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Evidence for fine-scale genetic structure and estuarine colonisation in a potential high gene flow marine goby (*Pomatoschistus minutus*)

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Marine fish seem to experience evolutionary processes that are expected to produce genetically homogeneous populations. We have assessed genetic diversity and differentiation in 15 samples of the sand goby *Pomatoschistus minutus* (Pallas, 1770) (Gobiidae, Teleostei) from four major habitats within the Southern Bight of the North Sea, using seven microsatellite and 13 allozyme loci. Despite its high dispersal potential, microsatellite loci revealed a moderate level of differentiation (overall $F_{ST} = 0.026$; overall $R_{ST} = 0.058$). Both hierarchical analysis of molecular variance and multivariate analysis revealed significant differentiation ($P < 0.01$) between estuarine, coastal and marine samples with microsatellites, but not with allozymes. Comparison among the different estimators of differentiation (F_{ST} and R_{ST}) pointed to possible historical events and contemporary habitat fragmentation. Samples were assigned to two breeding units in the estuary and coastal region. Despite this classification, there were indications of a complex and dynamic spatiotemporal structure, which is, most likely, determined by historical events and local oceanic currents.
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Introduction

One of the most interesting challenges in marine evolutionary biology is to diagnose the processes responsible for genetic differentiation of distantly or closely related populations. Consequently, the genetic structure of numerous organisms has been assessed using several kinds of estimators, resulting in the discovery of disparate levels of genetic differentiation for different markers (Pogson *et al*, 1995; Lemaire *et al*, 2000; but see, Allendrof and Seeb, 2000). Although allozymes are still widely used, microsatellites have gained in importance due to their high levels of polymorphism, which facilitates the discovery of subtle differentiation (Ruzzante *et al*, 1998; Shaw *et al*, 1999). In addition, they are useful tools for the inference of historical dispersal and gene flow events, due to molecular insights into the nature of alleles and their mutation models (Balloux and Lugon-Moulin, 2002).

Populations of marine fishes encounter essentially two major homogenising forces. They usually exhibit a high effective population size and produce a large number of eggs and larvae capable of dispersal via passive or active mechanisms over vast distances, thus limiting population divergence (Wirth and Bernatchez, 2001; Hoarau *et al*, 2002). The marine environment also tends to be physically less structured than continental systems and to exhibit fewer constraints on gene flow, rendering marine fishes poor candidates for genetic studies on a small geographic scale. Nevertheless, fronts, local and global oceanic current patterns, bottom topography, the influence of estuaries 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).

Species that inhabit marine, as well as coastal and estuarine regions, are thought to develop a mechanism of 'divergence-with-gene-flow' through local adaptation (Beheregaray and Sunnucks, 2001). To test such hypotheses, we chose a system including various types of habitats to see whether an annual noncommercial marine fish, with high reproductive effort and dispersal capability, is able to develop and maintain genetic structure.

The sand goby, *Pomatoschistus minutus* (Pallas, 1770) (Gobiidae, Teleostei), a small bottom-dwelling fish, is well suited to test these hypotheses. It occurs in several European regions and especially within the Southern Bight of the North Sea, where it lives in estuarine (Oosterschelde and Westerschelde), coastal and marine habitats. It reproduces from May to July (Fonds, 1973). Males build nests and attract females to obtain eggs (Lindström, 1992). A male then defends his nest until the hatching of the larvae. The larvae are pelagic for 4–6 weeks and adopt a demersal lifestyle after metamorphosis. Adults are thought to have limited swimming abilities, yet they carry out inshore spawning migrations on a scale of 10 km (Pampoulie *et al*, 1999). Given its high dispersal capabilities, we might expect only slight or no

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genetic differentiation among populations in geographic proximity. On the other hand, the geomorphology of the Belgian Continental Shelf, characterised by a combination of sand banks and gullies swept by strong tidal currents (De Moor and Lanckneus, 1990), and by an inshore/offshore gradient under the influence of the Schelde estuary (Nihoul and Hecq, 1984; Offringa *et al*, 1996; Dewicke, 2001), might limit dispersal and promote small-scale interpopulation differentiation.

The scope of this study is to assess: (1) whether a small marine fish species, exhibiting a high dispersal rate and living in diverse and dynamic environments, could have developed any reproductive barriers in such a heterogeneous area, and (2) whether, as suggested by Beheregaray and Sunnucks (2001), those specific hydrodynamic systems lead to a 'divergence-with-gene-flow' system that might be favourable to incipient speciation.

Materials and Methods

Sampling

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 in the Coastal area (coastal and Flemish banks: Sb, Ht, and K), the Estuary (Westerschelde) and the marine area (Of1, Of2) over a distance of at most 120 km (Figure 1). In addition, four samples were taken in the Schelde estuary (Oosterschelde, The Netherlands) for microsatellite analysis only. The latter area consists of a unique marine environment and is used as a nursery 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 exchanges between the ecosystems (Hamerlynck and Hostens, 1994). One distant population has been sampled in Texel (Tx; The Netherlands) as an 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 genotyping

Allelic variation was assayed for nine populations at eight enzymes coding for 13 loci (Table 1), 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).

The liver, eye and muscle tissues were dissected and ground in distilled water. The samples were subjected to cellulose acetate gel electrophoresis (Richardson *et al*,

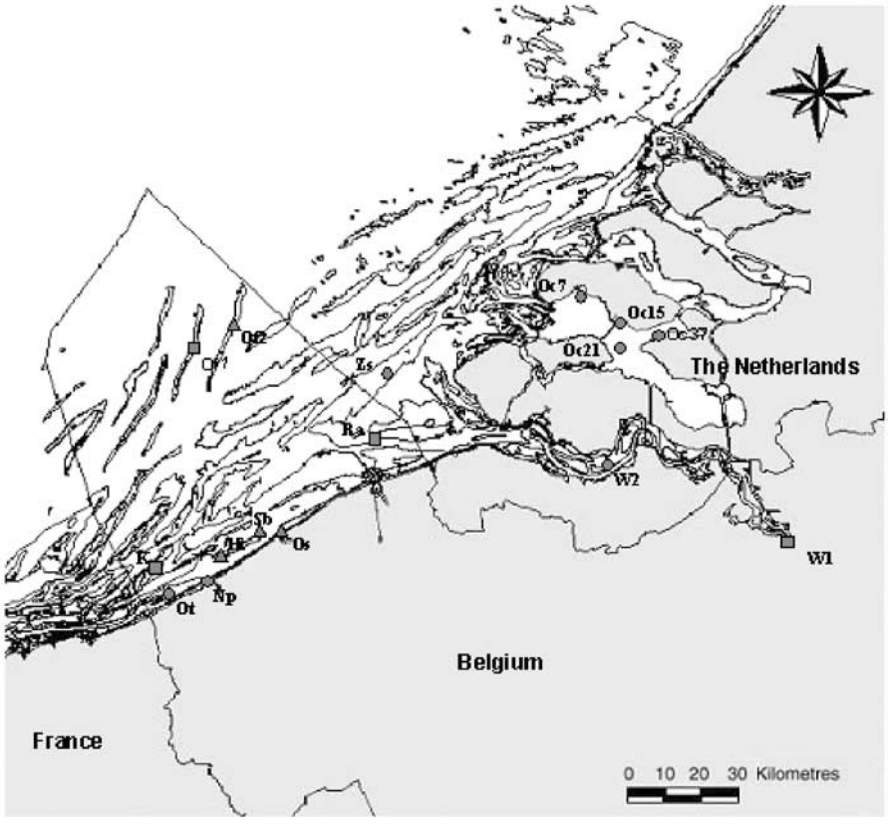

Figure 1 Sampling locations and codes of the sampling sites of *P. minutus* within the Southern Bight of the North Sea. Triangles: allozyme samples; squares: microsatellite and allozyme loci; circles: microsatellite samples. For the code designation, see Table 1.

1986) using two continuous buffer systems: Tris-maleate (pH 7.8) and Tris-glycine (pH 8.8) as described by Hebert and Beaton (1989). 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 genotyping

Allelic variation was assayed at seven microsatellite loci, *Pmin-01*, *Pmin-05* and *Pmin-10* (described by Jones *et al*, 2001a, b), and the newly developed loci, *Pmin-06*, *Pmin-07*, *Pmin-08* and *Pmin-11* (Table 2). A total of 15 samples were assayed with a sampling size of 36–54 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 primer sets used, PCR was conducted in a 10 μl reaction volume containing specific amounts of primers and $MgCl_2$ ranging, respectively, from 1 to 2 μM 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 of: 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% and 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.

Table 1 Sampling locations of *P. minutus* in the Southern Bight of the North Sea

Habitat	*Sampling site*	*Code*	*Period*	*Allozymes*	*Microsatellites*
Westerschelde	Doel 10	W1	October 1998	71	54
	Westerschelde	W2	August 2001	—	52
Oosterschelde	Oosterschelde 7	Oc7	August 2001	—	52
	Oosterschelde 15	Oc15	August 2001	—	52
	Oosterschelde 21	Oc21	August 2001	—	45
	Oosterschelde 37	Oc37	August 2001	—	52
Coast	Kwintebank 2	K2	February 1997	68	—
	Stroombank	Sb	February 1997	83	—
	Kwintebank 10	K1	October 1997	161	58
	Raan 1	Ra1	March 1997	50	—
	Ostend	Os	October 1997	52	—
	Weststroombank	Ht	October 1998	35	—
	Kwintebank	K12	August 2000	—	53
	Oostduinkerke	Ot1	August 2000	—	54
	Zuid-Steenbank	Zs	August 2000	—	53
	Raan 2	Ra2	August 2000	—	53
	Niewpoort	Np	August 2000	—	52
	Oostduinkerke	Ot2	August 2001	—	52
	Texel	Tx	September 1999	—	51
Marine	Oosthinder	Of1	October 1997	51	36
	Bligh Bank	Of2	February 1998	28	—

Table 2 Characteristics of the microsatellite DNA markers scored on 15 populations of sand goby (768 individuals were scored)

Locus	*Primer sequence (5′→3′)*	*Repeat Sequence*	*Size range*	*No. of alleles*	*Accession number*
Pmin-01	R: CACAAAGTCAATCCTAAATA F: CCAAACTGTTTAGCACTG	(GT)	158–418	86	AF516896
Pmin-05	R: TTTCCCCCGAACAACACAAC F: TTCCCATGCCTCCTTTTGTC	(GT)	118–276	88	AF516897
Pmin-06	R: CGCATTAGAATTATTAGGCC F: TCANTNCTACTCACTAACCT	(CA)(AA)(CA)	91–199	43	AF516898
Pmin-07	R: TTTCAGCTGTATAGTCGCTGC F: TCGACAAACTCAAACTCACC	(CA)	162–178	9	AF516899
Pmin-08	R: GTTCGCCACCATGCACC F: AGTCTTCCACCGCTCACG	(CA)(CG)(CA)(CG)(CA)	152–286	45	AF516900
Pmin-10	R: AACCGCCCAATCCACAAC F: GAATGTCCCGAGAAACTGGAG	(GT)	142–202	45	AF516901
Pmin-11	R: CCGACCCAGAAATGGACAA F: GATTCGCCAACACAGATTCAA	(TGGA)	100–120	8	AF516902

4.02 (Belkhir *et al*, 1999). We used the software GENEPOP version 3.1 (Raymond and Rousset, 1995) to test for deviations from Hardy–Weinberg equilibrium (HWE). When appropriate, significant levels were adjusted with a sequential Bonferroni test (Rice, 1989). Wright's single-locus F-statistics (Wright, 1969) were calculated from allele frequencies for 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 jackknifing over all populations according to Weir (1990). The 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) were calculated in GENETIX assuming genetic drift-mutation equilibrium and a constant population size over time for both allozyme and microsatellite loci. 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. We performed a Mantel test (Mantel, 1967) to test for correlation between geographical and genetic distance between samples (isolation-by-distance) as implemented in GENETIX (after 1000 permutations). We carried out a multidimensional scaling (MDS) approach on pairwise Nei (1978) genetic distance using Statistica 5.1 (Statsoft Inc., 1997). An analysis of molecular variance (AMOVA) was carried out in ARLEQUIN version 2.0 (Schneider *et al*, 2000) to assess the hierarchical partitioning of genetic variability within and among populations, and among *post hoc* defined regions (Oosterschelde, Westerschelde, coastal and marine), using the observed structure in the MDS analysis.

Each individual microsatellite genotype was used to estimate the proportion of an individual's genotype originating from one or the other of the studied populations (STRUCTURE; Pritchard *et al*, 2000). A two-population model was chosen based on the ecological knowledge of the sand goby in the Southern Bight and on the results found in the MDS, that is, based on two known breeding units (Oosterschelde and coastal area) and on the presence of a putative admixed populations (Westerschelde). First the model was forced *a priori* for the structure identified in the descriptive analyses, and second run without forcing any population structure.

Results

Allozyme genetic diversity

Nine out of 13 scored allozyme loci were polymorphic in the nine samples 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 interpopulation 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 samples 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).

Allozyme population structure

As no clear differentiation was observed between all samples separately, samples were grouped by season (summer and winter) to assess temporal variation and variation of the F-estimates. The multilocus F_{ST} value (0.01) for 'summer–autumn' samples (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' samples (Ra3, Of2, Sb and K2) were less differentiated ($F_{ST} = 0.005$) than the 'summer' samples (F_{ST} not significant). No differentiation at locus *LDH-C** was observed in this group.

Pairwise genetic distances (Nei, 1978) calculated over all loci between the samples of sand goby were not significant. 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, 1995) showed no differences.

The MDS analysis revealed a slight differentiation between marine, estuarine (Westerschelde) and coastal samples (Figure 2a). When applying AMOVA, within-population effects explained all the observed variation and did not show any consistent differentiation between the samples (Table 4).

Microsatellite genetic diversity

Although the seven microsatellite loci studied exhibited a high level of polymorphism (Table 2; $P_{(0.95)} = 1$), two of them might be considered highly polymorphic (*Pmin-01* and *Pmin-05*), while three loci are moderately polymorphic (*Pmin-06*, *Pmin-08* and *Pmin-10*) and two slightly polymorphic (*Pmin-07* and *Pmin-11*) compared to values found in the literature for marine fishes. The number of alleles per locus across all samples ranged from eight (*Pmin-11*) to 88 (*Pmin-05*). Observed heterozygosity averaged over all loci ranged from 0.62 to 0.76 in the 15 samples 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 all samples except for K1. The overall excess of homozygotes for all loci combined (F_{IS}), as quantified by the correlation of alleles within individuals 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 different pairs of loci in different populations, thus suggesting

Table 3 Number of individuals scored (*n*), number of alleles (*A*), mean number of alleles (MNA), expected heterozygosity (H_E), observed heterozygosity (H_O) and F_{IS} according to Weir and Cockeram (1984) for the polymorphic allozyme loci of sand goby

Locus	*Samples*								
	K1	*K2*	*Os*	*Ra1*	*Sb*	*Ht*	*Of1*	*Of2*	*W1*
*LDH-A**									
n	155	60	51	50	79	35	44	28	57
A	2	2	2	1	1	1	2	1	2
H_E	0.013	0.017	0.019	0.000	0.000	0.000	0.023	0.000	0.017
H_O	0.013	0.017	0.020	0.000	0.000	0.000	0.023	0.000	0.017
F_{IS}	−0.003	0	0	—	—	—	0	—	0
*LDH-B**									
n	146	56	51	49	77	35	36	28	66
A	1	2	2	2	1	1	1	2	1
H_E	0.000	0.018	0.038	0.020	0.000	0.000	0.000	0.069	0.000
H_O	0.000	0.018	0.039	0.020	0.000	0.000	0.000	0.070	0.000
F_{IS}	—	0	0	0	—	—	—	−0.019	—
*LDH-C**									
n	144	54	51	47	66	35	19	28	64
A	4	3	3	3	3	3	3	3	3
H_E	0.549	0.561	0.573	0.517	0.481	0.231	0.460	0.428	0.424
H_O	0.403	0.500	0.373	0.298	0.439	0.171	0.105	0.214	0.281
F_{IS}	**0.150**	**0.307**	**0.288**	**0.284**	**0.298**	**0.311**	**0.502**	**0.513**	**0.239**
*MDH-1**									
n	156	57	52	49	56	35	43	28	71
A	2	1	2	1	2	1	3	1	1
H_E	0.013	0.000	0.019	0.000	0.018	0.000	0.068	0.000	0.000
H_O	0.013	0.000	0.019	0.000	0.018	0.000	0.070	0.000	0.000
F_{IS}	−0.003	—	0	—	0	—	−0.006	—	—
*PGM-1**									
n	153	56	49	49	61	31	40	24	65
A	3	3	3	3	2	3	3	3	3
H_E	0.284	0.180	0.250	0.265	0.290	0.297	0.258	0.284	0.242
H_O	0.307	0.161	0.184	0.245	0.246	0.290	0.300	0.333	0.215
F_{IS}	−0.045	0.076	0.131	0.021	0.147	0.029	−0.077	−0.154	0.071
*PGM-2**									
n	105	47	29	38	44	25	38	9	51
A	5	3	4	4	3	3	4	3	6
H_E	0.595	0.567	0.620	0.606	0.600	0.547	0.630	0.512	0.571
H_O	0.476	0.532	0.552	0.658	0.728	0.360	0.579	0.222	0.549
F_{IS}	0.204	0.072	0.128	−0.073	−0.201	0.360	0.095	0.605	0.049
*PGI-1**									
n	156	61	51	50	66	34	46	24	70
A	3	1	1	1	1	2	2	1	2
H_E	0.013	0.000	0.000	0.000	0.000	0.057	0.022	0.000	0.042
H_O	0.013	0.000	0.000	0.000	0.000	0.059	0.022	0.000	0.043
F_{IS}	0	—	—	—	—	−0.016	0	—	−0.015
*PGI-2**									
n	157	61	51	50	72	34	45	24	70
A	2	2	2	3	2	1	2	1	2
H_E	0.056	0.016	0.019	0.078	0.027	0.000	0.022	0.000	0.056
H_O	0.057	0.016	0.020	0.080	0.028	0.000	0.022	0.000	0.043
F_{IS}	−0.026	0	0	−0.011	−0.007	—	0	–	0
*GOT**									
n	114	45	47	28	64	35	44	26	71
A	1	1	1	2	1	2	1	1	2
H_E	0.000	0.000	0.000	0.035	0.000	0.028	0.000	0.000	0.014
H_O	0.000	0.000	0.000	0.036	0.000	0.029	0.000	0.000	0.014
F_{IS}	—	—	—	0	—	0	—	—	0
Total									
MNA	2.15	1.69	1.85	1.77	1.54	1.62	1.92	1.58	2.08
H_E	0.118	0.104	0.118	0.117	0.109	0.089	0.114	0.099	0.105
H_O	0.099	0.096	0.093	0.103	0.112	0.070	0.086	0.065	0.090

Bold values: F_{IS} values deviating significantly from HWE after sequential Bonferroni corrections. For sampling codes, see Table 1.

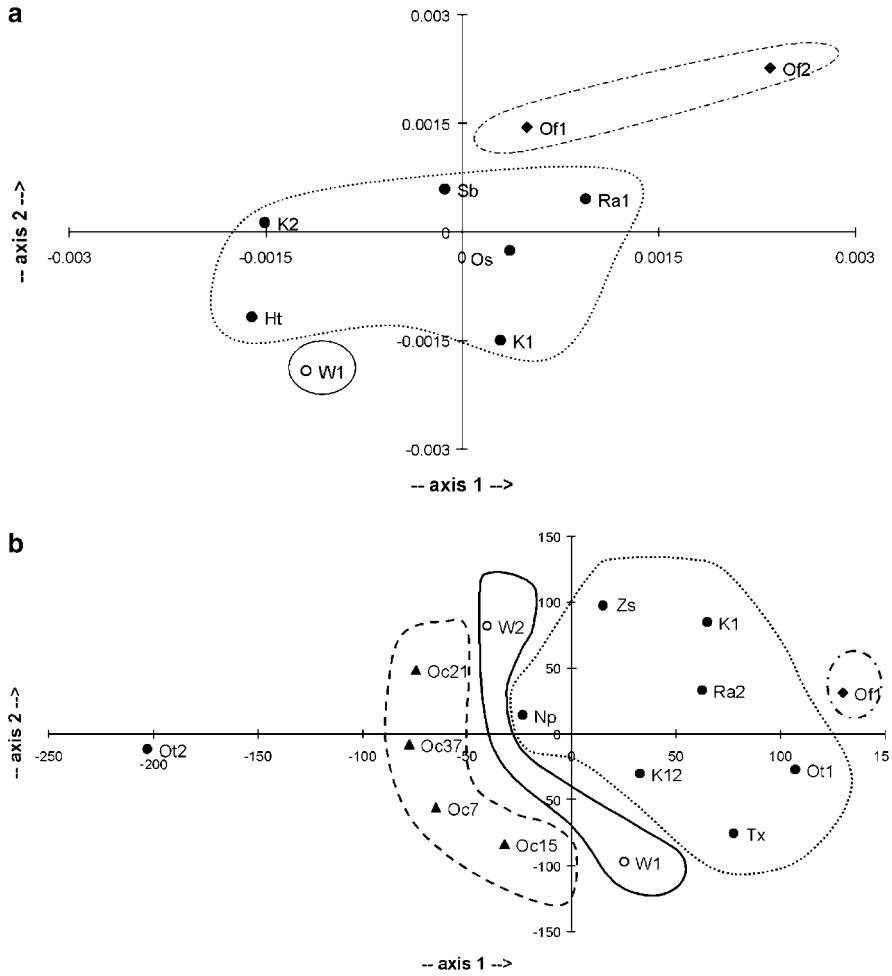

Figure 2 Multidimensional scaling analysis on populations of *P. minutus* based on Nei's distances (1978). (**a**) Results observed for allozyme markers on nine populations: and (**b**) results observed for microsatellite markers on 15 populations. -----Marine. · · · · · ·Coastal, **----** Oosterschelde and —— Westerschelde group.

Table 4 Hierarchical AMOVA among nine populations of *P. minutus* grouped in three regional groups for the allozyme data (Marine, Coastal and Westerschelde) and 15 populations grouped in four regional groups for the microsatellite data (Marine, Coastal, Westerschelde and Oosterschelde)

Loci	*Source of variation*	*df*	*Variance components*	*% variation*	*Fixation indices*	P-*value*
Allozymes	Among groups	2	−0.003	−0.83	CT = −0.008	0.536
	Among samples within groups	6	0.035	8.93	SC = 0.089	<0.0001
	Within samples	899	0.356	91.90	ST = 0.081	<0.0001
	Total	907	0.387	100		
Microsatellites	Among groups	3	0.017	0.60	CT = 0.006	<0.01
	Among samples within groups	11	0.066	2.28	SC = 0.022	<0.0001
	Within samples	1519	2.79	97.12	ST = 0.029	<0.0001
	Total	1533	2.88	100		

df: degree of freedom, *p*: significance level.

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).

Microsatellite 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 an mean R_{ST} value of 0.058 and an R_{IS} of 0.197 (Table 6). Pairwise differentiation between populations yielded significant F_{ST} values for all comparisons after sequential Bonferroni adjustment, while not all values were significant for the R_{ST} estimator (Table 7). The highest pairwise F_{ST} and R_{ST} values were observed between the marine populations and the other populations. The total differentiation for both estimators was

Table 5 Number of individuals scored (*n*), number of alleles (*A*), mean number of alleles (MNA), expected heterozygosity (H_E), observed heterozygosity (H_O) and F_{IS} according to Weir and Cockeram (1984) for the microsatellite loci

Locus	*Samples*														
	K1	*K12*	*Zs*	*Ra2*	*Ot1*	*Ot2*	*Np*	*Tx*	*Of1*	*W1*	*W2*	*Oc7*	*Oc15*	*Oc21*	*Oc37*
Pmin-01															
n	56	53	49	52	53	48	49	49	36	52	47	50	50	41	52
A	45	33	34	37	49	39	43	46	18	49	46	46	38	25	46
H_E	0.967	0.939	0.953	0.954	0.971	0.958	0.965	0.967	0.940	0.968	0.963	0.966	0.962	0.936	0.970
H_O	0.696	0.623	0.674	0.750	0.868	0.834	0.898	0.776	0.833	0.808	0.787	0.860	0.960	0.927	0.904
F_{IS}	0.288	**0.345**	**0.303**	0.223	0.116	0.141	0.08	**0.208**	0.105	0.175	**0.193**	0.120	**0.012**	0.022	0.079
Pmin-05															
n	57	50	52	53	54	51	50	51	36	54	51	49	49	43	52
A	56	37	32	39	44	33	40	39	22	46	38	31	40	39	26
H_E	0.976	0.956	0.947	0.962	0.966	0.959	0.962	0.964	0.940	0.967	0.964	0.950	0.964	0.961	0.942
H_O	0.860	0.660	0.635	0.755	0.926	0.843	0.860	0.765	0.889	0.815	0.843	0.714	0.755	0.977	0.689
F_{IS}	0.128	**0.319**	**0.338**	**0.224**	0.051	**0.131**	**0.116**	**0.216**	**0.069**	**0.166**	**0.135**	**0.258**	**0.227**	−0.004	**0.279**
Pmin-06															
n	57	52	53	53	54	51	51	49	36	53	52	52	48	44	49
A	29	27	23	25	22	26	20	24	19	27	24	13	13	24	30
H_E	0.911	0.914	0.919	0.925	0.909	0.930	0.904	0.920	0.882	0.886	0.871	0.723	0.833	0.902	0.863
H_O	0.860	0.679	0.736	0774	0.722	0.846	0.843	0.612	0.750	0.887	0.904	0.750	0.750	0.818	0.694
F_{IS}	0.065	**0.266**	**0.208**	**0.173**	**0.214**	**0.100**	**0.077**	**0.344**	0.164	0.009	−0.028	−0.028	**0.109**	**0.104**	**0.205**
Pmin-07															
n	57	53	52	53	54	50	47	46	36	53	50	52	51	43	52
A	6	6	6	6	5	5	5	7	3	7	5	6	6	6	6
H_E	0.697	0.702	0.607	0.700	0.652	0.634	0.607	0.764	0.538	0.704	0.607	0.691	0.641	0.499	0.606
H_O	0.386	0.660	0.442	0.697	0.611	0.680	0.500	0.717	0.679	0.453	0.500	0.500	0.451	0.465	0.673
F_{IS}	**0.453**	**0.069**	0.280	0.039	**0.072**	−0.069	**0.207**	0.072	0.239	**0.365**	0.186	**0.286**	**0.305**	0.079	−0.102
Pmin-08															
n	56	52	53	49	52	51	46	47	36	54	50	48	48	45	52
A	29	28	25	25	22	26	26	25	20	23	24	21	30	27	29
H_E	0.936	0.894	0.900	0.924	0.879	0.913	0.914	0.928	0.888	0.926	0.918	0.923	0.946	0.930	0.940
H_O	0.857	0.884	0.849	0.816	0.692	0.922	0.804	0.872	0.889	0.833	0.900	0.771	0.792	0.910	0.810
F_{IS}	**0.094**	0.020	0.064	0.127	**0.221**	0.001	0.131	0.070	0.013	0.110	0.030	**0.175**	**0.174**	**0.031**	**0.149**
Pmin-10															
n	58	53	53	53	54	52	50	51	36	53	50	52	50	45	52
A	28	32	14	26	24	26	22	21	19	28	28	18	22	30	24
H_E	0.931	0.931	0.853	0.929	0.931	0.947	0.941	0.905	0.879	0.941	0.939	0.909	0.903	0.929	0.931
H_O	0.828	0.660	0.556	0.577	0.860	0.706	0.726	0.580	0.667	0.648	0.686	0.689	0.680	0.591	0.583
F_{IS}	0.120	**0.368**	**0.345**	**0.387**	**0.086**	**0.299**	**0.278**	**0.368**	**0.255**	**0.319**	**0.278**	**0.100**	**0.257**	**0.374**	**0.382**
Pmin-11															
n	58	53	53	53	54	52	50	51	36	53	50	52	50	45	52
A	7	5	6	7	6	7	7	6	5	6	5	5	6	7	6
H_E	0.596	0.543	0.518	0.552	0.598	0.587	0.598	0.585	0.157	0.513	0.439	0.623	0.550	0.533	0.470
H_O	0.552	0.566	0.547	0.547	0.648	0.592	0.700	0.412	0.167	0.418	0.460	0.712	0.600	0.511	0.500
F_{IS}	0.083	0.305	−0.047	−0.051	−0.074	−0.090	−0.160	**0.305**	−0.045	0.089	−0.039	−0.132	−0.081	0.052	−0.057
Total															
MNA	28.57	24.00	20.00	23.57	24.57	23.00	24.29	24.00	15.14	26.57	24.29	20.00	22.14	22.57	22.43
H_E	0.859	0.840	0.813	0.849	0.844	0.846	0.834	0.862	0.743	0.843	0.814	0.827	0.828	0.813	0.817
H_O	0.720	0.676	0.636	0.705	0.761	0.764	0.726	0.676	0.659	0.702	0.726	0.615	0.713	0.743	0.693

Bold values: F_{IS} values deviating significantly from HWE after sequential Bonferronni corrections. For sampling codes, see Table 1.

mainly due to loci *Pmin-06* and *Pmin-07* (Table 7). Nei's (1978) distances exhibited significant values between all pairs of populations after Bonferroni correction (Table 8). The highest values were observed between marine populations and the others, and to a lesser extent between the estuary (Oosterschelde) and coastal populations.

A Mantel test failed to show any correlation between Nei's (1978) genetic distance and geographic distances ($P > 0.05$). Based on this genetic distance, MDS clearly clustered the populations of the coastal area (except Ot2) and the sample from Texel (Tx), the populations of the Oosterschelde, the populations of the Westerschelde and separated the marine populations from these three groups (Figure 2b; correlation values on axis 1 and 2: 0.311). The AMOVA analysis revealed a weak but highly significant inter-regional pattern of genetic structure of the Belgian population of sand gobies, which was composed of four different groups, namely, the marine, the Oosterschelde, the Westerschelde and the coastal populations (Table 4).

Table 6 F-statistics and R-statistics over loci for seven microsatellite loci in 15 populations of *P. minutus*

Locus	*F-statistics*			*R-statistics*		
	F_{IS}	F_{IT}	F_{ST}	R_{IS}	R_{IT}	R_{ST}
Pmin-01	0.165	0.174	0.011	0.192	0.226	0.044
Pmin-05	0.179	0.191	0.015	0.191	0.227	0.050
Pmin-06	0.134	0.174	0.046	0.078	0.167	0.106
Pmin-07	0.170	0.221	0.061	0.109	0.240	0.148
Pmin-08	0.096	0.102	0.007	0.108	0.120	0.015
Pmin-10	0.330	0.351	0.031	0.451	0.462	0.021
Pmin-11	−0.007	0.011	0.012	0.072	0.086	0.016
Total	0.163	0.185	0.026	0.197	0.233	0.058
CI 95%	0.151–0.172	0.172–0.194	0.024–0.028			

Table 7 Estimates of F_{ST} and R_{ST} among pairs of populations of *P. minutus*

	K1	*K12*	*Zs*	*Ra2*	*Ot1*	*Ot2*	*Np*	*Tx*	*Of1*	*W1*	*W2*	*Oc7*	*Oc15*	*Oc21*	*Oc37*
K1	0	0.017	0.025	0.014	0.014	0.018	0.035	0.017	0.049	0.010	0.021	0.032	0.020	0.022	0.022
K12	0.085*	0	0.025	0.014	0.014	0.013	0.036	0.014	0.051	0.015	0.019	0.032	0.021	0.026	0.021
Zs	0.060*	0.013	0	0.017	0.022	0.017	0.035	0.036	0.060	0.021	0.023	0.040	0.025	0.018	0.025
Ra2	0.048*	0.030*	0.013	0	0.008	0.017	0.033	0.016	0.034	0.014	0.018	0.023	0.014	0.027	0.019
Ot1	0.034*	0.047*	0.025*	0.000	0	0.027	0.040	0.013	0.037	0.015	0.025	0.032	0.020	0.035	0.028
Ot2	0.082*	−0.003	0.023*	0.040*	0.063*	0	0.018	0.019	0.064	0.015	0.018	0.024	0.019	0.014	0.016
Np	0.107*	0.049*	0.100*	0.103*	0.099*	0.043*	0	0.029	0.085	0.031	0.031	0.036	0.037	0.021	0.025
Tx	0.074*	0.014	0.041*	0.041*	0.049*	0.008	0.020	0	0.042	0.019	0.023	0.030	0.023	0.039	0.025
Of1	0.099*	0.071*	0.038*	0.027*	0.043*	0.092*	0.148*	0.097*	0	0.042	0.057	0.059	0.051	0.073	0.055
W1	0.022*	0.032*	0.023*	0.010	0.008	0.037*	0.082*	0.053*	0.044*	0	0.022	0.017	0.011	0.019	0.014
W2	0.053*	0.019	0.041*	0.036*	0.048*	0.022*	0.049*	0.027*	0.107*	0.024	0	0.045	0.027	0.025	0.022
Oc7	0.112*	0.056*	0.096*	0.077*	0.084*	0.080*	0.100*	0.100*	0.126*	0.055*	0.065*	0	0.018	0.034	0.026
Oc15	0.085*	0.042*	0.076*	0.068*	0.080*	0.056*	0.072*	0.081*	0.124*	0.038*	0.020	0.017	0	0.027	0.016
Oc21	0.033*	0.029*	0.031*	0.032*	0.034*	0.029*	0.074*	0.033*	0.104*	0.009*	0.013	0.078*	0.053*	0	0.014
Oc37	0.081*	0.005	0.037*	0.041*	0.055*	0.008	0.055*	0.019	0.105*	0.031*	0.024	0.044*	0.040*	0.015	0

F_{ST} estimates for seven microsatellite loci are above the diagonal and R_{ST} are below the diagonal.*Significant values of R_{ST}.

Table 8 Estimates of Nei (1978) distances among pairs of populations of *P. minutus* for seven microsatellite loci

	K1	*K12*	*Zs*	*Ra2*	*Ot1*	*Ot2*	*Np*	*Tx*	*Of1*	*W1*	*W2*	*Oc7*	*Oc15*	*Oc21*	*Oc37*
K1	0														
K12	0.120	0													
Zs	0.148	0.148	0												
Ra2	0.101	0.100	0.102	0											
Ot1	0.100	0.096	0.129	0.058	0										
Ot2	0.132	0.087	0.098	0.120	0.183	0									
Np	0.245	0.240	0.205	0.222	0.273	0.117	0								
Tx	0.139	0.105	0.234	0.121	0.094	0.143	0.205	0							
Of1	0.216	0.233	0.272	0.135	0.146	0.309	0.448	0.178	0						
W1	0.073	0.098	0.126	0.099	0.098	0.099	0.204	0.136	0.176	0					
W2	0.126	0.111	0.126	0.107	0.142	0.100	0.181	0.135	0.251	0.129	0				
Oc7	0.220	0.206	0.239	0.152	0.210	0.155	0.229	0.211	0.274	0.107	0.268	0			
Oc15	0.132	0.133	0.145	0.089	0.125	0.078	0.233	0.154	0.224	0.069	0.155	0.110	0		
Oc21	0.128	0.155	0.099	0.164	0.207	0.119	0.119	0.257	0.342	0.113	0.135	0.200	0.153	0	
Oc37	0.136	0.124	0.140	0.113	0.169	0.093	0.145	0.153	0.245	0.082	0.121	0.150	0.093	0.076	0

All values remained significant after Bonferroni adjustment. For sampling codes, see Table 1.

Estimated population-level admixture proportions with the forced structure confirmed the results found with the MDS analysis and the AMOVA. The nonforced model showed a high level of admixture between the populations considered (Table 9).

Discussion

An unusual genetic diversity and heterozygote deficiency

The degree of heterozygosity as assayed with allozymes was comparable to values compiled for marine fish

Table 9 Estimation of population admixture assessed by the percentage of individuals clustered in the different populations using the program structure (Pritchard *et al*, 2000)

	Forced structure			*Non-forced structure*		
	1	*2*	*3*	*1*	*2*	*3*
Coastal area (1)	0.96	0.03	0.01	0.45	0.26	0.29
Westerschelde (2)	0.04	0.91	0.05	0.32	0.38	0.30
Oosterschelde (3)	0.01	0.02	0.97	0.26	0.31	0.43

species (Ward *et al*, 1994) and other gobioid fishes (Geertjes *et al*, 2001). Wallis and Beardmore (1984a) found slightly lower values in sand goby but studied more loci of which a fairly large proportion were 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. Stefanni *et al* (2003) compared the scoring of this locus also using starch gel electrophoresis (SGE) as cellulose acetate gel electrophoresis (CAGE), and concluded that the observed difference could be due to the weak staining of *LDH-C** on SGE, resulting in the observed differences between the studies.

The genetic diversity of the microsatellite loci, assessed as the expected level of heterozygosity H_E, exhibited a wide range (0.157–0.976) and was 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*. The level of polymorphism of the loci *Pmin-01* (86 alleles), *Pmin-05* (88 alleles) and *Pmin-10* (45 alleles) were striking in that they were much higher than in any other species studied (DeWoody and Avise, 2000). However, these high levels of polymorphism were also reported by Jones *et al* (2001a, b), indicating that they are not due to methodological errors.

Most microsatellite loci and the allozyme *LDH-C** locus clearly show a deficit in heterozygotes. This pattern is not unusual in populations of marine organisms, as has been shown elsewhere (Smith, 1987; García de León *et al*, 1997; De Innocentiis *et al*, 2001), and might find its origin in the Wahlund effect (Wahlund, 1928), cryptic species, inbreeding, groupings of relatives, scoring errors, the occurrence of null alleles or selection against heterozygotes.

The Wahlund effect, the most common explanation of heterozygote deficiency, should result in significant F_{IS} values at more than one allozyme locus, as drift causing population structuring should affect all polymorphic loci similarly (Pogson *et al*, 1995). For the microsatellites, we do not favour this hypothesis because we believe that the high observed F_{IS} values here are mainly due to structuring and mixing of sand goby populations.

Inbreeding remains an unlikely explanation for high F_{IS} in fish with large populations such as gobies that are not subject to drastic reduction in their effective population sizes like commercial fishes (Fu *et al*, 2001; Hoarau *et al*, 2002). Nevertheless, polygyny and polyandry, which are known to be common features in gobies (Lindström and Seppä, 1996), could be responsible for inbreeding because some of the parents might dominate the progeny (see Zeh and Zeh, 2001). 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. It seems highly improbable that all loci exhibit null alleles with such a constant frequency. On the other hand, we cannot reject this hypothesis for the *LDH-C** locus even if selective pressure could also be a possible explanation (Jollivet *et al*, 1995; Allegrucci *et al*, 1997).

Population differentiation over a small geographical scale

In general, our study suggests the existence of two spatially separated breeding units, namely the Oosterschelde estuary and coastal area, while other samples are related to one or the other unit. Comparison of our data with the work of Beheregaray and Sunnucks (2001) and Hoarau *et al*. (2002) leads us to conclude that the structure found in sand goby populations is more complex than the suggested four subunits detected in the AMOVA analysis. The admixture analysis with non-forced structure confirmed this conclusion, showing a high level of admixture between the considered populations. Nonetheless, gene flow appears to be sufficiently large to swamp any potential for large genetic differences detectable with F_{ST} estimates even if a small differentiation was revealed by our data. Moreover, estimates of gene flow and genetic distances based on F_{ST} values assume that population structure has been stable for sufficiently long 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, 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%) suggests that the allele shift between the two breeding units is large enough to be explained by isolation of these two populations during the formation of the Holocene coast. Thus, we assume that several processes such as historical events (colonisation of the Oosterschelde by coastal populations) and, to a lesser extent, restricted contemporary 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). Moreover, a discrepancy between allozyme and microsatellite markers has been described in several marine species (Lemaire *et al*, 2000; De Innocentiis *et al*, 2001). It is indicative of either differentiation in one or both markers (Lemaire *et al*, 2000; De Innocentiis *et al*, 2001), suggesting that the observed differentiation was either due to selection or stochastic events (drift–gene flow) (De Innocentiis *et al*, 2001; McLean and Taylor, 2001). In our data set, microsatellite loci exhibited higher F_{ST} values than allozyme loci, suggesting that the higher mutation rate in microsatellites has increased the allele frequency divergence among populations (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.

A model for the spatial dynamics of sand goby

Historical and contemporary factors seem to be at play in the spatial dynamics of the sand goby in the Southern Bight of the North Sea. Historically, the North Sea coast reached its current shoreline 7,500 years BP as the sea level rose at the end of the last Ice Age (Beets and Van der Spek, 2000). With the rising seawater, gobies must have invaded the area from the English Channel, which can be deduced from the phylogeographical homogeneity of gobies (Gysels, 2003) and plaice (Hoarau *et al*, 2002) in the North Sea. As soon as the water was rising, a mesotidal system became functional (van der Molen and de Swart, 2001a). During the Holocene, the wind–wave regime changed due to modifications in basin geometry with an increase in wave height (and mixing in the coastal zone) for a constant wave period (van der Molen and de Swart, 2001b). If the West Coast and Oosterschelde estuary represent historical evolutionary units for the sand goby, their separation might be linked to the geography of the estuaries of the Great Rivers (Schelde, Maas and Rhine), which enter the North Sea. For example, the Zeeland coast of the Netherlands only reached most of its current form some 700 years ago. Hence, the question arises as to whether the North Sea fauna and flora is currently in a gene flow – genetic drift – selection – mutation equilibrium.

In a contemporary perspective, sand gobies live in a very dynamic environment with strong tidal currents and experience heavy wave action due to the shallow water depth (Otto *et al*, 1990; Ozer *et al*, 2000). However, their preferred mating habitat seems to focus on two regions in the Southern Bight. Off the west coast of Belgium is a retention zone (Van den Eynde, 1994; Lanckneus *et al*, 2001) where the coastal flow from the English Channel 'collides' with the coastal flow from the Rhine/Schelde system. In spring, planktonic larval stages of numerous species can be found in this area (Dewicke, 2001). Later on, the postlarvae of many species, including gobies, drift to the nearby inshore nurseries. Off the Voordelta, a similar retention zone can be observed, this time driven by an SW coastal current and the NE current originating from the Rhine mouth (Hamerlynck and Mees, 1991). Hence the genetic structure of sand gobies and plaice (Hoarau *et al*, 2002) reflects historical and contemporary factors such as drift, homing and larval retention. Juveniles of the sand goby are known to undertake migrations from the coastal area and the Oosterschelde to the Westerschelde in fall, presumably to avoid predation and to find food (Maes *et al*, 1998). This might result in the mixing of genotypes from both breeding units with reduced pairwise differentiation. We are in the process of clarifying the regional dynamics of the seasonal migration, which are linked to feeding and growth, predation and natural mortality and habitat selection.

To conclude based on microsatellite loci, our study revealed the presence of two breeding units presumably connected by a low number of migrants. This pattern is consistent with secondary contact between partially or completely reproductively isolated units but not with a divergence-with-gene-flow model with incipient speciation. The genetic differentiation is most likely attributable to a combination of older historical events and restricted contemporary gene flow.

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