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Population structure of the common sole (*Solea solea*) in the Northeastern Atlantic and the Mediterranean Sea: revisiting the divide with EPIC markers

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Abstract:

Spatial and temporal population genetic structures of the common sole, Solea solea, were studied in Northeastern Atlantic and Mediterranean Sea populations, using three polymorphic exon-primed intron-crossing (EPIC) markers. Results demonstrated significant multilocus differentiation among Eastern Mediterranean and a group composed by Western Mediterranean and Atlantic populations ($\theta = 0.150$, $P < 0.001$), but also suggested unrecorded genetic differentiation of the Adriatic Sea population. No pattern of isolation-by-distance was recorded across the range covered by sampling, from the Kattegat to the Aegean Sea. Conversely to genetically structured Mediterranean populations, Atlantic populations ranging from Denmark to Portugal could be considered as representative of the same panmictic unit ($\theta = 0.009$, not significant). Results further demonstrated stability of multilocus genetic structure among temporarily replicated cohort samples [0+, 1+, subadults] from several coastal and estuarine locations from Bay of Biscay, excepted for the amylase locus Am2B3-2 at one location (Pertuis d'Antioche). Despite coherence of such observed patterns of multilocus differentiation with previous allozymic surveys in sole, and with patterns generally obtained for other marine fish species, single-locus results from EPICs indicated divergent coalescence schemes supporting a complex response to ecology and history of sole's populations. Results stress the use of nuclear genes such as EPIC markers to investigate population structure, but also historical, demographic, and possibly selective processes in marine fishes.

Keywords: common sole, introns, genetic structure, marine fish.

Introduction

Over the last two decades, studies of marine fish population genetic have accumulated, contributing to a better understanding of population structure and stock composition in the North Eastern Atlantic and the Mediterranean Sea (Borsa et al. 1997a; Bargelloni et al. 2003; Zardoya et al. 2004; and references therein). These studies questioned evolutionary mechanisms in the marine environment, the role of life-history traits (e.g. duration of the larval pelagic phase) in shaping patterns of genetic structure, and played a part in the improvement of guidelines for fish stocks exploitation. Phylogeographical studies demonstrated significant differentiation between the Atlantic and the Mediterranean among marine fish or invertebrates (review in, e.g. Borsa et al. 1997a). However, the geographical position of the transition between the two basins appeared to be different depending on the species considered. For instance a strong to moderate genetic divergence between the Atlantic and the Mediterranean Sea was reported in numerous fish species (e.g. sea bass: *Dicentrarchus labrax*, mackerel: *Scomber scombrus*, white anglerfish: *Lophius piscatorius*, and some Sparidae) whereas other species (e.g. chub mackerel: *Scomber japonicus*, black anglerfish: *Lophius budegassa*, other Sparidae, and *Trachurus* spp.) demonstrated no differentiation at all (see examples in e.g. Borsa et al. 1997a; Naciri et al. 1999; Bargelloni et al. 2003, 2005; Karaiskou et al. 2004; Zardoya et al. 2004; Charrier et al. 2006; and references therein). Moreover, it has been shown that the levels and patterns of genetic differentiation within each water body display marked differences. Atlantic shelf and North Sea populations were generally found broadly panmictic (e.g. Kotoulas et al. 1995; Hoarau et al. 2002; but see Hutchinson et al. 2001; Exadactylos et al. 2003), or reflecting patterns of isolation-by-distance (IBD) (e.g. Mariani et al. 2005; Gysels et al. 2004). On the other hand, non-shelf Atlantic populations (e.g. Nesbø et al. 2000; Hoarau et al. 2004), and Baltic Sea populations (e.g. Nielsen et al. 2003, 2004) were shown to be genetically differentiated from adjacent continental shelf Atlantic waters. Subtle local differentiation were observed to occur along Atlantic shorelines in some species (e.g. hake: *Merluccius merluccius*; Castillo et al. 2005), but due to limited sampling these studies were unable to clearly state whether this was due to a IBD pattern. Mediterranean populations of most investigated species were often found genetically differentiated, according to various models and reflecting a variety of demographic histories (e.g. Bahri-Sfar et al. 2000; Viñas et al. 2004; Magoulas et al. 2006). Most study published so far reported differentiation among the Western and Eastern Mediterranean basins of numerous invertebrate or vertebrate marine species (review in Borsa et al. 1997a), together with possible genetic distinctiveness of the Adriatic Sea (e.g. Borsa et al. 1997b; Tinti et al. 2002). Such patterns of genetic structure have been traditionally attributed to historical processes favouring vicariance, and to combined effects of contemporary gene flow modulated by ecological traits and hydrological features (e.g. Magoulas et al. 2006).

If such patterns of (phylo)geographic differentiation are then now well-established for numerous European marine fish species, most studies examining genetic differentiation of marine fish populations assumed temporal homogeneity of samples, and included sampling on just one occasion. This often translated in a “steady-state” picture of genetic structure, that was of very poor use for management. As populations fluctuate in terms of abundance, distribution, and recruitment, temporal heterogeneity is expected and should reflect in the inferred genetic population structure. Temporal changes in the genetic structure of marine population has been examined in some studies. For example, in a year (2001) with high inflow of North Sea waters into the Skagerrak, Knutsen et al. (2004) found that juvenile cod (*Gadus morhua*) caught along the Skagerrak coast are predominantly of North Sea origin, whereas in a year (2000) with low inflow juveniles appear to be of local origin. These findings indicate that offshore cod may influence coastal cod populations over large distances depending on the environment, then influence our perception of genetic structure and some management issues. Similar results have been described in herring (*Clupea harengus*) within the North Sea (Ruzzante et al. 2006). Conversely, only the use of temporarily replicated samples in eels (*Anguilla anguilla*) firmly proved the paradigm of panmixia reported for this species (Dannewitz et al. 2005), but also subtle temporal genetic differences across arrival waves (early *vs* late) of juveniles at a single site (Pujolar et al. 2006; Maes et al. 2006). Such kind of results were not only useful in terms of fishery management, but also for better understanding of mechanisms underlying temporal differentiation among cohorts at a single locale (‘chaotic genetic patchiness’ *sensu* Johnson and Black 1982), mainly including large variance in reproductive success that might shape temporal variation in the genetic composition of recruits (‘sweepstake hypothesis’, Hedgecock 1994; e.g. Chapman et al. 2002; Planes and Lenfant 2002; Pujolar et al. 2006), but also gene flow from different spawning populations with different allelic composition (e.g. Ruzzante et al. 1996), or potential selection on larval stages leading to differential mortality of genotypes before recruitment (Planes and Roman 2004). All three mechanisms might operate concurrently and are especially difficult to disentangle. In flatfish, temporal genetic differentiation was only assessed among different cohorts in plaice (*Pleuronectes platessa*). In this species, using

microsatellite loci, Hoarau et al. (2005) reported local panmixia across cohorts (juveniles *vs* adults) at each sampled nursery in a given year (2002; i.e. indicating no or few variance in local reproductive success), together with change in levels of inbreeding in each sampled population over decades (possibly indicating change in effective population sizes that influenced genetic composition of recruits). One older allozyme study by Beardmore and Ward (1977) indicated relationships between gene diversity, genotypic composition and growth in plaice, suggesting selective processes.

The common sole, *Solea solea*, is a highly valued commercial fish in both Atlantic and Mediterranean Europe. It represents a major resource in the Bay of Biscay, where 1800 tons are harvested yearly (Léauté and Caill-Milly, 2003). However, since 1993 the spawning biomass of the Bay of Biscay stock has been undergoing a constant decline and it is currently at risk, with the lowest level of recruitment of the last 20 years recorded for the year class 1999 (Anonymous, 2003). Together with more holistic approaches (e.g. ecosystem-based fishery management), management regime needs to consider intraspecific genetic diversity to investigate both temporally and spatially patterns of genetic diversity within- and among populations together with population structure (Kenchington et al. 2003). In sole, previous population genetics studies have been carried out, using different kinds of genetic markers (allozymes: Kotoulas et al. 1995, Exadactylos et al. 1998, Cabral et al. 2003; RAPDs, Exadactylos et al. 2003; mtDNA: Guarniero et al. 2002). In the most extensive of these studies, Kotoulas et al. (1995), using allozymes, reported multilocus genetic differentiation between Mediterranean and Atlantic populations, as well as among eastern and western Mediterranean samples. This result was later supported by Guarniero et al. (2002). Kotoulas et al. (1995) also indicated weak IBD across Atlantic populations ranging from Denmark to Portugal, whereas more local and regional studies indicated absence of population structure (Exadactylos et al. 1998, Cabral et al. 2003). Such findings need to be investigated with other markers to infer more thoroughly spatial genetic structure. Furthermore in sole, to our knowledge, only one study investigated the temporal genetic differentiation at a local scale (Vilaine estuary in the Bay of Biscay; Kotoulas et al., 1995), reporting moderate allozymic differentiation among samples due to a negative trend between heterozygote deficiency and age in sole. As no other samples than the Vilaine samples where temporarily replicated in Kotoulas et al.'s (1995) study , the reality of temporal genetic differentiation in sole cannot be extended further.

In that context, the present study meant to reach two objectives: *i*) to revisit, using three nuclear exon-primed intron-crossing (EPIC) markers, the established patterns of genetic structure across the North Eastern Atlantic and the Mediterranean Sea; *ii*) to examine the temporal stability of population genetic structure at selected coastal locations in the Bay of Biscay.

Materials and methods

Sample and data collection

Twenty four *Solea solea* (L.) samples of sizes comprised between 23 and 70 individuals (total sample size, $N = 749$), and collected between 2000 and 2002 in the Atlantic (along the shore of Portugal, Bay of Biscay, and English Channel) and the Mediterranean Sea were available to the present study (Fig. 1). A Danish sample originating from Kattegat at the junction of Baltic and North Sea was also studied. These samples were obtained from scientific cruises or commercial fishing operations, and covered not all, but most of the distribution area of the species on the continental shelf (Quéro et al. 1986). Details on sampling collection, sampling areas, and sampling dates are given in Table 1. Most populations of the Bay of Biscay were re-sampled up to three times on different sampling dates to investigate genetic differentiation over distinct cohorts (juveniles [0+, 1+], subadults) (Table 1). This point was scarcely investigated in previous genetic studies of sole (see Introduction). The Bay of Biscay is a convenient place to test temporal changes in genetic structure over cohorts because nurseries are well known in this area (e.g. Le Pape et al. 2003). Nurseries constitute isolated groups all along the year (Dorel et al. 1991; Koutsikopoulos et al. 1995).

Genetic analyses

Samples used in genetic analysis consisted of small pieces of muscle preserved either dried, frozen, or in ethanol. Samples (Table 1) were analysed for genetic variation at three nuclear-DNA intronic loci using a EPIC-PCR. The set of loci included the first intron of the metallothionein gene, and the third intron of two α-amylase genes. The two amylase (*Am2B3*) loci were amplified with primers defined in Hassan *et al.* (2002). Further cloning of alleles indicated that alignment of sequences of each amylase locus were not possible (JL Rolland and B Guinand, unpubl. results), indicating that we probably amplified two different genes of the multigenic α-amylase gene family (Bouneau et al. 2003). A new set of primers was defined for the metallothionein (*MT*) locus. The primers MetF (sense: 5'ATGA(CT)CCTTG(CT)GA(AC)TGCTC3') and MetR (antisense: 5'GCAGGA(GT)CCTCCGCAGTTGC3') were designed from the alignment of the metallothionein A gene of the rainbow trout (*Onchorhynchus mykiss*, GenBank M22487) with homologous cDNA sequences of sea bass (*Dicentrarchus*

labrax; GenBank AF199014). Individual PCR amplifications at each locus were carried out in a 10µl reaction mixture containing 1 µl DNA template (50ng), 0,8µ*M* of each primer (one primer from each set was 5'- labeled with fluorochrome CY5 (Eurogenetec, Strasbourg, France), 1.5 m*M* $MgCl_2$ (Promega, Madison WI, USA), 0.25m*M* each dNTP, and 0.05 *U Taq* polymerase (Promega, Madison WI, USA). Amplification conditions were 35 cycles of denaturation at 94°C for 1min, annealing at 50 °C (*MT*) or 48°C (*Am2B3-1*, *Am2B3-2*) for 1min and extension at 72°C for 10min. Amplification products were mixed directly with an equal volume of formamide loading dye (95% formamide, 20mM EDTA, 0.05% xylene cyanol and 0.05% bromophenol blue) and denatured at 94°C for 5 min. Two microliters of the mixture were loaded onto a 6 % denaturing polyacrylamide gel and run using 0.5X TBE buffer at 50 W for 3 hrs (*MT*), 5 Hrs (*Am2B3-1*) and (*Am2B3-2*). Gels were subsequently laser-scanned at 675 nm and CY5 fluorescent bands were visualised in an FMBio II fluorescence imaging apparatus (Hitachi Instruments, San José CA, USA).

Data analyses

Deviations from Hardy-Weinberg expectations within samples were investigated using Weir & Cockerham's (1984) *f* using Genetix v.4.05 (Belkhir et al. 1996-2001; available at http://www.univ-montp2.fr/~genetix/). Test of the null hypothesis of no significant departure from Hardy-Weinberg expectations (f=0) was carried by randomly permutating alleles from the original matrix of genotypes using the appropriate procedure in Genetix v.4.05. Levels of pairwise population differentiation were investigated using Weir & Cockerham's (1984) θ, an estimator of Wright's (1951) F_{st}, also using Genetix v.4.05. In all cases, critical significance levels for multiple testing were also corrected using sequential Bonferroni procedures. Reynold's et al. (1983) distance was used to construct the population phenogram using the neighbour-joining algorithm (Saitou and Nei 1987) implemented in Phylip v3.6.1 (Felsenstein 1993). Population phenogram was computed based on data at the three scored loci, but also for each locus separately. In the multilocus case, confidence in tree topology was assessed by bootstrapping data (1000 iterations). The unrooted trees was displayed using Treeview (Page 1996). Patterns of linkage disequilibrium were computed as Weir (1979), and significance testing carried out by permutations (1000) using Genetix v.4.05. Results were proved not being affected by the choice of distance (i.e., the shape and main relationships among samples in phenograms were found identical). The Reynold's et al. (1983) distance was retained since it is based on Weir & Cockerham's (1984) θ, offering a coherent metric to interpret phenograms with respect to estimation of (pairwise) genetic differentiation using θ.

Patterns of isolation by distance (IBD) looking at the relationship between pairwise population genetic differentiation (measured by $\theta/(1 - \theta)$; Rousset 1997) and geographical distance (log-transformed; measured as the shortest distance by sea) were computed using both the Mantel test and the Pearson correlation coefficient implemented in Genetix v.4.05. Tests (1000 iterations). Such test were computed over the whole range of samples (Atlantic and Mediterranean), then only for Atlantic samples, and both for the multilocus case and for each locus taken separately. For consistency, test were also computed only among subadult samples to avoid some 'noise' due to Bay of Biscay's replicated samples. Mediterranean samples were not considered alone due to low number of samples (Table 1).

Results

Allelic diversity , gene diversity and Hardy-Weinberg equilibrium

The *Am2B3-1*, *Am2B3-2* and *MT* loci were characterized by four, three and fourteen different alleles, respectively, with size varying from 354 to 381 base pairs (bp) for locus *Am2B3-1*, from 240 to 261bp for locus *Am2B3*-2, and from 106 to 112bp for locus *MT*. Mean observed heterozygosities across samples were 0.32, 0.53, and 0.63 for locus *Am2B3-1, Am2B3-2*, and *MT*, respectively. Alleles observed at locus *MT* were characterized by single-repeat nucleotidic changes (length variation of alleles due to poly-T variation; result not shown), making this locus a microsatellite-like locus. Allelic variation at each amylase loci was characterized by indels (not shown).

Allele frequencies at each locus and in each sample are reported in Table 2, together with expected (H_{exp}) and observed (H_{obs}) gene diversities, and f values. Only three samples over seventy two cases demonstrated significant departures of Hardy-Weinberg equilibrium at individual locus after Bonferroni correction for multiple testing. Only the *MT* locus was affected for samples both in the Atlantic (Loire 1+, PA 0+) and the Mediterranean Sea (Aegean) (Table 2). Multilocus estimate of f was significantly distinct from zero in only one population after Bonferroni correction testing (Loire Ad; Table 2). No case of significant linkage disequilibrium was reported for any couple of loci when sequential Bonferroni correction was applied (results not shown).

Population structure across the whole sample

Estimates of multilocus θ indicated significant population differentiation between Atlantic and Mediterranean populations of sole, but also significant differentiation among Mediterranean populations (Table 3). The associated tree based on all analysed loci clearly illustrated genetic distinctiveness of Mediterranean and Atlantic populations (Fig. 2A). From pairwise multilocus estimates of population differentiation (not reported), the population from the Adriatic appeared as the most genetically distinct from all other samples, including the Aegean sample. Conversely, no significant multilocus differentiation between populations was detected among Atlantic populations (Table 3).

Interpretation of results should be slightly qualified when considering each locus separately. It was shown that locus *Am2B3-1* displayed patterns found in the multilocus tree, with the Adriatic and Aegean populations being more distantly related to all other populations (Fig. 2B). For locus *Am2B3-1*, estimates of θ basically illustrated significant genetic differentiation between Eastern Mediterranean populations, and a group composed by Atlantic and Western Mediterranean populations of sole, whereas no differentiation among Atlantic and Western Mediterranean populations were observed (Table 3). As θ could level up 0.35, this locus had the strongest impact on the structure of the multilocus tree. As in the multilocus case, locus *Am2B3-2* reflected a clear genetic distinction between populations from the Atlantic and the Mediterranean Sea (Fig. 2C and Table 3). No significant differentiations were recorded among Atlantic or Mediterranean samples at this locus (Table 3). However, pairwise values of θ hence demonstrated significant Bonferroni-corrected genetic differentiation among some Atlantic populations from the Bay of Biscay, mainly involving the PA Ad sample (not reported; but see '*Genetic differentiation among samples*' for support of differentiation involving the Pertuis d'Antioche samples). Finally, estimates of θ at locus *MT* demonstrated no clear-cut distinction of the Mediterranean or of a set of Mediterranean populations with Atlantic populations (Table 3; see also Fig. 2D). Small but significant Bonferroni corrected genetic differentiation were found between samples from La Coubre and the subadults from the Loire River among of Bay of Biscay populations ($\theta = 0.078$; $P < 0.05$), but also between the Western Mediterranean Hérault and Rhône samples ($\theta = 0.066$; $P < 0.05$) (not reported).

No significant patterns of IBD were detected for the full set of populations or only Atlantic populations, both in the multi- or single-locus cases. Results were unchanged when considering all populations or only adult populations (details not given).

Genetic differentiation among replicated samples

No genetic differentiation was found among temporarily replicated sample (except once: locus *Am2B3-2* at Pertuis d'Antioche; Table 4), indicating stability in genetic structure at the local scale of Bay of Biscay's estuaries or bays. Nevertheless, results demonstrated that relative differentiation among samples expressed by the ratio of θ among replicated samples on θ at the scale of Bay of Biscay was often high (i.e. >100%), indicating more differentiation at a single site than over the Bay of Biscay. A role for chaotic genetic patchiness can then not completely discarded.

Discussion

This study provides a thorough genetic analysis on populations of the common sole, *Solea solea*, along the east European Atlantic continental shelf and across the Mediterranean Sea using three EPIC markers. It allows investigating patterns of genetic differentiation through time in the Bay of Biscay populations where several cohorts were sampled at the same location (Fig. 1; Table 1). Using three EPIC markers, the present study re-examined the genetic structure of sole populations along the European Atlantic continental shelf and across the Mediterranean Sea. For most populations of the Bay of Biscay, several cohorts were sampled at the same time, allowing a temporal analysis of the patterns of genetic differentiation. Heterozygosity values that we found (0.32, 0.53, and 0.63 for locus *Am2B3-1, Am2B3-2*, and *MT*, respectively) are much higher than those observed for allozyme loci (range: 0.03 to 0.11; Exadactylos et al. 1998). Polymorphism observed at the *MT* locus is based on single-repeat, microsatellite-like variation of alleles (not shown), and exhibited a level of diversity comparable to those obtained for microsatellite loci in sole (range: 0.55-0.83; Garoia et al. 2006).

Genetic differentiation using EPICs : multilocus vs single-locus interpretations

At the multilocus level, results using EPICs agreed with the differentiation reported among Atlantic and Mediterranean populations using allozymes (Kotoulas et al. 1995; Exadactylos et al. 1998). They also supported eastward-westward differentiation among Mediterranean populations of sole for allozymes (Kotoulas et al. 1995), and mtDNA (Guarniero et al. 2002). This Mediterranean division has been reported in other flatfish (flounder, *Platichthys flesus*, Borsa et al. 1997b; turbot, *Scophthlamus maximus:* Suzuki et al. 2004), or roundfish (e.g. sea bass, *Dicentrarchus labrax*: Bahri-Sfar et al. 2000; mackerel, *Scomber scombrus*: Zardoya et al.

2004). However, such a pattern of genetic differentiation within the Mediterranean Sea is not observed in every flatfish species (brill, *Scophthalmus rhombus*: Blanquer et al. 1992). Finally, the Adriatic population is differentiated from all other Mediterranean populations (Fig. 2A; Table 3). This pattern was already demonstrated in other species (e.g. *Sardina pilchardus*,Tinti et al. 2002; *Mullus barbatus*, Garoia et al. 2004; *Pomatoschistus minutus*, Steffani and Thorley 2003, Gysels et al. 2004), including a flatfish (flounder; Borsa et al. 1997b), but not in sole. Hence, observed multilocus differentiation among sole populations within the Mediterranean using EPICs is in accordance with previous studies that suggested different stocks. Differentiation among such populations could be due to the complex history of the Mediterranean that was strongly impacted during glacial episodes. This may lead to postulate the existence of distinct refuges in the Mediterranean as well as possible partial recolonisation by populations from the Atlantic that would explain westward-eastward differentiation (see details in, e.g. Zardoya et al. 2004; Magoulas et al. 2006). Maintenance of differentiation through time may also be favored by a much higher fragmentation than in the Atlantic due to other factors such as lower densities and lesser suitability of habitats for sole that may prevent gene flow (Sartor et al. 2002).

Conversely to significant multilocus patterns of population differentiation in the Mediterranean, observed genetic variation support large genetic homogeneity for sole populations in the Atlantic, from Portugal to Denmark (Table 3; Fig. 2A). This further extends results from Kotoulas et al. (1995) that indicated genetic homogeneity for Bay of Biscay and English Channel samples, but not for Atlantic samples further south or north that were not previously considered. Hence, as for other flatfishes such as plaice (Hoarau et al. 2002, 2004), flounder (Borsa et al. 1997b), turbot (Nielsen et al. 2004), and sand sole (*Solea lascaris*, Pinheiro et al. 2005), the Atlantic population of sole could function as one single panmictic unit. Exadactylos et al. (2003) indicated however differentiation of sole populations around the British Isles.

If interpretation of the multilocus patterns of spatial genetic differentiation in sole using EPICs was consistent with previously published studies using allozymes and mtDNA in sole, and with results pertaining to many other fish species, separate results from each single EPIC marker highlighted a very particular pattern of differentiation (Fig. 2B, C, D). Namely, locus *Am2B3-2* revealed a clear-cut Mediterranean-Atlantic differentiation, but no significant differentiation within the Mediterranean or Atlantic populations was observed (Table 3; Fig. 2C). Such a pattern was reported for the bonito (*Sarda sarda*, Pujolar et al. 2001; Viñas et al. 2004), and the brill (Blanquer et al. 1992). On the other hand, locus *Am2B3-1* demonstrated differentiation within the Mediterranean basin, separating the Eastern Mediterranean populations from all the other populations (Fig. 2B). This pattern was also reported in the mackerel, the bluefin tuna (*Thunnus thynnus thynnus*) and various sparids (Bargelloni et al. 2003; Carlsson et al. 2004; Zardoya et al. 2004), and was interpreted as a results of secondary contact after allopatric divergence. At locus *MT*, genetic differentiation between the Mediterranean and Atlantic was not supported. Absence of genetic differentiation among Atlantic and Mediterranean populations was also recorded in chub mackerel (*Scomber japonicus*, Zardoya et al. 2004), white anglerfish (*Lophius piscatorius*, Charrier et al. 2006), and blue jack and horse mackerels (*Trachurus picturatus* and *T. trachurus*, respectively, Karaiskou et al. 2004). In sole, the absence of differentiation could be due to effect of the microsatellite-like genetic diversity scored at locus *MT* (H_{obs} = 0.63). Theory demonstrated that microsatellite-like markers with high gene diversity exhibited lower and often not significant levels of population differentiation (see O'Reilly et al. 2004 for details). Using fourteen microsatellite loci, O'Reilly et al. (2004) demonstrated such a relationship in walleye pollock (*Theragra chalcogramma*). Nevertheless, this hypothesis is rather unlikely at locus *MT* in sole as this phenomenon was primarily recorded for locus with $H_{obs} > 0.7$ (Olsen et al 2004; O'Reilly et al 2004).

Whatever the mechanisms underlying genetic divergence at particular locus such as locus *MT*, the concordance of pattern of differentiation across multiple loci within a species are essential to the definition of true phylogeographical boundaries (Avise 1998). Such a concordance is then clearly not reach in sole, and the disparate single-locus patterns of genetic differentiation - hence the different genealogies and levels of gene flow - observed in this study indicate that history of sole in the Mediterranean Sea and the Eastern Atlantic is complex and that any multilocus summary as described above should be cautiously considered. Because most recent studies dealing with population structure of marine fishes in the Atlantic and the Mediterranean were mostly based on patterns of variation at one mtDNA locus, they very often revealed only one of the aforementioned pattern at the species level. For instance, comparative studies of population structure for five sparids provided by Bargelloni et al. (2003) revealed that each species exhibited one unique pattern of mtDNA differentiation between the Atlantic and the Mediteranean (i.e. presence or absence of significant genetic variation between water bodies; hence mostly the presence of one or two mtDNA clades). Similar results were found by Zardoya et al. (2004) and Charrier et al. (2006). As results based on mtDNA data are aimed to investigate historical demography of species, they might be incomplete and complementary analyses of variation at nuclear genes. EPICs (e.g. Palumbi and Baker 1994; Durand et al. 2005; Hickerson and Cunningham 2005; this study), allozymes (e.g.

Bargelloni et al. 2003), or microsatellites (e.g. Carlsson et al. 2004) can provide reliable information to interpret evolutionary history of the species more thoroughly (Hare 2001; Buonaccorsi et al. 2001). Studies that jointly used different markers to investigate differentiation within and among the Eastern Atlantic and the Mediterranean Sea division are scarce (De Innocentiis et al. 2001; Bargelloni et al. 2003; Carlsson et al. 2004; Lemaire et al. 2005). To our knowledge, only two studies in this area considered variation at one intronic marker (Ely et al. 2002; Nakadate et al. 2005). Among those studies, only Bargelloni et al. (2003) reported discordance in patterns of genetic differentiation among markers (i.e., allozymes and mtDNA) for two over five sparids species they studied (*Pagrus pagrus*, *Pagellus bogaraveo*). Data for sole are the first ones to report such differences in patterns of genetic differentiation within a single class of polymorphic nuclear loci (even though microsatellite-like polymorphism to locus *MT*).

Such distinct signals of genetic differentiation among EPIC markers may be due to stochastic variation occurring among gene tree topologies because of different, but possibly all confirming to neutral expectations, demographic histories of each gene. Nevertheless, as levels of differentiation could reach quite distinct values across loci, we may hypothesize that drift and gene flow are not the only causes for the observed genetic variation, but that effect of late and recent population history combined with selection in shaping the genetic structure of sole populations. Testing for selection is difficult as most reliable tests for selection imply more loci (≥ 10; review in Beaumont 2005), and/or specific type of loci (e.g. microsatellites; Schlötterer 2002). EPIC markers are considered as being neutral, but they may be indirectly subject to selection by the so-called Hill-Robertson effect (e.g. Barton 2000). In such a case, selection truly acts on neighbouring coding nucleotidic sites, but its effects may also be detectable across a stretch of DNA sequence whose length depends on recombination rate and effective population size. Selective hypothesis in shaping observed of Atlantic-Mediterranean differentiation is long-standing, but is traditionally overcome by purely neutral scenarios implying historical and demographic processes. However, Cimmaruta et al. (2005) recently demonstrated clinal variation matching temperature and salinity variations for hake (*Merluccius merluccius*) across the Mediterranean was very probably selective and not resulting from secondary contact (see also Launey et al. 2002 in flat oyster, *Ostrea edulis*).

Temporal differentiation within Bay of Biscay estuaries

Temporal differentiation among cohorts at single locales has been poorly investigated in sole. Only Kotoulas et al. (1995) addressed this issue and demonstrated moderate differentiation when comparing one adult (sampled in 1981) and three juveniles (successively sampled in 1986) cohorts in the Vilaine estuary. In this study, whatever the location considered, no genetic multilocus differentiation was found among temporally replicated samples of the Bay of Biscay (Tables 3 and 4). Only locus *Am2B3-2* indicated significant differentiation among juveniles and adult samples, but due to Pertuis d'Antioche samples only (Table 4). Absence of multilocus temporal genetic differentiation supports the expectation drawn from biological data because of the mixing in spawning areas of mature individuals originating from adjacent nurseries, and because of probably passive diffusion of eggs and larvae to nursery areas (Dorel et al. 1991; Koutsikopoulos et al. 1991). Hoarau et al. (2005) also reported absence of genetic differentiation among cohorts in plaice, both for one North Sea and one Icelandic populations. As sole and plaice share most main life-history features (e.g. high fecundity), observed panmixia at both a local (among samples taken at one location; Table 4) and a more global (Atlantic; Table 4) scale is not really surprising. Nevertheless, we demonstrated that in most cases, genetic variation among temporal samples within estuarine/coastal population clearly exceeded the geographical component (i.e., among Bay of Biscay's subadult samples) (Table 4). This might indicate that despite one overall context that favoured panmixia, other mechanisms promoting subtle genetic patchiness a local scale might act in the genetic structuring of populations (Maes et al. 2006). As noted by Dannewitz et al. (2005), the statistical power in the within-population (temporal) analyses becomes low because of small sample sizes. This might participate to the many non-significant comparisons observed in sole (Table 4). Interestingly, it was shown in flounder that some alleles and/or allelic combinations of few allozymic loci were favored in estuaries of Loire, Vilaine and Gironde, possibly indicating genetic patchiness due to selective effects, whereas alleles at other loci were not (Marchand et al. 2003). Results pertaining to locus *Am2B3-2* at Pertuis d'Antioche can be interpreted in this way, and both non-selective and selective hypothesis still need to be thoroughly investigated.

Hence, further works at the spatial (i.e., investigation about patterns of differentiation of the distinct loci) and the temporal (i.e., stability of genetic structure across cohorts at a given location) levels should also help to define more precisely how drift and/or selection combine in sole populations.

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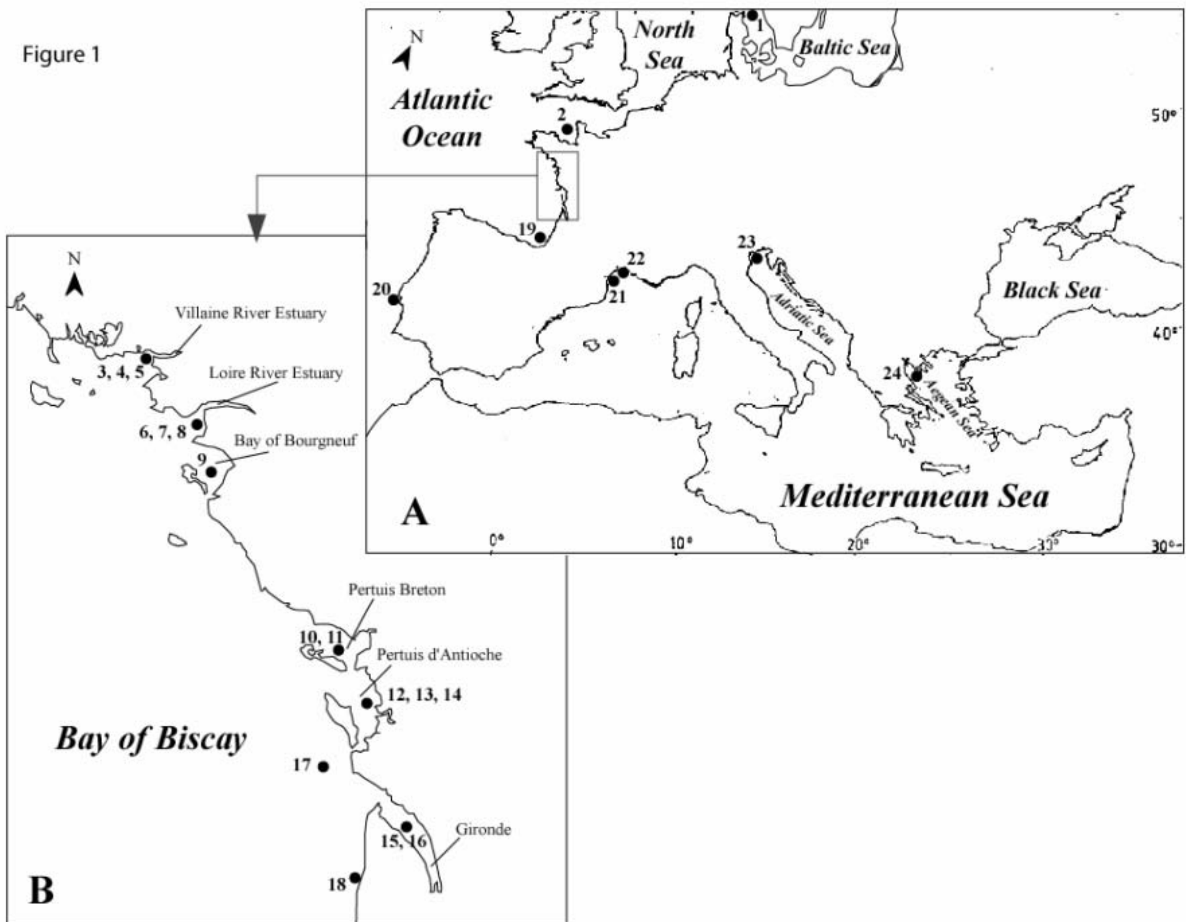

Figure 1: Sampling locations for common sole, *Solea solea*, in Northeastern Atlantic and Mediterranean Sea. **A -** Overall distribution of sampling locations; **B -** Bay of Biscay samples. Several numbers located close a single dot indicate sampling of distinct samples at the same place . Details are provided in Table 1.

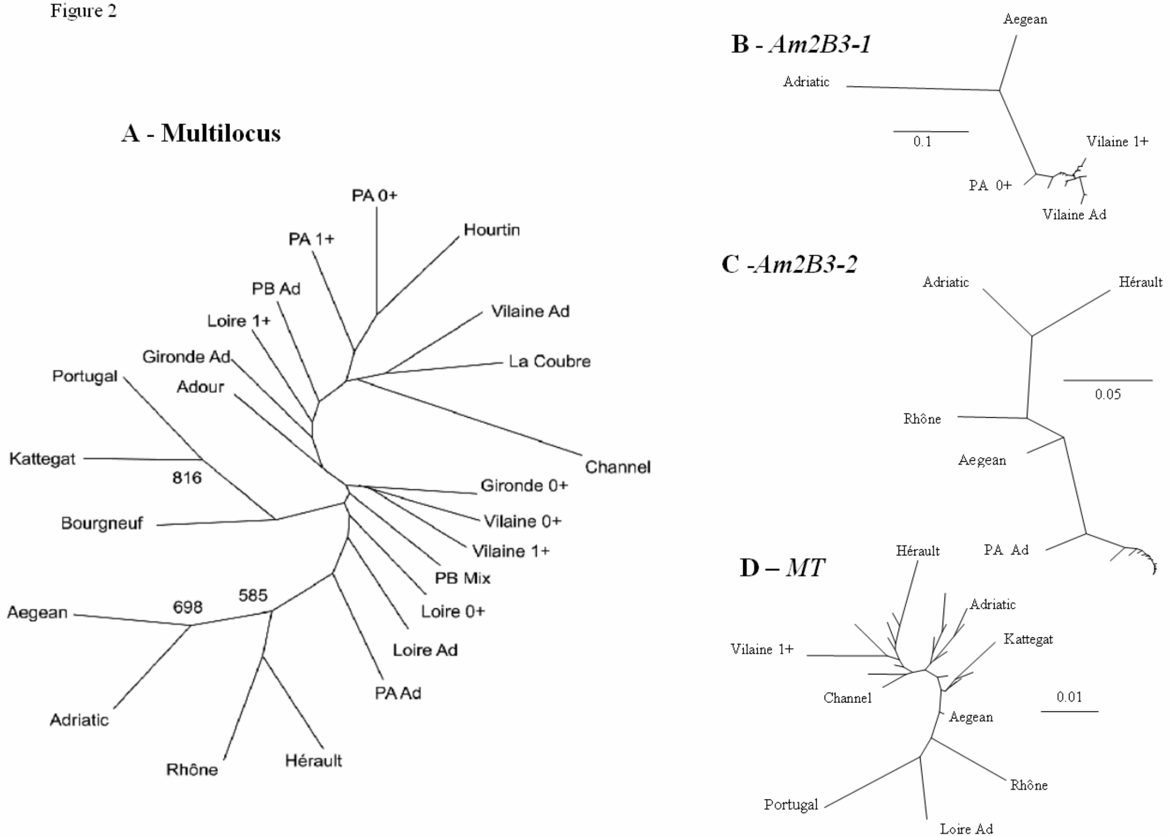

Figure 2: **A** - Multilocus neighbour-joining tree of the 24 sole samples based on distance proposed by Reynolds et al.'s (1983). The set of Mediterranean populations is highlighted. Names of samples as in Table 1. Bootstrap values of more than 0.50 are given. Neighbour-joining tree of sole samples for: **B** - locus *Am2B3-1*. **C** - locus *Am2B3-2*, and **D** - locus *MT*. In single locus analyses, names of most samples were not indicated because of space requirement.

Table 1: Sole samples considered in this study. Dates of sampling and age of individuals in each sample are given. *n* represents the individual sample size.

Sample	Label	Specific location		Sampling date	*n*	Age
North Sea–Baltic Sea	1	Kattegat	Denmark, North Sea–Baltic Sea transition	2003	30	Subadults
Atlantic	2	English Channel	St Brieuc Bay	2003	30	Subadults
	3	Vilaine	Bay of Biscay, Vilaine estuary	2000	30	Subadults
	4	Vilaine	Bay of Biscay, Vilaine estuary	2001	30	0+
	5	Vilaine	Bay of Biscay, Vilaine estuary	2001	30	1+
	6	Loire	Bay of Biscay, Loire estuary	2000	30	Subadults
	7	Loire	Bay of Biscay, Loire estuary	2001	30	0+
	8	Loire	Bay of Biscay, Loire estuary	2001	30	1+
	9	Bourgneuf	Bay of Biscay, Bay of Bourgneuf	2000	30	Subadults
	10	Pertuis Breton Mix	Bay of Biscay	2001	70	Mix 0+, 1+
	11	Pertuis Breton	Bay of Biscay	2000	30	Subadults
	12	Pertuis d'Antioche	Bay of Biscay	2000	30	Subadults
	13	Pertuis d'Antioche	Bay of Biscay	2001	30	0+
	14	Pertuis d'Antioche	Bay of Biscay	2001	30	1+
	15	Gironde	Bay of Biscay, Garonne River estuary	2000	29	Subadults
	16	Gironde	Bay of Biscay, Garonne River estuary	2000	30	0+
	17	Gironde	Bay of Biscay, off Gironde estuary (La Coubre)	2001	30	Subadults
	18	Gironde	Bay of Biscay, south Gironde estuary (Hourtin)	2001	30	Subadults
	19	Adour	Bay of Biscay, Adour River estuary	2001	30	Subadults
	20	Portugal	Algarve, off Faro	2002	23	Subadults
Mediterranean Sea	21	Hérault	Gulf of Lions, off Hérault River estuary	2001	30	Subadults
	22	Rhône	Gulf of Lions, off Rhône River estuary	2002	30	Subadults
	23	Adriatic	Adriatic Sea, off Venice	2002	29	Subadults
	24	Aegean	Aegean Sea, off Thessaloniki	2002	27	Subadults

Dates of sampling and age of individuals in each sample are given. *n* represents the individual sample size

Table 2: Allele frequencies recorded at each individual locus in the 24 sole samples considered in this study (AD: subadults). Alleles are labeled alphabetically. *n*: number of individuals studied; H_{exp}: expected heterozygosity, H_{obs}: observed heterozygosity. *f*: Weir & Cockerham's (1984) fixation index measuring departure from theoretical Hardy-Weinberg expectations in the single-locus and the multilocus cases. HWE indicates departures from equilibrium (corrected for multiple test; *: $P<0.05$; ***: $P<0.001$; NS: not significant).

	1 Kattegat	2 English Channel	3 Vilaine Ad.	4 Vilaine 0+	5 Vilaine 1+	6 Loire Ad.	7 Loire 0+	8 Loire 1+	9 Bourgneuf	10 Pertuis Breton Mix	11 Pertuis Breton Ad.	12 Pertuis Antioche Ad.
MT locus												
n	29	27	27	30	27	26	30	30	23	70	27	26
A	0.0172	0.0185	0.0556	0.0500	0.0000	0.0385	0.0833	0.0000	0.0217	0.0071	0.0185	0.0000
B	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
C	0.0000	0.0000	0.0185	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
D	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
E	0.0000	0.0556	0.0000	0.0167	0.0741	0.0385	0.0167	0.0333	0.0217	0.0500	0.0370	0.0000
F	0.0000	0.0000	0.0185	0.0000	0.0556	0.0000	0.0000	0.0167	0.0000	0.0000	0.0000	0.0000
G	0.0345	0.0370	0.0370	0.0833	0.1111	0.2500	0.1000	0.0167	0.1087	0.0857	0.1481	0.1346
H	0.6552	0.4074	0.2963	0.4167	0.4074	0.3846	0.3833	0.3667	0.4783	0.3000	0.2593	0.2500
I	0.1897	0.4074	0.3889	0.3000	0.2037	0.1923	0.3000	0.4000	0.2174	0.4000	0.2963	0.3654
J	0.0517	0.0370	0.0926	0.0667	0.1296	0.0577	0.0500	0.0833	0.0435	0.0929	0.1481	0.1346
K	0.0517	0.0370	0.0741	0.0667	0.0185	0.0000	0.0667	0.0667	0.0870	0.0571	0.0741	0.0962
L	0.0000	0.0000	0.0000	0.0000	0.0000	0.0385	0.0000	0.0167	0.0000	0.0000	0.0185	0.0192
M	0.0000	0.0000	0.0185	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0071	0.0000	0.0000
N	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0217	0.0000	0.0000	0.0000
H_{exp}	0.5372	0.6730	0.7554	0.7299	0.7687	0.7594	0.7514	0.7040	0.7169	0.7334	0.8085	0.7730
H_{obs}	0.5172	0.5556	0.5926	0.6667	0.5185	0.4615	0.6667	0.4000	0.5652	0.5857	0.5926	0.6923
f	0.038	0.177	0.219	0.088	0.330	0.397	0.115	0.436	0.215	0.203	0.271	0.106
HWE	NS	NS	NS	NS	NS	***	NS	***	NS	NS	NS	NS
Am2B3-1 locus												
n	30	30	26	30	30	30	30	30	30	69	30	29
A	0.1167	0.1333	0.1154	0.1167	0.1833	0.0833	0.0667	0.1333	0.1333	0.1014	0.1667	0.1207
B	0.6167	0.6333	0.6731	0.6333	0.4833	0.5333	0.6833	0.5500	0.5333	0.6522	0.7000	0.6379
C	0.2167	0.1833	0.2115	0.2333	0.2333	0.3167	0.1667	0.2833	0.3167	0.1812	0.1333	0.2069
D	0.0500	0.0500	0.0000	0.0167	0.1000	0.0667	0.0833	0.0333	0.0167	0.0652	0.0000	0.0345
H_{exp}	0.5661	0.5542	0.4985	0.5395	0.6797	0.6141	0.5023	0.6085	0.6073	0.5312	0.4723	0.5439
H_{obs}	0.5667	0.5000	0.3846	0.6000	0.7667	0.5333	0.5333	0.5333	0.6000	0.5072	0.6000	0.5517
f	−0.001	0.099	0.232	−0.114	−0.131	0.134	−0.063	0.125	0.012	0.045	−0.276	−0.015
HWE	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Am2B3-2 locus												
n	30	30	26	29	30	30	30	30	30	68	30	29
A	0.0000	0.0000	0.0000	0.0172	0.0000	0.0167	0.0167	0.0000	0.0000	0.0147	0.0000	0.0172
B	0.7833	0.9167	0.9231	0.8621	0.9333	0.7833	0.7667	0.8167	0.8500	0.8382	0.8000	0.7241
C	0.2167	0.0833	0.0769	0.1207	0.0667	0.2000	0.2167	0.1833	0.1500	0.1471	0.2000	0.2586
H_{exp}	0.3452	0.1554	0.1448	0.2462	0.1266	0.3520	0.3712	0.3045	0.2593	0.2776	0.3254	0.4156
H_{obs}	0.3000	0.1667	0.1538	0.2759	0.1333	0.3000	0.4000	0.3000	0.2333	0.2941	0.3333	0.5517
f	0.133	−0.074	−0.064	−0.123	−0.055	0.150	−0.079	0.015	0.102	−0.060	−0.025	−0.335
HWE	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Multilocus												
f	0.045	0.118	0.194	−0.018	0.101	0.254	0.016	0.240	0.119	0.101	0.051	−0.037
HWE	NS	NS	NS	NS	NS	*	NS	NS	NS	NS	NS	NS

Table 2: continued

	13 Pertuis Antioche 0+	14 Pertuis Antioche 1+	15 Gironde Ad.	16 Gironde 0+	17 Gironde Ad. La Coubre	18 Gironde Ad. Hourtin	19 Adour	20 Portugal	21 Hérault	22 Rhône	23 Adriatic	24 Aegean Sea
MT locus												
n	30	30	27	29	28	29	30	18	28	26	24	24
A	0.0000	0.0000	0.0370	0.0000	0.0179	0.0000	0.0167	0.0556	0.0000	0.0769	0.0000	0.0208
B	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0278	0.0000	0.0000	0.0000	0.0000
C	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
D	0.0000	0.0000	0.0000	0.0000	0.0000	0.0172	0.0000	0.0000	0.0000	0.0192	0.0000	0.0000
E	0.0000	0.0000	0.0000	0.0345	0.0000	0.0172	0.0167	0.0000	0.0000	0.0000	0.0000	0.0000
F	0.0500	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0769	0.0208	0.0000
G	0.0833	0.0833	0.0741	0.1552	0.0357	0.0862	0.1500	0.1389	0.0893	0.1923	0.0625	0.1250
H	0.3000	0.3500	0.2963	0.2931	0.3214	0.2931	0.3167	0.5278	0.3214	0.2885	0.4375	0.4583
I	0.4833	0.4333	0.4074	0.3276	0.5000	0.3793	0.4000	0.2500	0.2143	0.3077	0.3542	0.3333
J	0.0667	0.0500	0.0556	0.1207	0.0893	0.1207	0.0333	0.0000	0.3214	0.0192	0.1042	0.0417
K	0.0167	0.0833	0.1111	0.0172	0.0357	0.0690	0.0667	0.0000	0.0536	0.0192	0.0208	0.0208
L	0.0000	0.0000	0.0000	0.0172	0.0000	0.0172	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
M	0.0000	0.0000	0.0185	0.0345	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
N	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
H_{exp}	0.6734	0.6847	0.7372	0.7786	0.6474	0.7556	0.7232	0.6540	0.7500	0.7873	0.6817	0.6746
H_{obs}	0.4000	0.4333	0.8148	0.6897	0.6786	0.6897	0.6667	0.5556	0.6786	0.6154	0.7083	0.9167
f	0.410	0.371	–0.107	0.116	–0.049	0.089	0.079	0.154	0.097	0.222	–0.040	–0.369
HWE	***	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	**
Am2B3-1 locus												
n	30	30	30	30	29	30	30	22	30	29	29	27
A	0.0333	0.0833	0.0333	0.1000	0.1034	0.0833	0.0833	0.1364	0.0667	0.0517	0.0345	0.0185
B	0.6000	0.6500	0.7333	0.5500	0.6897	0.6000	0.5833	0.6136	0.6667	0.7414	0.0517	0.3889
C	0.3500	0.1833	0.2167	0.2500	0.1724	0.2833	0.2333	0.2045	0.1833	0.1724	0.8621	0.5370
D	0.0167	0.0833	0.0167	0.1000	0.0345	0.0333	0.1000	0.0455	0.0833	0.0345	0.0517	0.0556
H_{exp}	0.5249	0.5390	0.4209	0.6254	0.4912	0.5610	0.5983	0.5740	0.5192	0.4241	0.2547	0.5674
H_{obs}	0.6667	0.6000	0.3667	0.6000	0.5517	0.4667	0.5667	0.5909	0.4000	0.3793	0.2759	0.4815
f	–0.276	–0.115	0.131	0.040	–0.126	0.171	0.054	–0.030	0.233	0.107	–0.085	0.154
HWE	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Am2B3-2 locus												
n	30	30	30	30	29	30	30	23	30	29	29	27
A	0.0000	0.0000	0.0000	0.0167	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0172	0.0000
B	0.9000	0.8833	0.8000	0.8500	0.8793	0.9333	0.8167	0.7826	0.5500	0.6034	0.4655	0.6111
C	0.1000	0.1167	0.2000	0.1333	0.1207	0.0667	0.1833	0.2174	0.4500	0.3966	0.5172	0.3889
H_{exp}	0.1831	0.2096	0.3254	0.2638	0.2160	0.1266	0.3045	0.3478	0.5034	0.4870	0.5245	0.4843
H_{obs}	0.2000	0.2333	0.4000	0.3000	0.2414	0.1333	0.3667	0.3478	0.5667	0.5172	0.5517	0.4815
f	–0.094	–0.115	–0.234	–0.140	–0.120	–0.055	–0.208	–0.000	–0.128	–0.063	–0.053	0.006
HWE	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

Table 2: continued

	13 Pertuis 0+	Antioche	14 Pertuis 1+	Antioche	15 Gironde Ad.	16 Gironde 0+	17 Gironde Ad. La Coubre	18 Gironde Ad. Hourtin	19 Adour	20 Portugal	21 Hérault	22 Rhône	23 Adriatic	24 Aegean Sea
Multilocus														
f	0.084		0.118		–0.067	0.048	–0.088	0.108	0.016	0.053	0.073	0.112	–0.052	–0.091
HWE	NS		NS		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

Alleles are labeled alphabetically. HWE indicates departures from equilibrium (corrected for multiple test)

n Number of individuals studied, H_{exp} expected heterozygosity, H_{obs} observed heterozygosity, *f* Weir and Cockerham's (1984) fixation index measuring departure from theoretical Hardy–Weinberg expectations in the single-locus and the multilocus cases, *NS* not significant

$^{*}P < 0.05$

$^{***}P < 0.001$

Table 3: Estimates of multilocus and single-locus genetic differentiation (θ) among various sets of sole samples. When computing genetic differentiation among area, populations of each area were previously pooled.

Table 3 Estimates of multilocus and single-locus genetic differentiation (θ) among various sets of sole samples

	Multilocus	*Am2b1*	*Am2b2*	*MT*
All populations	0.043**	0.061**	0.075**	0.015
Atlantic	0.009	0.002	0.010	0.014
Mediterranean	0.176***	0.313***	–0.001	0.031
Atlantic *vs* Mediterranean	0.068***	0.211***	0.057***	0.002
Atlantic *vs* Western Mediterranean only	0.049**	0.006	0.198***	0.011
(Atlantic + Western Mediterranean) *vs* Eastern Mediterranean	0.150***	0.272***	0.200***	0.009
Western Mediterranean *vs* Eastern Mediterranean	0.135***	0.354***	–0.006	0.018

When computing genetic differentiation among area, populations of each area were previously pooled

$^{**}P < 0.01$; $^{***}P < 0.001$

Table 4: Relative genetic differentiation among Bay of Biscay samples expressed as the ratio of spatial genetic differentiation over the subadult samples to temporal genetic differentiation expressed at a single site. *: $P < 0.05$; - : no data because of negative θ estimates among temporarily replicated samples.

	θ among Bay of Biscay subadult samples (n = 9)		θ among replicated samples at a single site	Percentage of variation of θ over time at a single site relative to θ across Bay of Biscay's subadult samples
Multilocus	0.00836	Vilaine (n = 3)	0.00508	60.76
		Loire (n = 3)	0.00653	78.11
		Pertuis Breton (n = 2)	−0.00135	–
		Pertuis d'Antioche (n = 3)	0.00847	101.32
		Gironde (n = 2)	0.00889	106.34
Am2b3-1	0.00468	Vilaine (n = 3)	0.01168	249.57
		Loire (n = 3)	0.00687	146.80
		Pertuis Breton (n = 2)	0.00135	28.85
		Pertuis d'Antioche (n = 3)	0.00829	177.14
		Gironde (n = 2)	0.02539	542.52
Am2b3-2	0.01498*	Vilaine (n = 3)	0.00167	11.15
		Loire (n = 3)	−0.01443	–
		Pertuis Breton (n = 2)	−0.00377	–
		Pertuis d'Antioche (n = 3)	0.06342*	423.36
		Gironde (n = 2)	−0.00118	–
MT	0.00810	Vilaine (n = 3)	0.00347	42.84
		Loire (n = 3)	0.01385	170.99
		Pertuis Breton (n = 2)	−0.00223	–
		Pertuis d'Antioche (n = 3)	−0.00706	–
		Gironde (n = 2)	0.00110	13.58