ANNEX

Subtle genetic differentiation in a high gene flow marine species (*Pomatoschistus minutus*) living in a dynamic environment

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We assessed genetic differentiation and diversity in 15 populations of sand goby (*Pomatoschistus minutus*) within the Southern Bight of the North Sea, using seven microsatellite and thirteen allozyme loci. While no significant differentiation was observed with allozymes, microsatellite loci revealed a moderate level of differentiation (overall F_{ST}=0.026; overall R_{ST}=0.058). The hierarchical analysis of molecular variance revealed a significant differentiation (p<0.01) between Oosterschelde, Westerschelde, coastal and offshore samples with microsatellites but not with allozymes. This structure was confirmed by multidimensional scaling analysis based on Nei and $\partial\mu^2$ genetic distances. The comparison of the different estimators (F_{ST} and R_{ST}) of differentiation lead us to conclude that this genetic structure was mainly due to an historical event, namely colonisation of the estuary during its establishment, and to restricted actual gene flow. Therefore, using all of these loci, we found that the 15 natural populations of the sand goby can be clustered into two different breeding units, namely the Oosterschelde and coastal populations. Despite this clear distinction, there were indications of an actual complex dynamic local structure that would bear further examination to understand the processes involved in the differentiation of these two breeding units.

INTRODUCTION

One of the most interesting challenges in evolutionary biology is to assess the processes responsible for genetic differentiation of distantly or closely related populations without any obvious barriers. In general, genetic drift and adaptation to local conditions are known to counteract gene flow, but recently other factors such as complex mating systems (Ross, 2001), sex-biased gene flow (Scribner *et al*, 2001) and chaotic processes (Huisman & Weissing, 2000) were shown to influence the genetic structure of populations. Given the potential association of high dispersal capabilities and barriers to gene flow of adults or larvae of marine species, it remains unclear to understand how divergence can arise and be maintained in the marine environment. Marine organisms with a pelagic larval stage have a high potential for gene flow resulting in a lower degree of genetic differentiation than freshwater or anadromous species (Ward *et al*, 1994; DeWoody and Avise, 2000). Indeed, many marine species show little or no intraspecific genetic differentiation even on a scale of thousands of kilometres (Suk *et al*, 1996; Lundy *et al*, 1999). However, the marine environment, although physically less structured than freshwater or terrestrial ecosystems, is not homogeneous. Fronts, local and global oceanic current patterns, bottom topography and climatic barriers restrict the dispersal of pelagic larvae and adults and promote genetic differentiation within populations (Sinclair, 1988; Bowen and Grant, 1997; Lessios *et al*, 1999). In recent years this has been confirmed by high resolution genetic markers such as microsatellites, which have yielded evidence for a subtle but significant genetic structure within marine species

(Ruzzante *et al*, 1998; Lemaire *et al*, 2000; Wirth and Bernatchez, 2001). However, most studies have been carried out on a large geographic scale of hundreds or thousands of kilometres on species migrating over vast distances. Thus, little information is available as to whether small-scale systems of larval retention in the ocean exist and influence dispersal.

The Belgian Continental Shelf, situated in the Southern Bight of the North Sea, is characterised by a combination of sand banks and gullies which are continuously being swept by strong tidal currents (De Moor and Lanckneus, 1990). A long-shore and inshore/off-shore gradient under the influence of the Schelde estuary is reflected in a number of abiotic factors (Nihoul and Hecq, 1984). Consequently, biotic factors vary according to distance from the coast (Govaere *et al*, 1980; Offringa *et al*, 1996; Dewicke, 2001). These physical and biological differences might structure local fish populations (Stepien *et al*, 2001; Rocha *et al*, 2002).

This paper focuses on the small-scale genetic differentiation of a marine fish species, the sand goby *Pomatoschistus minutus*. It is one of the most common fishes along the area and it plays an important role in the food web as predator of benthos (Hamerlynck and Cattrysse, 1994) and prey for larger fish (Doornbos and Twisk, 1987). This annual species reproduces from May to July (Fonds, 1973) along the coasts of Northwestern Europe. Males build a nest and attract females to obtain eggs (Lindström, 1992) that they defend until the hatching of the larvae. The larvae are pelagic for 4 to 6 weeks and adopt a demersal life-style after metamorphosis. Adults are thought to have poor swimming abilities, yet they carry out a spawning migration (Fonds, 1973; Pampoulie *et al*, 1999). Given the dispersal capabilities of this species, we might expect only slight genetic differentiation among populations on the scale of the Southern Bight of the North Sea. On the other hand, the geomorphology of the shelf and the presence of small-scale gyre systems might limit dispersal and promote small-scale inter-population differentiation.

Microsatellites are frequently used to detect subtle population structure on a small geographic scale as they evolve very quickly and are considered representative of the whole genome because of their independence and assumption of neutrality (Goldstein and Schlötterer, 1999). Allozymes on the other hand, have a lower resolution when discerning population differentiation but might be more subjected to historical events and to selective pressure as shown in numerous studies (Mitton, 1997). A combination of these two markers might enable us to gain more knowledge on genetic structure among sand gobies in the Southern Bight of the North Sea.

The scope of this study is to:

1) examine whether any evidence exists for genetic differentiation of a small marine fish species with an extended pelagic larval phase at a relatively small geographic scale.

2) assess the pattern of differentiation exhibited by two markers with a different rate of evolution to discriminate between long and short term evolution.

Materials and Methods

Sampling on the Belgian Continental Shelf was carried out with the oceanographic research vessels R/V ‘Belgica’ and R/V ‘Zeeleeuw’ along an inshore/offshore gradient on the Coastal Banks (Sb, Ht), the Flemish Banks (K), the Westerschelde, and the offshore banks (Of1, Of2) (Figure 1). In addition, four samples were taken in the Oosterschelde (The Netherlands) for microsatellite analysis only. The latter area consists of a unique marine environment and is used as a nursery area for fish from the adjacent North Sea. Although partly separated from the sea by a dike system, about 80% of the inflow passes through, thus conserving the exchanges between the ecosystems (Hamerlynck and Hostens, 1994). One distant population has been sampled in Texel (Tx; The Netherlands) as outgroup.

Fishes were either frozen in dry ice or liquid nitrogen immediately after capture and kept in a –80°C freezer until analysis. Gobies were identified morphologically on the basis of the dermal papillae of the head according to Miller (1986), and biochemically according to Wallis and Beardmore (1984a,b).

Allozyme electrophoresis

Allelic variation was assayed for 9 populations at 8 enzymes coding for 13 loci, namely Creatine kinase (*CK-1**, EC 2.7.3.2), Lactate dehydrogenase (*LDH-A**, EC 1.1.1.27; *LDH-B**, EC 1.1.1.27; *LDH-C**, EC 1.1.1.27), Malate dehydrogenase (*MDH-1**, EC 1.1.1.37; *MDH-2**, EC 1.1.1.37), Phosphoglucomutase (*PGM-1**, EC 5.4.2.2; *PGM-2**, EC 5.4.2.2), Glucose phosphate isomerase (*GPI-1**, EC 5.3.1.9; *GPI-2**, EC 5.3.1.9), Glutamate oxaloacetate transferase (*GOT**, EC

2.6.1.1), Adenylate kinase (*AK**, EC 2.7.4.3) and Fumarate hydratase (*FH**, EC 4.2.1.2).

Liver, eye and muscle tissues were dissected and ground in distilled water. The samples were subjected to cellulose acetate allozyme electrophoresis (Richardson *et al*, 1986) using two continuous buffer systems: Tris-maleate (pH 7.8) and Tris-glycine (pH 8.8) as described by Hebert and Beaton (1989) with modification. Loci were stained according to recipes described by Hebert and Beaton (1989) and Richardson *et al* (1986). The fastest migrating locus was designated 1 or A according to the nomenclature of Shaklee *et al* (1990).

Microsatellite amplification

Allelic variation was assayed at seven microsatellite loci, *Pmin-01*, *Pmin-05* and *Pmin-10* described by Jones *et al* (2001a, b), and *Pmin-06*, *Pmin-07*, *Pmin-08* and *Pmin-11* (see sequences in Table 2). A total of 15 populations were assayed with an average sampling size of 50 individuals per population (Table 1). DNA samples were extracted from fin clips using a Chelex (Biorad, 10%) extraction protocol (Walsh *et al*, 1991).

For all primers sets used, PCR was conducted in a 10 µl reaction volumes containing specific amounts of primers and $MgCl_2$ ranging respectively from 10 to 20 mM and 0.6 to 2 mM. All PCR reactions were preceded by an initial denaturation step of 2 min at 95°C followed by 25 cycles extension steps: 1 min at 95°C, 1 min at the annealing temperature (60°C for *Pmin-01*; 62°C for *Pmin-05* and *Pmin-10*, 54°C for *Pmin-06*, 57°C for *Pmin-07*, 56°C for *Pmin-08* and 60°C for *Pmin-11*) and 1 min at 72°C. A final elongation step of 3 min at 72°C was performed.

PCR products were diluted with 5 µl (1:3) of stop-loading solution (Formamide 99%+Bromophenol blue) and were electrophoresed on 25 cm, 6% polyacrylamide gels and detected on an automatic sequencer (LI-COR, model 4200) using the software E-seq ver. 2.00 (LI-COR Inc., 2001). Products were scored using the software Gene ImagIR ver. 4.03 (Scanalytics Inc., 2001) several times to avoid scoring errors. Suspect individuals were deleted from the analysis.

Genetic data analysis

Allele frequencies, observed (H_O) and unbiased expected heterozygosity (H_E) were calculated in GENETIX ver. 4.0 (Belkhir *et al*, 1999). Hardy-Weinberg equilibrium (HWE) was calculated and tested for significance in GENEPOP version 3.1 (Raymond and Rousset, 1995). Significance levels were adjusted with a sequential Bonferroni test (Rice, 1989). Wright's single-locus *F*-statistics (Wright, 1969) were calculated from allele frequencies of all loci examined for each population according to Weir and Cockerham (1984) in GENETIX (θ). For the microsatellite loci, differentiation between populations was also quantified using the analogue *rho* of the R_{ST} of Slatkin (1995) following Goodman (1997) using the computer program RSTCALC (Goodman, 1997) and assuming the Stepwise Mutation Model (SMM; Kimura and Otha, 1978). Standard deviations of single-locus F_{ST} values were obtained by jack-knifing over all populations according to Weir (1990). Significance of multilocus F_{ST} and R_{ST} was assessed with permutation tests (1000 replicates). Pairwise genetic distances corrected for bias in sampling (Nei, 1978) was calculated in GENETIX assuming genetic drift-mutation equilibrium and a constant population size over time for both allozyme and microsatellite loci. For microsatellite loci, genetic distance between populations was also assessed by the specific distance $\partial\mu^2$ of Goldstein *et al* (1995) obtained with RSTCALC. A graphical representation of R_{ST} values was computed using the neighbour joining algorithm (Saitou and Nei, 1987) in the NEIGHBOUR program inferred in the PHYLIP software package (Felsenstein, 1995) in conjunction with TREEVIEW (Page, 1996). Genetic linkage disequilibrium between locus pairs was estimated according to Weir and Cockerham (1979) and tested on contingency tables under the null hypothesis of independence. A microsatellite multilocus estimate of the effective number of migrants (*Nm*) according to Slatkin's private allele model (1985) was assessed with GENEPOP. The effective number of migrants was also assessed according to the "Island model" (Wright, 1969). When significant genetic differentiation was observed between populations, a Mantel test (Mantel, 1967) was performed in GENETIX to test for isolation-by-distance. An Analysis of Molecular Variance (AMOVA) was carried out in ARLEQUIN version 2.0 (Schneider *et al*, 2000) to assess hierarchical partitioning among and within populations of genetic variability. We carried out a principal component analysis on the allele frequencies and a multidimensional scaling (MDS) approach on pairwise genetic distance (Nei, 1978; Goldstein *et al*, 1995) using respectively PCA-GEN version 1.2.1 (Goudet, 1999) and Statistica 5.1 (Statsoft Inc, 1997).

Results

Allozymes

Hardy-Weinberg Equilibrium

*Nine out of 13 scored allozyme loci were polymorphic in the nine populations analysed (*LDH-A**, *LDH-B**, *LDH-C**, *MDH-1**, *PGM-1**, *PGM-2**, *GPI-1**, *GPI-2* *and* GOT**; Table 3). The observed heterozygosity averaged over all loci ranged from 0.09 to 0.11. No inter-population differences in mean heterozygosity, number of alleles per locus or levels of polymorphism were observed (Table 3).*

All polymorphic loci were in HW equilibrium after Bonferroni correction with the exception of *LDH-C**, where a strong heterozygote deficit across all populations examined was observed, independent of sample size (ranging from 30 to 200 individuals). Neither a trend nor gradient in allele frequencies across sampling sites was observed at this locus. A Mantel test failed to show any correlation between Nei's (1978) genetic distances and geographic distances (p>0.05 under null hypothesis after 1000 permutations).

Population structure

As no clear differentiation was observed between all populations separately, samples were grouped according to the season (summer and winter) to assess temporal variation and variation of the *F*-estimates. The multilocus F_{ST} value (0.01) for 'summer-autumn' populations (Of1, Os, K1, W1 and Ra1) was significant (p<0.05), which was entirely due to a differentiation at locus *LDH-C** (F_{ST}=0.028, p<0.05). Excluding *LDH-C**, the multilocus F_{ST} was only 0.003 (not significant). Exact tests confirmed the differentiation at *LDH-C** (p=0.0001). The 'winter' populations (Ra3, Of2, Sb and K2) were less differentiated (F_{ST}=0.005) than the ‘summer’ populations (F_{ST} not significant). No differentiation at locus *LDH-C** was observed in this group.

Pair-wise genetic distances (Nei, 1978) calculated over all loci between the populations of sand goby were not significant. AMOVA revealed that within-population effects explained all the observed variation. Temporal variation in allele frequencies was assessed by comparing samples taken at approximately the same site in two different seasons. K2 was compared with K1, Sb with Ht and Oh with Of2 (Figure 1). Exact tests for allelic homogeneity (Raymond and Rousset, 1995a) showed no differences.

Neither the hierarchical analysis of molecular variance (Table 4) nor the PCA-GEN (data not shown) analysis showed consistent differentiation between the samples while the MDS revealed the presence of slight differentiation between offshore, Westerschelde and coastal populations (Figure 2a).

Microsatellite loci

Hardy-Weinberg Equilibrium

Although the seven microsatellite loci studied exhibited a high level of polymorphism (Table 2; $P_{(0.95)}$=1), two of them can be considered as highly polymorphic (*Pmin-01* and *Pmin-05*) while three are moderately polymorphic (*Pmin-06*, *Pmin-08* and *Pmin-10*) and two slightly polymorphic (*Pmin-07* and *Pmin-11*) compared to values found in literature. The number of alleles per locus across all populations ranged from 8 (*Pmin-11*) to 88 (*Pmin-05*). Observed heterozygosity averaged over all loci ranged from 0.62 to 0.76 in the 15 populations and tended to be lower than the expected heterozygosity. Genotypic proportions in 55 of 105 exact tests were out of HWE (Table 5). In particular, at *Pmin-10* no HWE was observed in any population excepted for K1. The overall excess of homozygotes for all loci combined, as quantified by the correlation of alleles within individuals (F_{IS}) was 0.163 (Table 6). Based on permutation tests (1000 replicates), the F_{IS} values were significant for six out of seven loci ($0.001<p<0.01$).

Exact tests for linkage-disequilibrium yielded several significant values ($0.01<p<0.05$) involving several pairs of loci without any consistency, thus suggesting that the results were not due to physical linkage of the marker loci. No linkage disequilibrium was observed between allozyme and microsatellite loci in the three common sampling sites (Of1, K1 and W1).

Population structure

The partitioning of genetic variance among and within the 15 populations as estimated by *F*-statistics showed a mean F_{ST} value of 0.026 and a F_{IS} of 0.163 while *R*-statistics showed a mean R_{ST} value of 0.058 and a R_{IS} of 0.197 (Table 6). Pair-wise comparisons between populations exhibited significant F_{ST} values following sequential Bonferroni adjustment, while R_{ST} values were not all significant (Table 7). The highest F_{ST} and R_{ST} values were observed between the off-shore populations and the other populations. The total observed differentiation was essentially due to *Pmin-06*, *Pmin-07* and *Pmin-10* for F_{ST} and to *Pmin-06* and *Pmin-07* for R_{ST} (Table 7). Without these three loci the mean F_{ST}, F_{IT} and F_{IS} were respectively 0.011, 0.133 and 0.120 while R_{ST} values were respectively 0.0271, 0.156 and 0.143. Nei's (1978) and $\partial\mu^2$ (Goldstein *et al*, 1995) distances exhibited significant values between all pairs of populations (Table 8). All pair-wise comparison remained significant after Bonferroni correction. The highest values were observed between offshore populations and the others, and to a lesser extent between Oosterschelde and coastal populations.

Based on Slatkin's private allele model (1985), the corrected number of migrants (*Nm*) was 7.07 while it varied between 2.7 and 24.6 based on Wright (1969).

A Mantel test failed to show any correlation between Nei's (1978) and $\partial\mu^2$ genetic distances and geographic distances (p>0.05 under null hypothesis after 1000 permutations).

Based on Nei's (1978) genetic distances, MDS clearly clustered the populations of the coastal area (K1, K2, Zs, Ra8, Ot1, Np) (except Ot2) and also the sample from Texel (Tx), the populations of the Oosterschelde (Oc7, Oc15, Oc37, Oc 21), the populations of the Westerschelde (W1, W2) and separated the offshore populations (Of1) from these three groups (Figure 2b; correlation values on axis 1 and 2: 0.311). Based on the $\partial\mu^2$ distance of Goldstein *et al*. (1995), MDS clearly clustered the Oosterschelde populations (except Oc21), the populations of the coastal area and separated the offshore population from these two groups (Figure 3; correlation values on axis 1 and 2: 0.1511). The principal component analysis carried out on the allele frequencies using PCA-GEN confirmed the presence of three clusters (with the exception of Ot2 which did not cluster with the coastal populations) as Westerschelde populations clustered with the coastal populations. (total inertia: 0.21; percent inertia per axis: 1:21.48, 2:13.72; F_{ST} per axis: 1:0.008, p=0.045, 2:0.005, p=0.046; global F_{ST}=0.035; data not shown). The obtained phenogram using the neighbour joining tree based on the R_{ST} values was concordant and indicated a separation between estuarine and coastal populations (Figure 4). Inter-annual temporal variation within sites (K and Ot) showed to be non-significant (see Figure 4).

The AMOVA analysis revealed a weak but highly significant interregional pattern of genetic structure of the Belgian population of sand gobies which was composed of four different groups, namely the offshore, the Oosterschelde, the Westerschelde and the coastal populations (Table 4).

DISCUSSION

Genetic diversity of sand goby populations

The degree of heterozygosity as assayed with allozymes was comparable to values found by Ward *et al* (1994) for marine species and other gobioid fishes (Suk *et al*, 1996; Geertjes *et al*, 2001). Wallis and Beardmore (1984a) found slightly lower values in the sand goby but studied more loci of which a fairly large proportion was monomorphic, thus decreasing overall values of heterozygosity. Polymorphism at most allozyme loci was comparable to the results of Wallis and

Beardmore (1984a), with the exception of the highly polymorphic *LDH-C*,* which was completely monomorphic in the former study, probably due to the use of different electrophoresis techniques.

The genetic diversity assessed as the expected level of heterozygosity H_E exhibited a wide range for the microsatellite loci (0.157-0.976) and were generally comparable to other marine fish species (García de León *et al*, 1997; Ruzzante *et al*, 1998; De Innocentiis *et al*, 2001) except for loci *Pmin-07* and *Pmin-11*. On the contrary, the polymorphism of the loci *Pmin-01* (86 alleles), *Pmin-05* (88 alleles) and *Pmin-10* (45 alleles) is higher than in any other species studied (DeWoody and Avise, 2000). This high polymorphism could be explained by the complex mating system (Ross, 2001) of the studied species, in which males can breed with several females during a season and defend nest where 6 females have laid eggs (Jones *et al*, 2001a,b).

Hardy-Weinberg equilibrium

Most microsatellite loci and the allozymatic *LDH-C** locus clearly show a deficit in heterozygotes. This pattern is not unusual in populations of marine organisms as shown in numerous studies (Smith, 1987; García de León *et al*, 1997; De Innocentiis *et al*, 2001), and could find its origin in scoring errors, Wahlund effect (Wahlund, 1928), inbreeding, the occurrence of null alleles, assortative mating or selection against heterozygotes.

The Wahlund effect should result in significant F_{IS} (or R_{IS} for microsatellites) values at more than one allozyme locus, as drift causing population structuring should affect all polymorphic loci equally (Pogson *et al*, 1995). For microsatellites this hypothesis can be rejected as well, because the level of differentiation observed between samples (F_{ST}=0.026; R_{ST}=0.058) was by far smaller than the mean F_{IS} value (F_{IS}=0.163; R_{IS}=0.197) (García de León *et al*, 1997). Moreover, when populations were grouped together following the AMOVA defined structure, the overall F_{IS} value did not decrease, confirming that the Wahlund effect can be rejected.

Inbreeding in gobies is highly improbable, mainly because this phenomenon is usually observed when populations are of limited size and isolated (Hartl, 1988).

For microsatellite loci, we do not favour the hypothesis of null alleles because all F_{IS} estimates were positive, significant and relatively consistent across loci (F_{IS}=0.165 to 0.330; R_{IS}=0.072 to 0.0451). It seems highly improbable that all these loci exhibited null alleles with such a constant frequency. On the contrary, we do not reject this hypothesis for the *LDH-C** locus even if selective pressure could also be a possible explanation for heterozygote deficit in allozyme loci (Jollivet *et al*, 1995; Allegrucci *et al,* 1997). On the contrary, selection against heterozygotes has never been proven using microsatellite loci which are considered representative of the whole genome.

Assortative mating has been suggested in *Pomatoschistus* species closely related to the sand goby but has never been empirically demonstrated (Lindström, pers.com.). Nevertheless, we believe that the complex mating system of this species involving sexual selection by both sexes is mainly responsible for the high F_{IS} values we observed (see Ross 2001).

Spatial patterns of differentiation

Allozyme electrophoresis yielded no significant population structure except at locus LDH-C*. However, this locus deviates strongly from Hardy-Weinberg proportions in all samples and thus caution is warranted when interpreting results. Indeed, AMOVA results showed that all of the observed variation in sand goby is due to within-population effects and no spatial structure is observed, suggesting balancing selection as a homogenizing force (Pogson et al, 1995).

On the other hand, our results at microsatellite loci lead us to conclude that the null hypothesis, namely that panmixia occurs among populations of sand goby, could be rejected. Estimates of fixation indices were mostly significant between pairs of populations leading to a highly significant overall F_{ST} of 0.026 and R_{ST} of 0.058. In marine fish, such as dusky grouper, the observed level of differentiation detected was 0.0179 (De Innocentiis *et al*, 2001), while it amounted to 0.007 in European sea bass (García de León *et al*, 1997), and 0.0084 in cod (Ruzzante *et al*, 1998). Higher pair-wise F_{ST} (0.044-0.151) values were found for the silverside fish (Beheregaray and Sunnucks, 2001) suggesting the existence of a ‘divergence-with-gene-flow’ system.

In general our study suggests the existence of two spatially separated breeding units, namely the Oosterschelde and coastal area, while other populations are more related to one or the other unit. The comparison of our data with the work of Beheregaray and Sunnucks (2001) leads us to conclude that the structure found in sand goby populations is more complex than the suggested four sub-units detected in the AMOVA analysis. The fixation indices, showing either high level of differentiation with *R*-statistics (6%, SE=0.002) or moderate level of differentiation with *F*-statistics (3%, SE=0.042), suggest that differences in allele sizes between populations were far more pronounced than differences in allele frequencies (see review of Balloux and Lugon-Moulin (2002) for the significance of both fixation indices), and especially between the Oosterschelde and coastal populations (F_{ST}=0.008 and R_{ST}=0.022). Nonetheless, gene flow ($7.07<Nm<24.6$) appears to be sufficiently large to swamp any potential for large genetic differences detectable with F_{ST} estimates within the Southern Bight of the North Sea even if a small differentiation was put forward with our data. Moreover, estimates of gene flow and genetic distances based on F_{ST} assume that population structure has been stable for sufficient time to allow an equilibrium between drift and migration. As a consequence, small F_{ST} values can be observed due to high migration rates in the past years, despite little or no current gene flow. Under a strict SMM, R_{ST} will be more sensitive to historical events and to restricted or pronounced gene flow (Balloux and Lugon-Moulin, 2002). The observed R_{ST} value (6% of the differentiation) suggests that the allele shift between the Oosterschelde and coastal populations is large enough to be explained by isolation of these two populations during the formation of the Holocene coast, including the estuary some 700 years ago. Thus, we assume that several processes such as historical events (colonisation of the

Oosterschelde by coastal populations) and to a lesser extent restricted actual gene flow due to the geomorphology of the studied area, contributed to the observed differentiation.

Congruence between allozyme and microsatellite loci

The variation of fixation indices among types of markers is one of the most powerful methods for examining whether natural selection has played a role in the observed genetic divergence (see Allendorf and Seeb, 2000). Consequently, discrepancy between allozyme and microsatellite markers has been described in several marine species (Lemaire *et al*, 2000; De Innocentiis *et al*, 2001). Various results have been observed, showing either differentiation in one or both markers, attributed either to selection or stochastic events (drift-gene flow) (De Innocentiis *et al*, 2001; McLean and Taylor, 2001). In our data set, microsatellite exhibited higher F_{ST} values than allozyme, suggesting that the highest mutation rate in microsatellite has increased allele frequency divergence among populations (Allendorf and Seeb, 2000). In addition, none of the alleles scored occur at high frequency in one population and are absent in the others, as would be observed if new mutations drift to high frequencies within their population of origin before being distributed to others by migration (Allendorf and Seeb, 2000). Thus, our results suggest that the observed differentiation was mainly due to drift and restricted gene flow between the distinct breeding populations (coastal and Oosterschelde groups) and the other samples, but not to any type of selection (see allozymes).

A model for the spatial pattern of sand goby in the Southern Bight of the North Sea

In the Southern Bight of the North Sea, sand gobies breed along the coast and disperse either during the larval or adult phase (Fonds, 1973). During summer time, breeding units were essentially observed on the east coast in June-July (Dewicke, 2001) while they were observed on the west coast in July-August (Pampoulie, pers. obs.; Ot1 and Ot2 sampling sites) suggesting a temporal variation in the location of the breeding sites. Moreover, a breeding population is known to occur in the Oosterschelde but not in the Westerschelde. Unfortunately, the ecological literature focused on the coastal populations (Fonds, 1973). So far, we assume that the high level of differentiation observed between the Oosterschelde and coastal populations may be the result of historical events and drift between two breeding sub-units (the mean pair-wise F_{ST} value between these two groups is 0.008 while the R_{ST} value is 0.022). Hence, the population structure is quite similar to the one observed by Beheregaray and Sunnucks (2001), namely a divergence-with-gene-flow-system between the Oosterschelde and coastal area (Figure 5). However, we do not have any precise ecological information about these two putative breeding units to assess whether ecological data would be consistent with genetic data. Marine juveniles of the sand goby are known to undertake migration from the coastal area and the Oosterschelde to the Westerschelde, supposedly to avoid the high level of predation and to find a high amount of food (Maes *et al*, 1998). This may result in the pooling of genotypes from both breeding units with subsequent low pair-wise differentiation. In addition, the offshore populations seemed to be more related to the coastal populations (see Figure 4) suggesting a coastal origin.

To conclude, based on microsatellite loci, our study clearly revealed the existence of two breeding units presumably connected with a low amount of migrants, consistent with a divergence-with-gene-flow system. This differentiation is partly attributable to a combination of older historical events, restricted actual gene flow and the complex mating system of sand gobies. Additional temporal (summer and winter) investigations should be done in order to increase the knowledge on the ecology and genetics of the two distinct breeding units (coastal area and Oosterschelde) and to assess the spatio-temporal stability and dynamics of both units.

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References

Allegrucci G, Fortunato C, Sbordoni V (1997) Genetic structure and allozyme variation of sea bass (*Dicentrarchus labrax* and *D. punctatus*) in the Mediterranean Sea. *Marine Biology*, **122**, 347-358.

Allendorf FW, Seeb LW (2000). Concordance of genetic divergence among Sockeye salmon populations at allozyme, nuclear DNA, and mitochondrial DNA markers: *Evolution* **54**: 640-651.

Balloux F & Lugon-Moulin N (2002) The estimation of populations differentiation with microsatellite markers. *Molecular Ecology*, **11**, 155-165.

Beheregaray LB, Sunnucks P (2001) Fine-scale structure, estuarine colonization and incipient speciation in the marine silverside fish *Odontesthes argentinensis*. *Molecular Ecology*, **10**, 2849-2866.

Belkhir, K, Borsa P, Goudet J, Bonhomme F (1999) Genetix: logiciel sous Windows pour la genetique des populations Version 3.0. Laboratoire Génome & Population, CNRS-UPR, Université de Montpellier II, Montpellier (France).

Bowen BW, Grant WS (1997) Phylogeography of the sardines (*Sardinops* spp): assessing biogeographic models and population histories in temperate upwelling zones. *Evolution*, **51**, 1601-1610.

Cowen RC, Kamazima M, Lwiza M, Sponaugle S, Paris CB, Olson DB (2000) Connectivity of marine populations: open or closed? *Science*, **287**, 857-859.

De Innocentiis S, Sola L, Cataudella S, Bentzen P (2001) Allozyme and microsatellite loci provide discordant estimates of population differentiation in the endangered dusky grouper (*Epinephelus marginatus*) within the Mediterranean Sea. *Molecular Ecology*, **10**, 2163-2175.

De Moor G, Lankneus J (1990) Stabilité et apports sédimentaires sur les bancs de Flandre. *Annales de la Société Géologique du Nord*, **109**, 129-139.

Dewicke A (2001) Hyperbenthic communities of the North Sea. PhD University of Gent, 219p.

DeWoody JA, Avise JC (2000). Microsatellite variation in marine, freshwater and anadromous fishes compared with other animals. *Journal of Fish Biology*, **56**, 461-473.

Doornbos G, Twisk F (1987) Density, growth and annual food consumption of gobiid fish in the saline lake Grevelingen, The Netherlands. *Netherlands Journal of Sea Research*, **21**, 45-74.

Felsenstein J (1995) Phylogeny inference package (Phylip). University of Washington.

Fonds M, 1973. Sand gobies in the Dutch Wadden sea (*Pomatoschistus*, Gobiidæ, Pisces). *Netherlands Journal of Sea Research* 6, 417-478.

García de León FJ, Chikhi L & Bonhomme F, 1997. Microsatellite polymorphism and population subdivision in natural populations of European sea bass *Dicentrarchus labrax* (Linnaeus, 1758). *Molecular Ecology*, **6**, 51-62.

Geertjes GJ, Kamping A, van Delden W & Videler JJ 2001. Genetic relationship among one non-endemic and two endemic Mediterranean triplefin blennies (Pisces, Blennidae). *Marine Ecology*, **22**, 255-265.

Goodman SJ (1997) Rst Calc: a collection of computer programs for calculating estimates of genetic differentiation from microsatellite data and a determining their significance. *Molecular Ecology*, **6**, 881-885.

Goldstein DB, Ruiz-Linares A, Cavalli-Sforza LL, Feldman MW (1995) Genetic absolute dating based on microsatellites and the origin of modern humans. *Proceedings of the National Academy of Sciences USA*, **92**, 6723-6727.

Goldstein DB, Schlötterer C (1999) Microsatellites: Evolution and application. Oxford University Press, 352p.

Goudet J (1999) PCA-GEN, version 1.2. Institute of Ecology, Biology Building, University of Lausanne.

Govaere JCR, Van Damme D, Heip C, De Coninck LAP (1980) Benthic communities in the Southern Bight of the North Sea and their use in ecological monitoring. *Helgoländer Meeresuntersuchungen*, **33**, 507-521.

Hamerlynck O, Cattrysse A (1994) The food of *Pomatoschistus minutus* (Pisces, Gobiidæ) in Belgian coastal waters and a comparison with the food of its potential competitor *P. lozanoi*. *Journal of Fish Biology*, **44**, 753-771.

Hamerlynck O, Hostens K (1994) Changes in the fish fauna of the Oosterschelde: a 10-year time series of fyke catches. *Hydrobiologia*, **283**, 497-507.

Hartl DL, 1988. Génétique des populations. Flammarion (1994), 305p.

Hebert PDN, Beaton MJ (1989) *Methodologies for allozyme analysis using cellulose acetate electrophoresis: A practical handbook*. Helena Laboratories Incorporation, 31p.

Huisman J, Weissing FJ (2000) Reply: Coexistence and resource competition. *Nature*, **407**, 694-694.

Jollivet D, Desbruyères D, Ladrat C, Laubier L (1995) Evidence for differences in the allozyme thermostability of deep-sea hydrothermal vent polychaetes (Alvellidae): a possible selection by habitat. *Marine Ecology Progress Series*, **123**, 125-136.

Jones AG, Walker D, Kvarnemo C, Lindström K, Avise JC (2001a) How cuckoldry can decrease the opportunity for sexual selection: Data and theory from a genetic parentage analysis of the sand goby, *Pomatoschistus minutus*. *Proceedings of the National Academy of Sciences, USA*, **98**, 9151-9156.

Jones AG, Walker D, Lindström K, Kvarnemo C, Avise JC (2001b) Surprising similarity of sneaking rates and genetic mating patterns in two populations of sand goby experiencing disparate sexual selection regimes. *Molecular Ecology*, **10**, 461-468.

Kimura M, Otha T (1978) Stepwise mutation model and distribution of allelic frequencies in a finite population. *Proceedings of the National Academy of Sciences USA*, **75**, 2868-2872.

Lemaire C, Allegrucci G, Naciri M *et al*. (2000) Do discrepancies between microsatellite and allozyme variation reveal differential selection between sea and lagoon in the sea bass (*Dicentrarchus labrax*)? *Molecular Ecology*, **9**, 457-467.

Lessios HA, Kessing BD, Robertson DR, Paulay G (1999) Phylogeography of the pantropical sea urchin *Eucidaris* in relation to land barriers and ocean currents. *Evolution*, **53**, 806-817.

LI-COR Inc. (2001) E-seq Version 2.0. LI-COR Incorporation, USA.

Lindström K (1992) Female spawning patterns and male mating success in the sand goby *Pomatoschistus minutus. Marine Biology*, **113**, 475-480.

Lundy CJ, Moran P, Rico C, Milner RS, Hewitt GM (1999) Macrogeographical differentiation in oceanic environments: a case study of European hake (*Merluccius merluccius*), a commercially important fish. *Molecular Ecology*, **8**, 1889-1898.

Maes J, Pas J, Taillieu A, Van Damme PA, Ollevier F (1998) Diel changes in the vertical distribution of juvenile fish in the Zeeschelde estuary. *Journal of Fish Biology*, **54**, 1329-1333.

McLean JE, Taylor EB (2001) Resolution of population structure in a species with high gene flow: microsatellite variation in the eulachon (Osmeridae: *Thaleichthys pacificus*). *Marine Biology*, **139**, 411-420.

Mantel N (1967) The detection of disease clustering and generalized regression approach. *Cancer Research*, **27**, 209-220.

Miller PJ (1986) Gobioidei. In Fishes of the North–eastern Atlantic and the Mediterranean. Whitehead P. J. P., Bauchot M. L., Hureau J. C., Nielsen J., Tortenese E., éd., U. N. E. S. C. O., III, pp1019-1085.

Mitton JB (1997) Selection in natural populations. Oxford University Press. pp 240

Nei M (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics*, **89**, 583-590.

Nihoul J, Hecq JH (1984) Influence of the residual circulation on the physico-chemical characteristics of water masses and the dynamics of ecosystems in the Belgian coastal zone. *Continental Shelf Research*, **3**, 167-174.

Offringa H, Seys J, Van den Bossche W, Meire P (1996) Seabirds on the Channel doormat. *Le Gerfaut*, **86**, 3-71.

Page RDM (1996) TREEVIEW: an application to display phylogenetic trees on personal computers. *Computers Applications in the Biosciences*, **12**, 357-358.

Pampoulie C, Rosecchi E, Bouchereau JL, Crivelli AJ (1999) Life history traits of *Pomatoschistus minutus* (Teleostei, Pisces) in the Rhone delta, France. *Journal of Fish Biology*, **55**, 892-896.

Pogson GH, Mesa KA, Boutilier RG (1995) Genetic population structure and gene flow in the Atlantic cod *Gadus morhua*: a comparison of allozyme and nuclear RFLP loci. *Genetics*, **139**, 375-385.

Raymond R, Rousset F (1995) GENEPOP (version 1.2): population genetic software for exact tests and ecumenism. *Journal of Heredity*, **86**, 248-249.

Rice WR (1989). Analyzing table of statistical tests. *Evolution*, **43**, 223-225.

Richardson PJ, Baverstock PR, Adams M (1986) *Allozyme electrophoresis: A handbook for animal systematics and population studies*. Academic Press Incorporation, London 410p.

Rocha LA, Bass AL, Robertson R & Bowen BW (2002) Adult habitat preferences, larval dispersal, and the comparative phylogeography of three Atlantic surgeonfishes (Teleostei: Acanthuridae). *Molecular Ecology*, **11**, 243-252.

Ross KG, 2001. Molecular ecology of social behaviour: analyses of breeding systems and genetic structure. *Molecular Ecology*, **10**, 265-284.

Ruzzante DE, Taggart CT, Cook D (1998) A nuclear DNA basis for shelf and bank scale population structure in northwest Atlantic cod (*Gadus morhua*): Labrador to Georges bank. *Molecular Ecology*, **7**, 1663-1680.

Saitou N, Nei M (1987) The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, **4**, 406-425.

Scanalytics (2001) Gene ImagIR version 4.03, Genotyping and DNA fragment analysis software. LI-COR Incorporation, USA.

Schneider S, Roessli D, Excoffier L (2000) ARLEQUIN ver 2.0: A software for population genetic analysis. Genetics and Biometry Laboratory, University of Geneva, Geneva

Scribner KT, Petersen MR, Fields RL et al. (2001) Sex-biased gene flow in spectacular eiders (Anatidae): inferences from molecular markers with contrasting modes of inheritance. Evolution, 55, 2105-2115.

Shaklee JB, Allendorf FW, Morizot DC, Whitt GS (1990) Gene nomenclature for protein-coding loci in fish. Transactions of the American Fisheries Society, 119, 2-15.

Sinclair M (1988) Marine populations: an essay on population regulation and speciation. University of Washington Press 252p.

Slatkin M (1985) Rare alleles as indicators of gene flow. Evolution, 39, 53-65.

Slatkin M (1995) A measure of population subdivision based on microsatellite allele frequency. Genetics, 139, 457-462.

Smith PJ (1987) Homozygous excess in the sand flounder, Rhombosoleia plebeia, produced by assortative mating. Marine Biology, 95, 489-492.

Statsoft Inc. (1997) Statistica version 6.0 for Windows (Computer program manual). Tulsa, Statsoft Incorporation.

Stepien CA, Rosenblatt RH, Bargmeyer BA (2001) Phylogeography of the spotted sand bass Paralabrax maculatofasciatus: divergence of Gulf of California and Pacific coast populations. Evolution, 55, 1852-1862.

Suk HY, Kim JB, Min MS, Yang SY (1996). Genetic differentiation and reproductive isolation among three types of the floating goby (Chaenogobius annularis) in Korea. Korean Journal of Zoology, 39, 147-158.

Wahlund S (1928) The combination of populations and the appearance of correlation examined from the standpoint of the study of heredity. Hereditas, 11, 65-106.

Wallis GP, Beardmore JA (1984a) An electrophoretic study of the systematic relationships of some closely related goby species (Pisces, Gobiidae). Biological Journal of the Linnean Society, 22, 107-123.

Wallis GP, Beardmore JA (1984b) Genetic differentiation between populations of Pomatoschistus minutus from the Bristol Channel and the Adriatic. Genetica, 62, 75-80.

Walsh PS, Metzger DA, Higuchi R (1991) Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques, 10, 506-513.

Ward RD, Woodwark M, Skibinski DOF (1994) A comparison of genetic diversity levels in marine, freshwater and anadromous fishes. Journal of Fish Biology, 44, 213-232.

Weir BS (1990) Genetic data analysis. Sinauer Sunderland, MA.

Weir BS, Cockerham CC (1984) Estimating F-statistics for the analysis of population structure. Evolution, 38, 1358-1370.

Wirth T, Bernatchez L (2001) Genetic evidence against panmixia in the European eel. Nature, 409, 1037-1040.

Wright S (1969) Evolution and the genetics of populations. Vol. 2. The theory of gene frequencies. University of Chicago Press, Chicago, 511p.