of too few haplotypes (see the case
22 of *Drosophila simulans*, Hamblin & Veuille, 1999). In the case of a recent admixture,
23 evolutionary divergent haplotypes are present within the same population and several
24 intermediates haplotypes are expected to be missing: for a given set of parameters (N , S), N_H
25 should thus be significantly smaller than N_{EH} (Hamblin & Veuille, 1999). A coalescent

- 1 simulation approach (program modified from a program developed and kindly provided by
- 2 M.-H. Muller) was used to compute confidence intervals and one-tailed P -values for N_H .
- 3

RESULTS

Molecular diversity over the dataset. A 533 base pair fragment of the COI gene was analyzed in each population (from 29 to 39 individuals according to the population, Table 1). Only synonymous substitutions were observed (Appendix). Over the whole dataset (N=262 individuals), 27 polymorphic sites and 22 haplotypes (GenBank accession numbers: AY789970-AY789991) were identified (Table 1, Appendix). The distribution of the 22 haplotypes is pictured in Fig. 1. Over the whole dataset, three haplotypes (A, B and C, see Fig. 1) were found at very high frequency, $f = 0.32, 0.25$ and 0.19 respectively (sum = 0.76), compared to the others ($0.004 < f < 0.06$). However, according to the population, large variations in gene and molecular diversity indices were observed (Table 1). In particular, the number of segregating sites (S) and the number of haplotypes (N_H) ranged from 0 (Thau and Faro populations) to 21 (Rivedoux) and from 1 (Thau and Faro) to 16 (Séné) respectively. Thau and Faro, located in the recognized natural range of the species, were the only monomorphic populations (Fig. 1). Hossegor showed low genetic diversity compared to the other French Atlantic and English Channel (FAEC) populations: only 2 haplotypes were observed and nucleotide diversity ($\pi = 0.00050$) was 2 to 3-times lower than in other populations ($\pi = 0.00102$ to 0.00153). Genetic variability was comparable in the other five FAEC populations with 14 (Séné) to 21 (Rivedoux) segregating sites and haplotype diversity values ranging from 0.589 to 0.911 (mean value $H_e = 0.743 \pm 0.120$). However, haplotype and molecular diversity estimates were not always agreeing. Séné showed the highest number of haplotypes and haplotype diversity ($N_H = 16, H_e = 0.911$) while the number of segregating sites and nucleotide diversity ($S = 14, \pi = 0.01024$) were the lowest of the FAEC populations. Hossegor and Séné, both different from Arguin, Ronce, Rivedoux and Stérec, may thus have

1 different histories and/or have been originated from different introduction processes in
2 comparison to the other recently settled French populations.

3

4 **Haplotypic network and genetic structure at the population level.** The network
5 (Fig. 2) pictures the most likely genealogies between haplotypes. The three most frequent
6 haplotypes (i.e. A, B and C) corresponded to three main clusters (cluster 1: A, J and H, cluster
7 2: B, D, E, F and R and cluster 3: C, U and V). The genetic distances between these clusters
8 were large (13 to 15 mutational steps) compared to genetic distances within clusters (1 to 5
9 mutational steps). The haplotype G, present only in the population of Rivedoux (6 individuals
10 out of 39; Fig. 1) was found to be very distant from the other clusters (14 to 18 mutational
11 steps according to the cluster). The other 10 haplotypes (I, K-Q, S and T) found at very low
12 frequencies (over the whole dataset, $f = 0.015$ and 0.008 for I and K respectively, and $f =$
13 0.004 for the others) were close to each other, constituting an unresolved torso in the middle
14 of the network.

15 Interesting features were observed when comparing the geographical distribution of
16 haplotypes (Fig. 1) and their location in the network (Fig. 2). In five out of the six FAEC
17 populations, namely Arguin, Ronces, Rivedoux, Séné and Stérec, haplotypes belong to two or
18 all three of the clusters identified through the network analysis. This co-occurrence of
19 individuals characterized by evolutionary divergent haplotypes is corroborated by the
20 mismatch distribution constructed for each polymorphic population (Fig. 3). Arguin, Ronces,
21 Rivedoux and Stérec all showed at least two major peaks featuring two or more groups of
22 genetically divergent haplotypes co-occurring at the population level. This is exemplified by
23 the haplotypic tests as the observed number of haplotypes (N_H) was significantly lower than
24 expected under mutation-drift equilibrium (N_{EH}) in each of the four populations (Fig. 3). A
25 different situation was observed for Hossegor and Séné for which the observed distribution

1 appeared to fit the expected curve constructed under a demographic equilibrium hypothesis.
2 In Hossegor, the individuals appeared to be evolutionary closely related to each other. The K -
3 test was irrelevant in this population since only one segregating site was found (N_H always
4 equals to 2). In Séné, a more uniform and continuous mismatch distribution than in Hossegor
5 was observed. This may be related to the occurrence of a larger number of haplotypes that
6 differ from each other over a large range of nucleotide divergence (from 1 to 14). In this
7 population, a larger number of haplotypes than expected was observed (Fig. 3) suggesting a
8 recent population expansion or ancient balanced polymorphism (Depaulis & Veuille, 1998).

9

10 **Genetic divergence across populations and isolation by distance models.** Over
11 the whole study, the genetic differentiation was highly significant ($P < 10^{-5}$) and \hat{F}_{ST} was
12 equal to 0.382. Obviously, this substantial genetic structure was partly due to Faro and Thau,
13 monomorphic for two divergent haplotypes (A and B respectively). However, when removing
14 these populations, F_{ST} estimate decreased to 0.225 only and was still significant ($P < 10^{-5}$).
15 Interestingly, not all populations were equally genetically differentiated with each other.
16 Population pairwise \hat{F}_{ST} values indeed ranged from 0.042 to 0.458 (Table 2). While not
17 significant when all six FAEC populations were considered ($P = 0.162$), IBD was revealed
18 after removing Hossegor from the analysis ($P = 0.016$) highlighting an increase of pairwise
19 F_{ST} estimates when more distant populations were considered (Table 2; Fig. 4). For instance,
20 when the population of Arguin is compared to populations located more and more northerly,
21 i.e. Ronce, Rivedoux, Séné and Stérec, \hat{F}_{ST} values increased (0.053, 0.147, 0.176 and 0.276
22 respectively; Table 2).

23

DISCUSSION

ADMIXTURE PATTERNS AND HUMAN-MEDIATED INTRODUCTIONS

In this study, *C. neritea* populations sampled in the natural range of the species (Thau and Faro) were found to be monomorphic. On the contrary, five of the six FAEC populations (i.e. Arguin, Ronce, Rivedoux, Séné and Stérec) exhibited high levels of genetic diversity (e.g. mean $H_e = 0.743 \pm 0.120$ and $\pi = 0.01343 \pm 0.00210$). This result is in agreement with an increasing number of genetic study of terrestrial, freshwater and marine NIS indicating that the expected decrease of genetic polymorphism is lacking for numerous introduced species (poaceae (Novak *et al.*, 1993), brown mussel (Holland, 2001), thiarid snail (Facon *et al.*, 2003), slipper limpet (Dupont *et al.*, 2003) or Cuban lizard (Kolbe *et al.*, 2004)). As underlined by Kolbe *et al.* (2004), when natural populations are monomorphic, the occurrence of multiple introductions could promote the settlement of NIS by transforming among-population variation in native ranges to within-population variation in introduced areas.

The level of polymorphism (27 segregating sites defining 22 haplotypes over 262 individuals) is similar with that observed in many studies carried out with COI at the intra-species level (e.g. Gopurenko *et al.*, 1999; Kirkendale & Meyer, 2004; Wilding *et al.*, 2000). The striking difference in genetic polymorphism between Thau and Faro and the other French populations suggests that (i) at the population level, low polymorphism is an attribute of *C. neritea* in its native range and (ii) recurrent human-mediated non-intentional introductions may be at the origin of the settlement of the majority of the French populations recorded since the 1970's. In addition, divergent haplotypes were found within these five populations. For instance, haplotypes belonging to clusters 1, 2 and 3 were observed in Arguin, Ronce and Stérec. This particular pattern is unlikely for populations at mutation-drift equilibrium but is

1 characteristic of recent admixture events (i.e. mixing of individuals coming from populations
2 that have evolved independently). For four populations (i.e. Arguin, Ronce, Rivedoux and
3 Stérec), this is illustrated by: (i) multi-peak mismatch curves and (ii) highly significant *K*-tests.
4 The first result showed that these populations were characterized by the co-occurrence of at
5 least two groups of genetically divergent individuals. The *K*-test confirmed that populations
6 are not under a demographic equilibrium and that recent admixture events have occurred.
7 Admixture implies that introductions did not originate from one given source but from several
8 genetically differentiated sources. Oyster exchanges, known to be responsible for numerous
9 NIS introductions along the Atlantic coasts of Europe (Gouletquer *et al.*, 2002; Wolff &
10 Reise, 2002), may have played a major role in the colonization of the FAEC coasts by *C.*
11 *neritea* by introducing, in the vicinity of oyster beds, large numbers of individuals coming
12 from various genetically differentiated populations. Such mechanisms and vectors have been
13 hypothesized for other coastal marine introductions (see for instance *Ocinebrellus inornatus*,
14 Martel *et al.*, 2004). Séné and Hossegor appeared to be in a different situation. In Séné, a
15 larger haplotype number than expected under the equilibrium hypothesis was observed (*K*-test)
16 and these haplotypes were differing from each other over a large range of nucleotide
17 divergence (from 1 to 14). At the population level, a star-like genealogy of haplotypes was
18 observed suggesting a recent demographic expansion either in Séné or in the source
19 population of Séné. Except if the species had been present but unnoticed for a long time in
20 Séné, local expansion after the settlement of *C. neritea* seems unlikely since the species was
21 recorded for the first time in this site only twenty years ago (1984, Le Roux *et al.*, 1988). The
22 alternative hypothesis of a limited number of source populations for Séné, one of them having
23 recently undergone a large demographic expansion in the native range therefore seems more
24 likely. Because our study did not aim at identifying the sources but looking at mechanisms for
25 the settlement of the species along the FAEC coasts, none of our analyses allow ascertaining

1 either the number or the origins of these introductions. Such an issue would have required a
2 different sampling scheme focusing on potential sources from the recognized native range of
3 the species.

4

5 **STATUS OF HOSSEGOR: NATURAL OR INTRODUCED POPULATION?**

6 The most peculiar genetic pattern was observed in Hossegor. This population
7 showed genetic characteristics closer to that of populations from the natural range than to the
8 other FAEC populations, suggesting a natural status for this population. Three pieces of
9 evidence support this hypothesis: first, the genetic diversity in this population was quite low
10 ($H_e = 0.265$, $\pi = 0.00050$) compared to other FAEC populations ($H_e = 0.743 \pm 0.120$ and $\pi =$
11 0.01343 ± 0.00210 mean values and associated standard deviations) whereas low diversity
12 was also observed in Thau and Faro. Second, of the two haplotypes found in Hossegor, the
13 most frequent, namely A, ($f = 0.85$) was characteristic of Faro, and the second (haplotype H)
14 was diverging from A by only one mutational step (Appendix). Finally, observed and
15 expected distribution of pairwise differences in the population of Hossegor matched perfectly,
16 indicating that the population was at demographic equilibrium (Li, 1977; Rogers &
17 Harpending, 1992; Slatkin & Hudson, 1991). The fact that Hossegor is not the result of a
18 recent introduction but a native population is consistent with the presumed history of *C.*
19 *neritea* in this geographical area. The species has been episodically recorded until 1950 along
20 French Basque coasts (Kisch, 1950; Morton, 1960) in particular in Socoa, only 50 km in the
21 South of Hossegor. The North edge of this area is located just south of the very exposed and
22 inhospitable for *C. neritea*, coasts of the Landes region. Moreover, as underlined by Sauriau
23 (1991), the southern coasts of the Bay of Biscay are close to a biogeographic boundary
24 between northern temperate species and southern sub-tropical species (Glémarec, 1979). Such
25 natural barriers are characterized by very specific patterns in term of species distributions,

1 hybridization between species (e.g. Bierne *et al.*, 2003) or genetic differentiation at the
2 population level (Jolly *et al.*, 2005; Luttkhuizen *et al.*, 2003). Hossegor may thus be the
3 historical northern range limit of *C. neritea* along the French Atlantic coasts and the existence
4 of a strong geographical barrier could have limited its natural spread northwards.

5

6 **LINKING HUMAN-MEDIATED INTRODUCTIONS AND NATURAL SPREAD**

7 An isolation-by-distance pattern (IBD) was clearly revealed between FAEC
8 populations when removing the “natural” population of Hossegor. The gradual appearance of
9 new FAEC populations of *C. neritea* could first be viewed as a consequence of the recent
10 warming of the Bay of Biscay (i.e. natural range expansion towards North) enhancing the
11 colonization along the French coasts. Indeed, oyster exchanges between aquaculture sites are
12 very unlikely to be responsible for such an IBD pattern since human-mediated exchanges
13 between shellfish culture sites occur randomly between very distant locations without
14 continuous progression. In particular, all oyster spats are produced in the bays of Arcachon
15 and Marennes-Oléron and transplanted from those bays to all the other FAEC and
16 Mediterranean oyster culture areas (Gouletquer & Heral, 1997). If random human-dispersal
17 was the only process structuring *C. neritea* populations, a random haplotype distribution
18 would thus have been observed.

19 To summarize, the genetic pattern observed in the FAEC populations (excluding the
20 “natural” population of Hossegor) was characteristic of human-mediated introductions from
21 multiple and divergent source populations and at the same time, a pattern of IBD that cannot
22 be related to human activities was also observed. Can we explain this discrepancy? One likely
23 explanation is the presence of *C. neritea* at low densities, and consequently unnoticed, on the
24 FAEC coasts, long before 1976. For instance, Hossegor is likely to be a natural *C. neritea*
25 population. However, in spite of continuous monitoring activities in this area (the Arcachon

1 Marine Station was created in the 1860's), official records of the population of Hossegor were
2 missing before our sampling. Similarly, in the Bay of Morlaix, the presence of *C. neritea* is
3 still officially unrecorded in spite of the observation of the first individuals in Stérec 8 years
4 ago (Ann Andersen, pers. comm.). The patchy distribution of *C. neritea* populations, even in
5 its natural range, makes it difficult to detect, even more when population densities are low. *C.*
6 *neritea* may thus have been present on the FAEC coasts as a cryptic species for decades and
7 could thus be classified as a cryptogenic species as defined by Carlton (1996). Such species
8 “not demonstrably native or introduced” represent probably a large part of newly recorded
9 species (Carlton, 1996). Such a category is of particular interest when dealing with species
10 close to their recognised native range. In the case of *C. neritea*, the most northern natural
11 population on the Atlantic coasts might have been a source for populations located further
12 North through natural migrations and/or more probably ancient and not recognized accidental
13 introductions. As an example, the oyster *Ostrea edulis* has been exploited in France since the
14 early twentieth century with exchanges between the Thau lagoon and the Bay of Marennes-
15 Oléron (Hinard & Lambert, 1928). However, the increase of accidental introductions probably
16 started only during the 1970's because of the intensification of oyster transfers between
17 Mediterranean and Atlantic aquaculture sites as well as among Atlantic oysters farms. At the
18 same time, ecological alterations (i.e. the rapid temperature increase in the Bay of Biscay)
19 may have favoured (i) the settlement of introduced individuals by weakening native
20 communities (Dukes & Mooney, 1999) and (ii) the increase of migrants coming from the
21 South, responsible for a demographic reinforcement of *C. neritea* French Atlantic populations.

22 Whatever the processes that played in the past (i.e. natural migration and accidental
23 introductions) they were apparently too rare to settle large and detectable populations of *C.*
24 *neritea*. The need of recurrent and massive introductions for a species to increase the chance
25 for a sustainable establishment in a new environment is well known: Sax & Brown (2000)

1 explained that species that become abundant and widespread often do so only after having
2 failed to establish following multiple earlier introductions and that the same phenomenon is
3 also true for natural colonization. The particular combination of factors, i.e. increase of
4 repeated accidental introductions associated with reinforcement by natural migrations, can be
5 responsible of the sustainable settlement of large (and thus detectable, from 400 to >1600
6 ind.m⁻² in Rivedoux, Tardy *et al.*, 1985) *C. neritea* populations along the Atlantic coasts of
7 France. Nevertheless, the population of Stérec has probably been founded exclusively because
8 of human-mediated introductions since several hundreds of kilometres of rocky shores are
9 isolating this site from the other French Atlantic populations and it is noteworthy that the
10 population is still at low density.

11

12 In conclusion, we showed that French populations shared common mechanisms for
13 their establishment. Our study ascertained the introduced status of *C. neritea* along the FAEC
14 coasts, showing that mixing of different evolutionary lineages is the rule and has been the
15 starting point for a rapid demographic expansion in sites where the species was absent or
16 unnoticed but present at low density. In a previous study, Bachelet *et al.* (2004) pointed out
17 that (i) *C. neritea* could compete with the native necrophagous nassariid, *Nassarius*
18 *reticulatus* and (ii) conversely to this native species, *C. neritea* did not show a heavy parasitic
19 load. These observations showed that this competitive gastropod may become definitively
20 integrated in the ecosystem where it has been introduced. The temperature increase may
21 facilitate this process by reinforcing the potential for this species to be established on a longer
22 term and even becoming a real invasive species. Altogether, since the natural or introduced
23 status for French Atlantic populations is not straightforward, *C. neritea* can be classified as a
24 cryptogenic species and a more comprehensive study of the species over its native range is
25 now required to understand short and long-term history of the species in Europe. Given the

1 limited polymorphism found in Thau and Faro, nuclear microsatellite markers recently
2 developed for *C. neritea* (Simon-Bouhet *et al.*, 2005) appear as promising markers. As
3 underlined by Davies *et al.*, (1999), assignment analyses based on multi-locus genotypes are
4 powerful to identify the limits of the natural range of species and sources for NIS. Since
5 ecological consequences of NIS introductions may be different when new populations are
6 introduced close to their previous native range (where the community is expected to be more
7 or less the same than in the native range of the NIS) or introduced far from their natural range
8 (where the NIS will find a totally different community), long-term ecological surveys are
9 needed to follow with a special interest those NIS located at the edge of their previous natural
10 range.

11

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FIGURE LEGENDS

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Fig. 1. Haplotype distribution among the eight study populations. Numbers of individuals analysed per population (N) are given.

Fig. 2. Haplotypic network picturing the phylogenetic relationships between haplotypes. Circle sizes are proportional to haplotype frequencies over the whole dataset. (|) Single mutational events. (●) Missing intermediate haplotypes between observed haplotypes.

Fig. 3. Mismatch distributions. For each polymorphic population, the observed distribution of pairwise nucleotide differences between sequences (continuous lines) is plotted. The expected distribution under the null hypothesis of demographic equilibrium is indicated by a dotted line. Results of the *K*-test (see Material & Methods) are indicated for each population except for Hossegor (since $S = 1$ and $N_H = 2$), 95% confidence intervals (CI) for the expected N_{EH} value are given together with the observed value (N_H). *P*-values (probability that N_{EH} is inferior to the observed value) were computed based on 5000 simulations. For Séné, *P*-value (P^*) is the probability that N_{EH} is higher than the observed value.

Fig. 4. Isolation by distance. Genetic distance $\frac{\hat{F}_{ST}}{1 - \hat{F}_{ST}}$ calculated for each FAEC population pair is plotted against geographic distance (D) in kilometres. Black squares represent pairwise values with the population of Hossegor. Regression lines are pictured with a dotted line when all populations are taken into account and a continuous line when Hossegor is removed from the analysis. *P*-values, associated to a Mantel test for a positive correlation between genetic and geographic distances, equals 0.162 and 0.016 with and without Hossegor respectively.

1 Table 1. Geographical location of the eight study populations. Numbers of individuals
 2 analysed (N) and genetic diversity estimators are given (N_H : number of haplotypes, S : number
 3 of segregating sites, H_e : haplotypic diversity, π : nucleotidic diversity)

	Latitude	Longitude	N	N_H	S	H_e	π
Thau (coastal lagoon)	43° 27' N	3° 38' E	29	1	0	0.000	0.00000
Faro (Ria Formosa)	37° 00' N	7° 58' W	31	1	0	0.000	0.00000
Hossegor (Basque coast)	43° 40' N	1° 25' W	33	2	1	0.265	0.00050
Arguin (Arcachon Bay)	44°35' N	1° 14' W	33	5	19	0.725	0.01253
Ronce les Bains (Marennes-Oleron Bay)	45° 48' N	1° 09' W	32	4	19	0.696	0.01520
Rivedoux (Isle of Ré)	46° 09' N	1° 16' W	39	5	21	0.796	0.01525
Séné (Gulf of Morbihan)	47° 35' N	2° 43' W	32	16	14	0.911	0.01024
Stérec (Bay of Morlaix)	48° 40' N	3° 52' W	33	3	18	0.589	0.01393
Total	-	-	262	22	27	0.793	0.01551

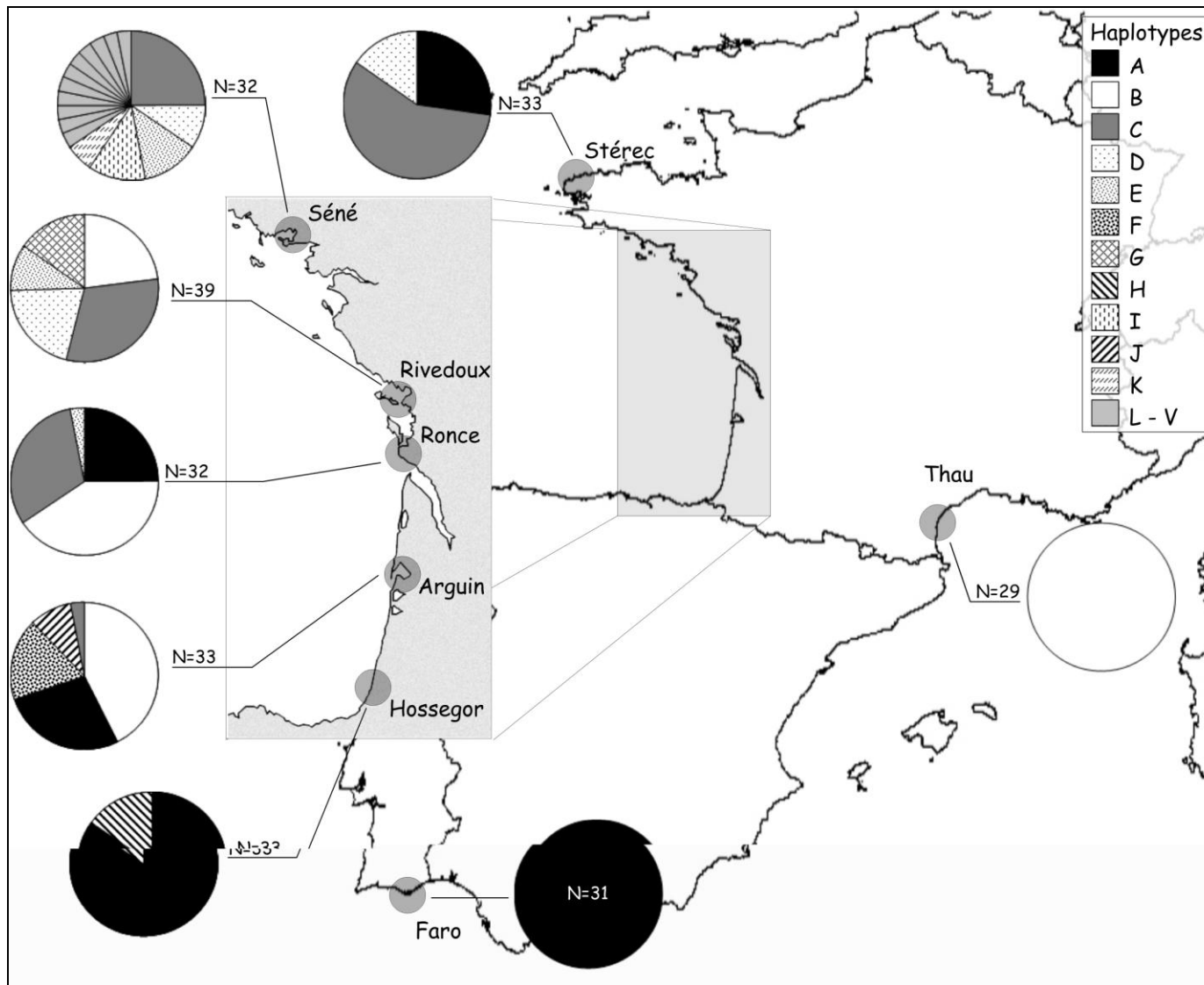
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- 1 Table 2. Pairwise F_{ST} matrix. The pairwise F_{ST} (below diagonal) and associated P -values
 2 (above diagonal) are given.

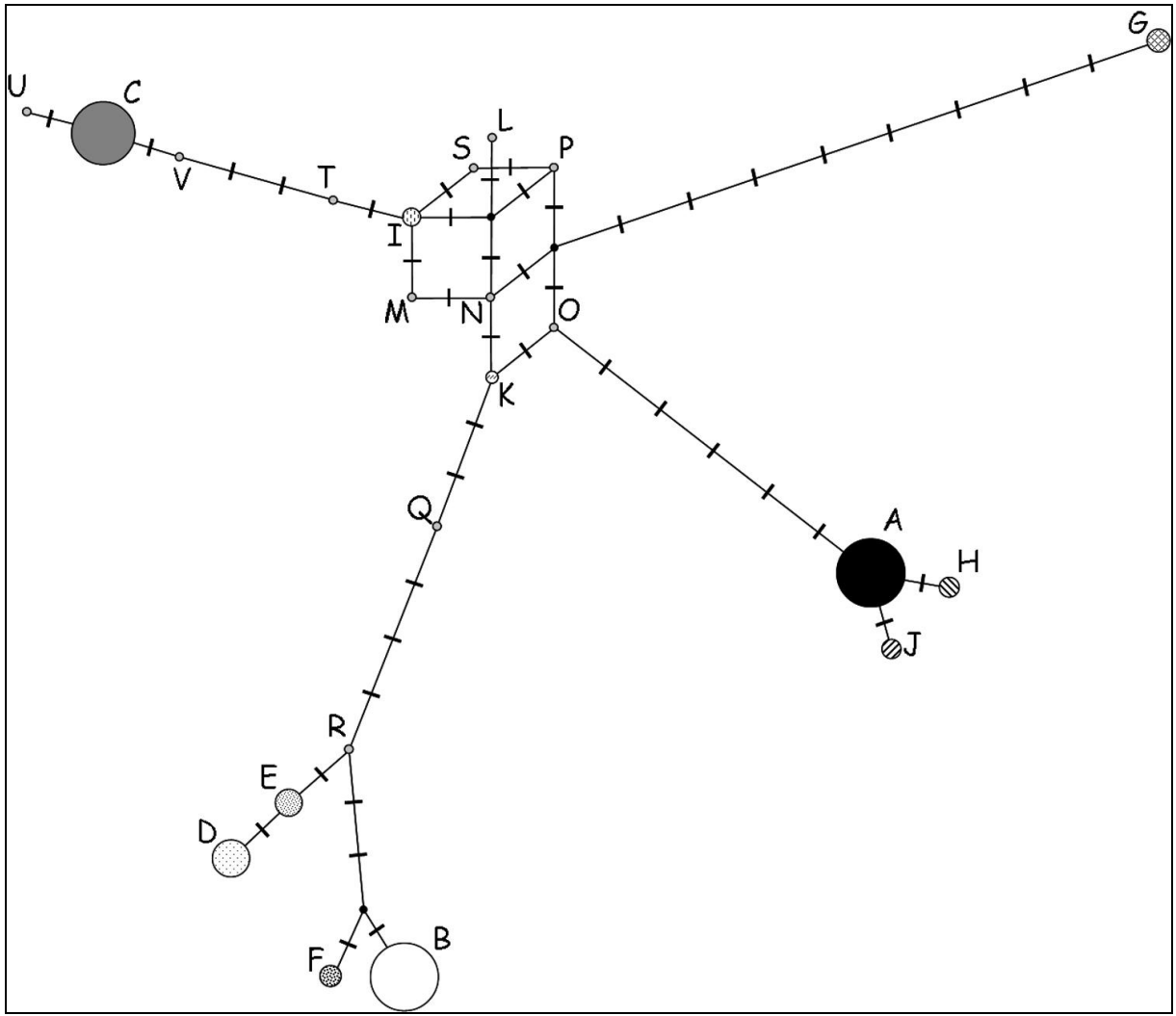
	Thau	Faro	Hossegor	Arguin	Ronce	Rivedoux	Séné	Stérec
Thau		$< 10^{-5}$	$< 10^{-5}$	$< 10^{-5}$	$< 10^{-5}$	$< 10^{-5}$	$< 10^{-5}$	$< 10^{-5}$
Faro	1.000		0.046	$< 10^{-5}$	$< 10^{-5}$	$< 10^{-5}$	$< 10^{-5}$	$< 10^{-5}$
Hossegor	0.860	0.120		$< 10^{-5}$	$< 10^{-5}$	$< 10^{-5}$	$< 10^{-5}$	$< 10^{-5}$
Arguin	0.353	0.493	0.356		0.041	$< 10^{-5}$	$< 10^{-5}$	$< 10^{-5}$
Ronce	0.401	0.532	0.392	0.053		0.007	$< 10^{-5}$	$< 10^{-3}$
Rivedoux	0.445	0.574	0.458	0.147	0.075		0.016	0.001
Séné	0.531	0.540	0.414	0.176	0.123	0.042		0.001
Stérec	0.692	0.587	0.444	0.276	0.146	0.123	0.109	

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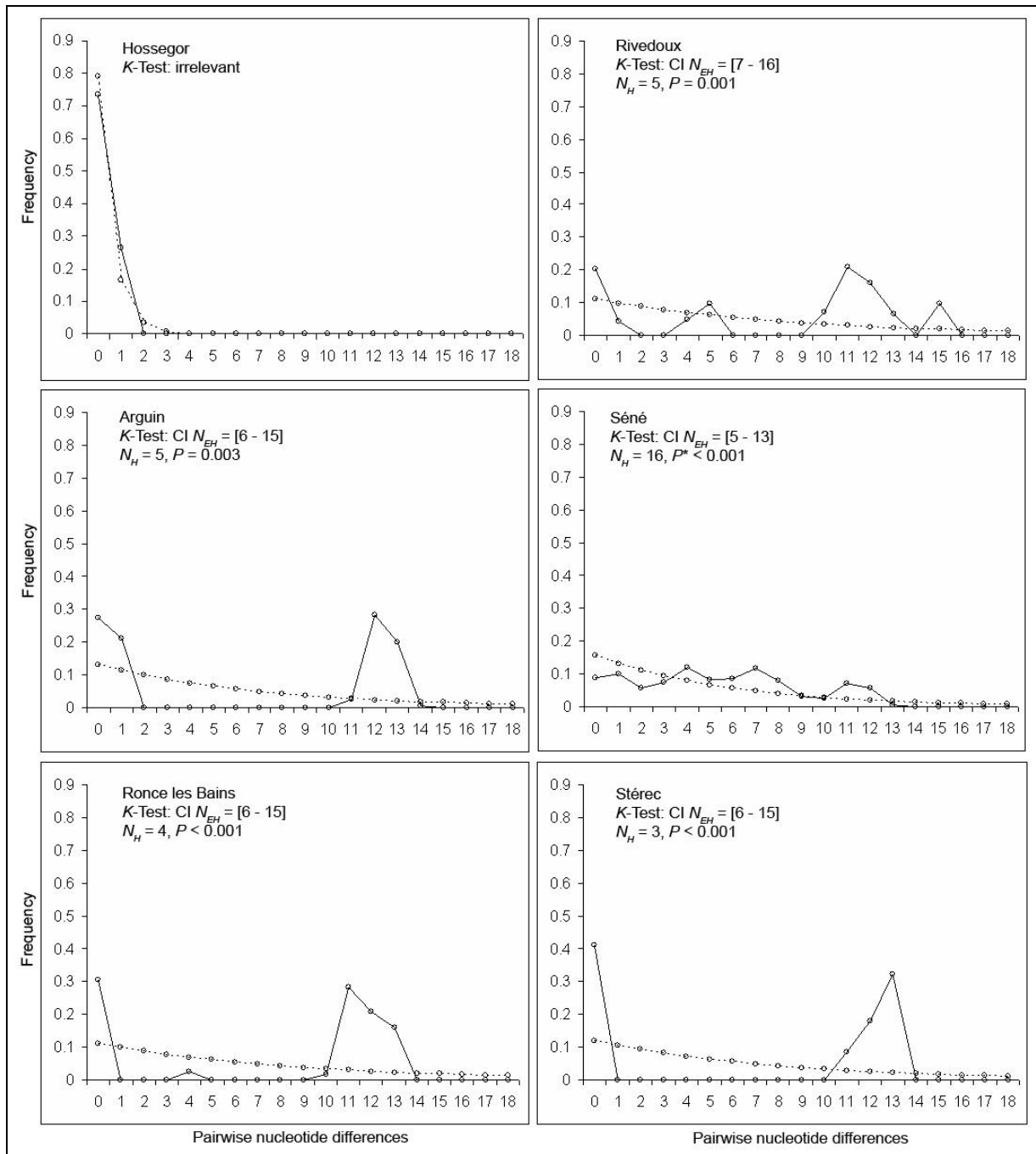
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2 Figure 1.



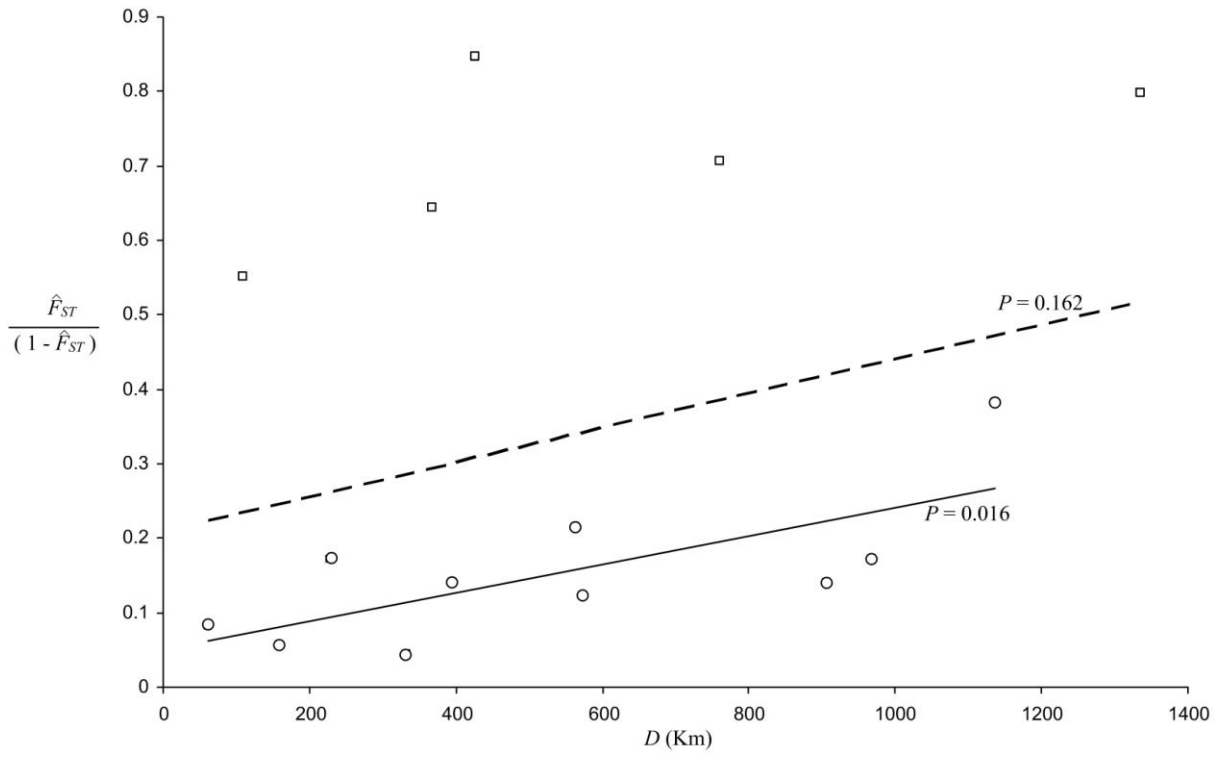
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2 Figure 2.



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2 Figure 3.



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2 Figure 4.

- 1 Appendix: Definition of haplotypes A to V (GenBank accession numbers: AY789970-AY789991) and number of occurrence over the whole
 2 dataset (N) are presented. Positions of mutated sites are given. “-” indicates that the same character is present in haplotype A.

Haplotype	N	Positions (bp)																										
		1	25	44	50	53	104	164	218	224	254	266	292	299	317	320	323	338	347	374	377	382	398	401	422	425	479	503
A	85	A	A	C	A	A	T	C	A	A	C	C	G	G	C	T	A	T	A	T	A	C	G	T	T	G	A	T
B	65	-	-	T	G	G	-	-	-	-	T	T	-	-	T	-	-	-	G	-	G	-	C	-	C	-	G	C
C	50	G	-	T	G	-	C	-	G	G	-	-	A	-	-	-	-	-	G	-	G	-	C	-	C	A	-	C
D	19	-	-	T	G	G	-	-	-	-	T	T	-	A	-	-	-	C	G	-	G	-	C	-	C	-	-	-
E	9	-	-	T	G	G	-	-	-	-	T	T	-	-	-	-	-	C	G	-	G	-	C	-	C	-	-	-
F	6	-	-	T	G	G	-	-	-	-	T	T	-	-	T	-	-	-	G	-	G	A	C	-	C	-	G	C
G	6	-	G	T	G	-	-	T	-	-	-	T	-	-	T	C	G	-	G	C	-	-	C	C	C	-	-	C
H	5	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
I	4	G	-	T	G	-	-	-	-	-	-	-	A	-	-	-	-	-	G	-	G	-	C	-	C	-	-	C
J	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-
K	2	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	G	-	C	-	C	-	-	C
L	1	G	-	T	G	-	-	-	G	-	-	-	-	-	-	-	-	-	G	-	G	-	C	-	C	-	-	C
M	1	-	-	T	G	-	-	-	-	-	-	-	A	-	-	-	-	-	G	-	G	-	C	-	C	-	-	C
N	1	-	-	T	G	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	G	-	C	-	C	-	-	C
O	1	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	C	-	C	-	-	C
P	1	G	-	T	G	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	C	-	C	-	-	C
Q	1	-	-	-	G	G	-	-	-	-	-	-	-	-	-	-	-	-	G	-	G	-	C	-	C	-	-	-
R	1	-	-	T	G	G	-	-	-	-	T	T	-	-	-	-	-	-	G	-	G	-	C	-	C	-	-	-
S	1	G	-	T	G	-	-	-	-	-	-	-	A	-	-	-	-	-	G	-	-	-	C	-	C	-	-	C
T	1	G	-	T	G	-	C	-	-	-	-	-	A	-	-	-	-	-	G	-	G	-	C	-	C	-	-	C
U	1	G	-	T	G	-	C	-	G	G	-	-	A	-	-	-	-	-	G	-	-	-	C	-	C	A	-	C
V	1	G	-	T	G	-	C	-	G	G	-	-	A	-	-	-	-	-	G	-	G	-	C	-	C	-	-	C