Biological Journal of the Linnean Society, 2004, **83**, 561–576. With 6 figures

Current and historic gene flow of the sand goby *Pomatoschistus minutus* on the European Continental Shelf and in the Mediterranean Sea

E. S. GYSELS[1]*, B. HELLEMANS[1], T. PATARNELLO[2] and F. A. M. VOLCKAERT[1]

[1]*Katholieke Universiteit Leuven, Laboratory of Aquatic Ecology, Ch. de Bériotstraat 32, B-3000 Leuven, Belgium*
[2]*University of Padova, Department of Biology, Via U. Bassi 58, I-35121 Padua, Italy*

Received 28 July 2003; accepted for publication 1 May 2004

Phylogeographical patterns of the sand goby *Pomatoschistus minutus* (Gobiidae, Teleostei) were studied by means of sequence and single-stranded conformational polymorphism analysis of a 283-bp fragment of the cytochrome *b* locus of the mtDNA. A total of 228 individuals sampled at 13 sites throughout the species's distributional range revealed a moderate level of diversity and a low but significant level of overall genetic differentiation at all but one site. The goby sample from the Adriatic Sea differed in sequence by approximately 10% from the Atlantic *P. minutus* and is thought to belong to a cryptic species of the genus *Pomatoschistus*. Limited genetic differentiation with a weak pattern of isolation-by-distance was recorded throughout the distributional range of the typical *P. minutus*. Phylogeographical analysis suggested a contiguous range expansion in the Atlantic and Baltic basins during the Eemian and evidence for a glacial refugium in the southern North Sea during the Weichselian. In *P. minutus* from the western Mediterranean Sea a high number of endemic haplotypes as well as the most common Atlantic haplotype were recorded in appreciable frequencies. This might be explained by secondary contact between different mitochondrial lineages, which evolved in allopatry.

ADDITIONAL KEYWORDS: Adriatic Sea – allopatric speciation – cytochrome *b* – genetic structure – phylogeography.

INTRODUCTION

The genetic structure of marine organisms has been shaped by several factors, such as: (1) behaviour and reproductive mode of the species, (2) oceanic currents as a means for either (larval) dispersal or retention (Lessios, Kessing & Robertson, 1999), and (3) past climatological and vicariance events (Bernatchez & Wilson, 1998). Disentangling the relative importance of each of these factors for explaining population structure in the marine environment remains a challenge, because it requires the integration of data from different fields such as palaeoclimatology, geology (pale)oceanography and the biology of the species. Most marine species have an extended pelagic larval phase, which potentially enables them to cover large distances via oceanic currents. Consequently, marine organisms reveal in general a low degree of population differentiation (for a review, see Ward, Woodwark & Skibinski, 1994) and phylogeographical patterns are often less resolved, in contrast to freshwater and terrestrial species. This may explain the paucity of data on phylogeographical patterns of the marine ichthyofauna, in particular from the north-eastern Atlantic coasts. Although numerous studies have dealt with evolutionary patterns in the anadromous salmonids (García-Marín, Utter & Pla, 1999; Verspoor, McCarthy & Knox, 1999; Consuegra *et al*., 2002), data on the phylogeography of truly marine fish along the European Atlantic coasts remain scarce, and are usually limited to highly vagile, commercially exploited species (Borsa, Blanquer & Berrebi, 1997; Nesbø *et al*., 1999).

The sand goby *Pomatoschistus minutus* (Pallas, 1770) is one of the most abundant fish species along the Atlantic coasts of western Europe (Healey, 1971).

*Corresponding author. E-mail: els.gysels@bio.kuleuven.ac.be

It occurs in the Black Sea, the Mediterranean Sea and the north-eastern Atlantic Ocean from the south of Spain to northern Norway (Tromsø) (Miller, 1986). It is present in estuaries as well as in the open sea (Claridge *et al*., 1985) but usually spawns near the coast (Fonds, 1973). Male *P. minutus* display courtship behaviour and establish a territory, build a nest under an empty bivalve shell and court a female (Fonds, 1973). A female deposits eggs, which are fertilized by the male, who protects and fans them for several weeks until they hatch. Females deposit batches of eggs in the nests of several males, while males may guard eggs of more than one female. Upon hatching, the larvae dwell in the plankton for at least 1 month before metamorphosis (Fonds, 1973). In northern regions *P. minutus* carries out a thermal migration towards deeper water when the water temperature drops below 4–5 °C (Fonds, 1973). Adults are considered poor swimmers and are adapted to a demersal life with pelvic fins fused into a suction disc (Miller, 1986).

Any passive large-scale larval dispersal of marine organisms, including *P. minutus*, in the north-eastern Atlantic Ocean is influenced by the extensive oceanic and coastal currents. The most important currents are the North Atlantic Current (NAC) and the Shelf Edge Current (SEC), flowing northward along the western coasts of the British Isles towards the Norwegian trench. An arm branches off at the Shetland Islands and flows southward along the eastern British coast and eastward towards the Skagerrak (Turrell, 1992). Atlantic water also enters the North Sea via the English Channel, resulting in a northward flow through the English Channel (Fig. 1). The strong tidal currents, especially in the southern North Sea and the English Channel, may also facilitate gene flow within coastal fish species such as *P. minutus*. Thus, if present-day gene flow between Atlantic *P. minutus* is maintained by passive larval drift via currents, we may expect limited genetic differentiation on a small scale, along with a cline of isolation-by-distance on a larger geographical scale throughout the north-eastern Atlantic basin.

The Mediterranean Sea is connected with the Atlantic Ocean through the Strait of Gibraltar, but the more significant interaction between Atlantic and Mediterranean water takes place at the Almería-Oran Front in the Alboran Sea (Tintore *et al*., 1988). This front acts as a barrier for dispersal to several marine species (Zane *et al*., 2000; Ríos *et al*., 2002) and may limit gene flow in *P. minutus*. Preliminary genetic studies of *P. minutus* based on allozymes (Stefanni *et al*., 2003) point to a high level of gene flow throughout the distributional range of the species, with little differentiation between the Atlantic Ocean and the western

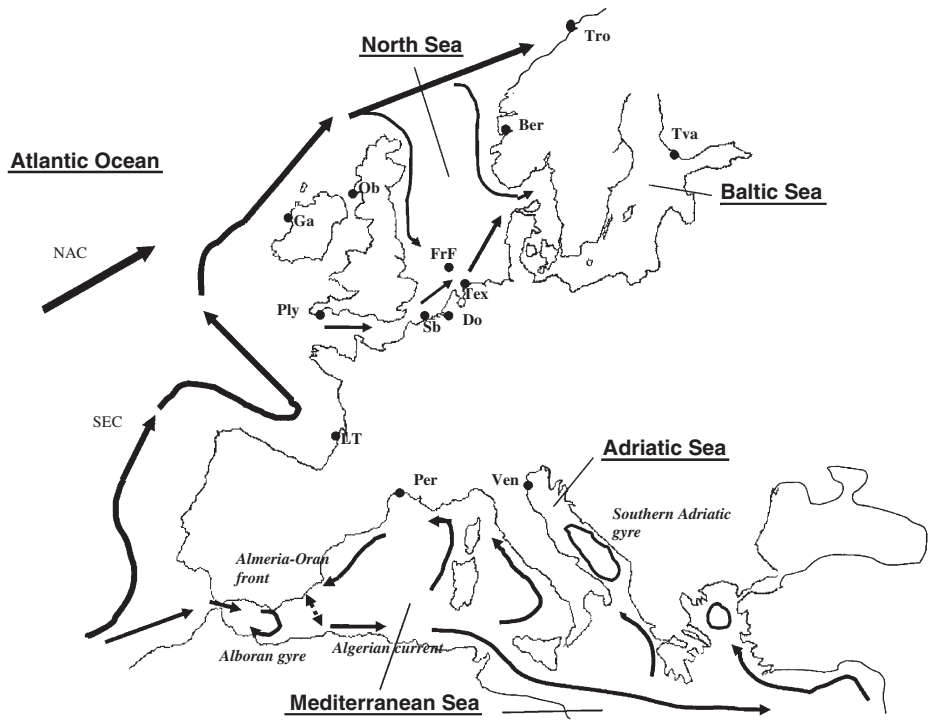

Figure 1. Sampling sites of *Pomatoschistus minutus* with main current patterns, compiled after Turrell (1992), Millot (1999) and Hansen & Østerhus (2000). Sampling sites are indicated with ●. Abbreviations of sampling sites are listed in Table 1. NAC, North Atlantic Current; SEC, Shelf Edge Current.

Mediterranean Sea. However, the resolution of allozymes for detecting population structure is limited, especially in highly vagile marine species. Thus, analysis of these samples with a more sensitive DNA marker, in combination with a larger number of sampling sites, should shed more light on the population genetic structure of this widely distributed goby.

Other major factors that may explain contemporary population structure are past geological and climatological events. The present distribution of *P. minutus* in the north-eastern Atlantic Ocean is the result of a northward population range expansion after the last glaciation (Weichselian), which ended about 10 kyr BP. During the last glacial maximum (22 kyr BP) an ice sheet covered Scandinavia and most of the British Isles (Lowe & Walker, 1997); the Southern Bight of the North Sea was dry (Van der Molen & de Swart, 2001), but a glacial lake has been proposed in the southern North Sea (Balson *et al*., 1991). The Iberian peninsula and the Mediterranean Sea have served as a southern refugium for many northern species (Bianchi & Morri, 2000; Consuegra *et al*., 2002), but additional refugia at the margins of the ice sheets in the southern North Sea and the Baltic region have been proposed (García Marin *et al*., 1999; Koljonen *et al*., 1999; Verspoor *et al*., 1999). After deglaciation the sea level rose and the ocean invaded the Southern Bight of the North Sea through the Strait of Dover (van der Molen & de Swart, 2001). By 7.5 kyr BP the present connection between the southern North Sea and the Atlantic Ocean was formed. The re-establishment of the North Atlantic Current around 10 kyr BP allowed marine organisms to migrate northward from their southern refugia. Whether *P. minutus*'s range expansion occurred from a southern refugium only, or isolated populations managed to survive in the north along the ice sheets and recolonized formerly glaciated areas, may be inferred from the geographical distribution of genetic variation (Templeton, Routman & Phillips, 1995).

The only phylogeographical analysis of *P. minutus* to date was carried out by Stefanni & Thorley (2003), who focused mainly on the distinctness of the Adriatic *P. minutus*. They postulated an evolutionary significant unit (ESU) in the northern Adriatic Sea of recent origin (5–10 kyr BP), while the Atlantic and western Mediterranean *P. minutus* would belong to a second ESU. However, allozyme electrophoresis revealed a similar degree of differentiation between the Adriatic *P. minutus* and the other sand goby samples as between *P. minutus* and its closest relative, *P. lozanoi* (de Buen, 1923), suggesting that the taxonomic status of the Adriatic *P. minutus* may have to be revised (Gysels, 2003). Moreover, no phylogeographical inferences about the Atlantic and Baltic *P. minutus* were made. Hence, we aim to: (1) complement the existing phylogeographical and population genetic information on *P. minutus* by including a larger number of sampling sites, and (2) assess the genetic differentiation between Venetian and other *P. minutus* by means of a second (mitochondrial) genetic marker in order to reinforce our conclusions regarding the hypothesis of allopatric speciation in the Adriatic Sea.

MATERIAL AND METHODS

Sequence and single-stranded conformational polymorphism (SSCP) analysis of mtDNA

Samples were taken either by hand net, beach seine or beam trawling. Sampling sites with coordinates and number of fish screened are listed in Table 1. A total of 228 *P. minutus* were subjected to sequence and single-

Table 1. Geographic location of the *Pomatoschistus minutus* sampling sites, codes, sampling periods and coordinates

Oceanic basin / Sampling site	Code	Period	Latitude	Longitude
Atlantic				
Trondheim (Norwegian Sea, central Norway)	Tro	September 2000	63°24′N	10°24 E
Bergen (North Sea, western Norway)	Ber	September 1997	60°23′N	5°21′E
Tvärminne (Baltic Sea, Finland)	Tva	July 2001	59°50′N	23°15′E
Oban (Atlantic Ocean, western Scotland)	Ob	August 1999	56°24′N	5°28′W
Galway (Atlantic Ocean, western Ireland)	Ga	August 1999	53°16′N	9°03′W
Frisian Front (North Sea, the Netherlands)	FrF	January 1999	53°30′N	4°00′E
Texel (Wadden Sea, the Netherlands)	Tex	December 1998	53°00′N	4°46′E
Doel (Schelde estuary, Belgium)	Do	October 1998	51°19′N	4°16′E
Stroombank (southern North Sea, Belgium)	Sb	February 1997	51°13′N	2°52′E
Plymouth (English Channel, UK)	Ply	November 1996	50°22′N	4°09′W
La Tremblade (Gulf of Biscay, France)	LT	October 2000	45°46′N	1°08′W
Mediterranean				
Pérols (Gulf of Lions, France)	Per	January 1998	43°34′N	3°57′E
Venice (Adriatic Sea, Italy)	Ven	October 1999	45°26′N	12°19′E

stranded conformational polymorphism (SSCP) analysis. *Pomatoschistus* sp. were identified morphologically on the basis of the dermal papillae of the head according to Miller (1986) and biochemically according to Wallis & Beardmore (1984). A fin clip was taken from each specimen for DNA extraction and stored in 100% ethanol. DNA extraction was carried out with the DNeasy tissue kit (Qiagen International) and with a standard phenol–choloroform protocol.

The universal cytochrome *b* primers (Kocher *et al*., 1989) did not work for *P. minutus*, but yielded a PCR product in the related species *P. lozanoi* (de Buen, 1923) and *P. microps* (Krøyer, 1838). Cytochrome *b* PCR products obtained for these two species were cloned with the TA cloning kit (Invitrogen) and both strands were sequenced with fluorescence-labelled M13 primers on a LICOR 4200 automated sequencer (Westburg). A BLAST search confirmed that the correct fragment had been amplified. Sequences of *P. microps* and *P. lozanoi* were aligned with cytochrome *b* sequences of other gobiid fishes retrieved from GENBANK *(Tridentiger bifasciatus* AB021254, *Proterhorhinus marmoratus* AF082969 and *Rhinogobius giurinus* AB018997). A new set of primers was designed for amplifying a fragment of the cytochrome *b* locus, based on conserved sequences across these goby species. These primers amplified a 283-bp fragment in *P. minutus*, which was used for SSCP analysis (Orita *et al*., 1989). Primer sequences were: GobycytbF: 5′-CCC ATC AAA CAT TTC TGC-3′ and GobycytbR: 5′- ACA TAG CCA ACG AAG GC-3′. PCR conditions for the SSCP reaction were as follows: denaturation at 97 °C for 3 min; 35 cycles of denaturation at 95 °C for 45 s, annealing at 60 °C for 45 s and elongation at 72 °C for 45 s, and a final elongation of 7 min at 72 °C. Both cytochrome *b* strands were sequenced. The MgCl2 concentration was 2 mM. Specimens of *P. lozanoi* and *P. microps* were employed as outgroup species. The same conditions as those for *P. minutus* were used for amplification and sequencing of *P. lozanoi*. For details on the sequence analysis of *P. microps* refer to Gysels *et al*. (2004).

The SSCP was carried out at a temperature of 4 °C with a run time of 2 h at 600 V; bands were visualized by silver staining. Each individual was run several times under identical conditions. A few individuals from each SSCP mobility group (depending on the mobility class frequency) were randomly chosen and their nucleotide sequence was determined. PCR conditions for the sequencing reaction were as follows: denaturation at 95 °C for 3 min; 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 15 s and elongation at 70 °C for 1 min, and a final elongation of 7 min at 70 °C. Both strands were sequenced. Sequences were analysed with the GeneReadIR DNA system and the AlignIR software (LICOR, Westburg).

Data analysis

Sequences were aligned in CLUSTALW version 1.7 (Thompson Higgens & Gibson, 1994). Genetic diversity, measured as haplotype (h) and nucleotide (π) diversity (Nei, 1987) were computed in DNASP version 3.51 (Rozas & Rozas, 1999). Because visual inspection of the sequences revealed an unequal distribution of mutation sites throughout the cytochrome *b* fragment, the program PUZZLE version 5.0 (Schmidt *et al*., 2002) was used for calculating the parameter á of the gamma distribution. A neighbour-joining haplotype dendrogram (Saitou & Nei, 1987) based on the Tamura-Nei genetic distances was computed in the program MEGA version 2.1 (Kumar *et al*., 2001). The model of Tamura & Nei (1993) takes into account unequal nucleotide frequencies and unequal mutation rates within the fragment. A sequence of each of the related species *P. microps* and *P. lozanoi* was employed as an outgroup in the dendrogram. Support for the nodes was assessed with bootstrapping (1000 replicates) (Felsenstein, 1985).

Pairwise genetic distances between samples were computed in ARLEQUIN version 2.0 (Schneider, Roessli & Excoffier, 2000) according to the model of Tamura & Nei (1993). Significance was assessed with permutation tests (1000 replicates). A sequential Bonferroni test was applied to correct significance levels for multiple testing (Rice, 1989). For assessing patterns of isolation-by-distance, a Mantel test (Mantel, 1967) on the geographical vs. genetic distances was carried out in GENETIX version 4.04. The distance matrix was constructed based on the shortest coastal distance between sites with the electronic atlas ENCARTA (Microsoft, 2001). In addition, a plot of geographical vs. genetic distances was constructed in STATISTICA version 6.0 (STATSOFT, 2001). The pairwise genetic distances were used as the basis for a multidimensional scaling analysis in the program STATISTICA for detecting indications of group structure. Subsequently, samples were grouped and subjected to a hierarchical analysis of variance (AMOVA) (Excoffier, Smouse & Quattro, 1992) in the program ARLEQUIN for assessing whether any significant geographical group structure could be detected.

For studying phylogeographical relationships we constructed a minimum spanning tree, which minimizes the connections between the haplotypes, in the program ARLEQUIN. This minimum spanning network was used as the basis for a nested design for performing a nested clade analysis (NCA). This analysis (Templeton *et al.*, 1995) tests for geographical associations between haplotypes and groups of haplotypes on different hierarchical levels and allows for distinguishing between historical events and contemporary gene flow to explain the observed genetic structure.

Significance of the clade distances and the nested clade distances was calculated with permutation tests as implemented in the program GEODIS (Posada, Crandall & Templeton, 2000) (1000 replicates) and interpreted with the inference key according to Templeton (1998). The parameters of the distribution of pairwise differences between haplotypes (mismatch distribution) (θ_0, θ_1 and τ) (Rogers & Harpending, 1992) were also calculated in the program ARLEQUIN. The parameter $\tau = \mu T$, where μ is the mutation rate and T the time since expansion in generations, was used for estimating the time of expansion of the *P. minutus* haplotypes.

RESULTS

Genetic diversity analysis

Twenty-eight haplotypes were recorded among the 228 individuals screened at 13 sampling sites. The various haplotypes with their EMBL accession numbers are listed in Appendix 1. The global haplotype and nucleotide diversity amounted to 0.627 and 0.0113, respectively. When the Venetian sample was excluded, haplotype and nucleotide diversity dropped to 0.593 and 0.0037, respectively. The lowest levels of haplotype and nucleotide diversity were recorded in Oban ($h = 0.054$, $\pi = 0.0002$) while the highest values were found in the western Mediterranean Sea ($h = 0.811$, $\pi = 0.0100$). These two samples differed by an order of magnitude in both haplotype and nucleotide diversity (Table 2). No indels were recorded. Forty-four segregating sites were recorded (Appendix 1), corresponding to 15% polymorphic sites. Thirty-two of the segregating sites were phylogenetically informative, while 12 mutational sites represented singletons. The transition/transversion ratio was 1.61 and parameter α of the gamma distribution was 0.23. Base frequencies were T = 0.198, C = 0.186, A = 0.324 and G = 0.291.

Haplotype A was the most widespread, being present in appreciable frequencies in all samples, with the exception of the Venetian sample, where it was absent (Table 2). The sample from Pérols was the most diverse, with 10 haplotypes recorded among 22 individuals. Eight haplotypes were unique to this sample and only two were shared with the Atlantic samples (A and K). All Venetian haplotypes were exclusive: none occurred in other samples and vice versa. Haplotypes F and G occurred commonly in the four samples of *P. minutus* collected in the southern North Sea (Doel, Stroombank, Texel and Frisian Front), but were not recorded in the samples north of the Frisian Front or south of the Belgian coast (Appendix 2). Only one haplotype was found in the samples from the Gulf of Biscay (two individuals) and Galway (four individuals), but the small sample numbers at these sites may have biased results. The least diverse samples containing a substantial number of individuals were Oban and Plymouth, where haplotype A occurred in at least 90% of the fish screened (Table 2).

The haplotype dendrogram (Fig. 2) clearly reveals (1) the complete isolation of the Venetian haplotypes,

Table 2. Number of *Pomatoschistus minutus* screened per sampling site (*N*), number of mtDNA haplotypes (n), number of unique haplotypes (nh), haplotype diversity (h) and nucleotide diversity (π), with standard deviation between parentheses and percentage of occurrence of the most common haplotype (MCH)

Site	*N*	n	nh	h	π	MCH	OH
Atlantic							
Tro	17	3	1	0.228 (0.129)	0.0080 (0.0005)	A (88.24%)	B, D
Ber	20	3	0	0.416 (0.116)	0.0015 (0.0005)	A (75.00%)	B,C
Tva	11	3	1	0.473 (0.026)	0.0018 (0.0007)	A (72.73%)	K, Q
Ob	37	2	1	0.054 (0.050)	0.0002 (0.0002)	A (97.28%)	AF
Ga	4	1	0	0	0	A (100.00%)	–
FrF	15	5	2	0.771 (0.072)	0.0043 (0.0006)	F (40.00%)	F, G, I, K
Tex	24	6	3	0.659 (0.085)	0.0037 (0.0009)	F (54.16%)	E, F, G,T, Z
Do	26	3	0	0.628 (0.065)	0.0031 (0.0004)	F (53.85%)	F, G
Sb	20	3	0	0.532 (0.100)	0.0024 (0.0005)	F (65.00%)	F, G
Ply	20	3	2	0.195 (0.0131)	0.0024 (0.0015)	A (90.00%)	R, S
LT	2	1	0	0	0	A (100.00%)	–
Mediterranean							
Per	22	10	8	0.844 (0.062)	0.0100 (0.0011)	A (36.36%)	H, K, U, V, W X, AA, AB, AC
Ven	10	5	5	0.822 (0.097)	0.0096 (0.0016)	N (40.00%)	L,M, O, P

Atl, Atlantic; MS, Mediterranean Sea; OH, other, less common haplotypes found at the sampling site. For sample codes see Table 1.

(2) the western Mediterranean as a separate cluster from the Atlantic *P. minutus*, (3) a shallow phylogeographical structure within the Atlantic basin, and (4) a higher level of divergence between the western Mediterranean haplotypes than between Atlantic haplotypes. The Adriatic *P. minutus* were grouped with *P. lozanoi* rather than with the other *P. minutus*. Bootstrap values between the Atlantic haplotypes were very low, usually lower than 30%. Furthermore, the dendrogram suggests that the Venetian lineage is even older than is the split between *P. minutus* and *P. lozanoi*. Pairwise genetic distances (not shown) between sequences varied between 0.007 and 0.018 for the Atlantic *P. minutus* haplotypes, while those between *P. lozanoi* and *P. minutus* ranged from 0.087 to 0.095. The genetic distance between the Adriatic *P. minutus* and the others was slightly higher, ranging from 0.104 to 0.117.

Genetic diversity and latitude

Linear regression of both haplotype and nucleotide diversity vs. latitude yielded a significantly negative correlation ($R^2 = 0.39$, $P = 0.040$ and $R^2 = 0.69$, $P = 0.001$, respectively) (Fig. 3). The samples from Galway and La Tremblade were not taken into account for the regression analysis due to the low number of individuals screened. Outliers in the regression were the British samples (Oban and Plymouth), showing a very low level of haplotype diversity. This is due to the almost exclusive occurrence of haplotype A, occurring in more than 90% of the samples screened (Table 2).

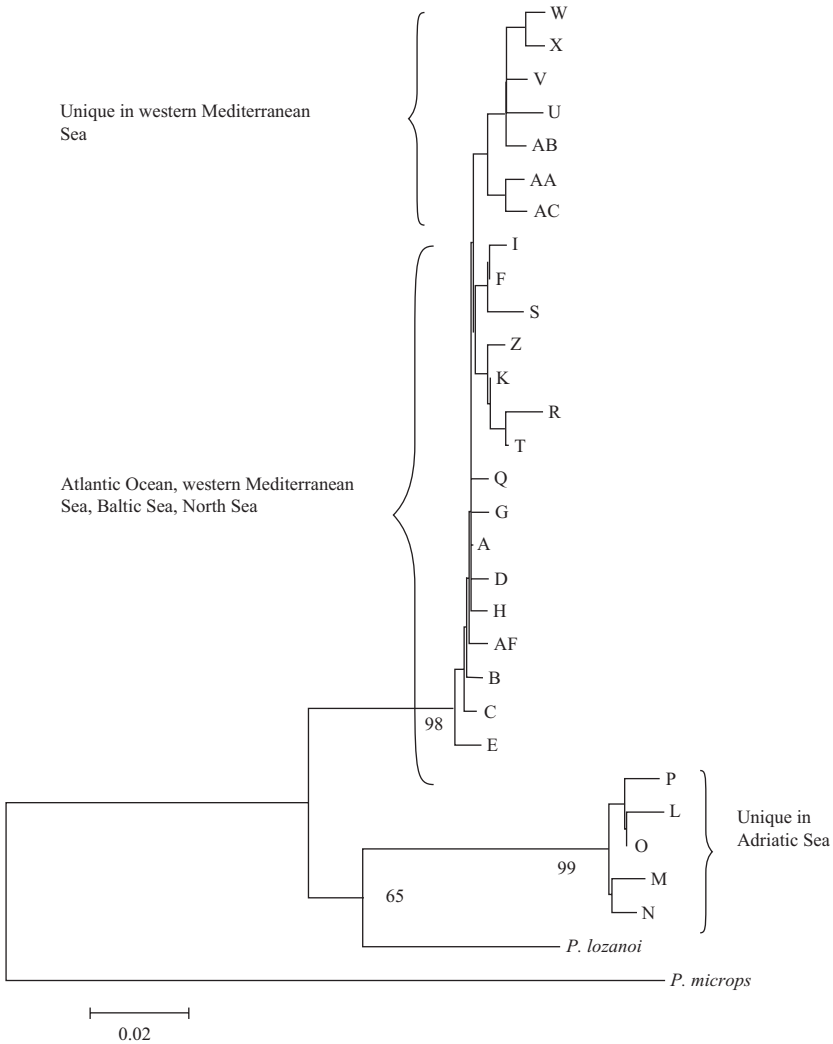

Figure 2. Neighbour-joining haplotype dendrogram for *Pomatoschistus minutus* based on Tamura-Nei genetic distances with *P. microps* and *P. lozanoi* as outgroups. Only bootstrap values higher than 60% are given. For haplotype designations see Appendix 1.

POPULATION DIFFERENTIATION OF *POMATOSCHISTUS MINUTUS* THROUGHOUT ITS DISTRIBUTIONAL RANGE

The values of the pairwise genetic distances are presented in Table 3; a sample of *P. lozanoi* has been added for comparison. The samples from Galway and La Tremblade were not included because of the small sample size (only four and two fish screened, respectively). Clearly, the largest values of pairwise distances were found between the Venetian sample and all the others. Furthermore, this differentiation was comparable to the genetic distance between *P. minutus* and that of *P. lozanoi* (Table 3). The sample from Pérols (Gulf of Lions, western Mediterranean Sea) was also significantly differentiated from the Atlantic *P. minutus*. Pairwise genetic distances within the north-eastern Atlantic Ocean and the Baltic Sea were generally not significant.

Because of the large differentiation between the *P. minutus* from the northern Adriatic Sea and the other samples (Fig. 1, Table 3), the Venetian *P. minutus* were omitted from further population genetic and phylogeographical analysis to avoid distortion of the results.

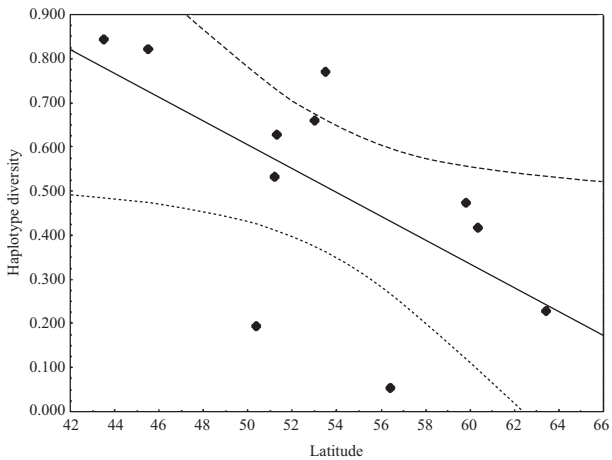

Figure 3. Regression of the haplotype diversity of *Pomatoschistus minutus* plotted against latitude. The dashed lines represent the 95% confidence interval. $R^2 = 0.39$, $P = 0.040$.

Isolation-by-distance

A Mantel test for all samples (except for Galway and La Tremblade) showed a significant correlation between geographical and genetic distances ($r = 0.76$, $P = 0.0062$), but when the Venetian sample was excluded, a significant effect was no longer observed ($r = 0.02$, $P = 0.249$). Yet, despite the lack of significance, some evidence for a pattern of isolation-by-distance was observed when geographical distance was plotted against genetic distance (Fig. 4).

GENETIC STRUCTURE OF *P. MINUTUS* WITHIN THE ATLANTIC AND WESTERN MEDITERRANEAN BASINS

A multidimensional scaling analysis was performed on all samples except those from Venice to see whether any indication for grouping structure could be found. Figure 5 shows that the samples from the southern North Sea (dominated by haplotype F) were grouped together, while the samples from Norway, Scotland and the English Channel (haplotype A-dominated) also formed a distinct group, indicating at least some degree of geographical structuring within the Atlantic

Table 3. Pairwise genetic distance (Tamura-Nei, 1993) for all samples of *Pomatoschistus minutus*

	Atlantic									Mediterranean		
	Tro	Ber	Ob	Tva	FrF	Tex	Do	Sb	Ply	Per	Ven	Pl
Tro	0.000											
Ber	0.021	0.000										
Ob	0.027	0.161**	0.000									
Tva	0.063	0.101	0.176	0.000								
FrF	0.273	0.279	0.423	0.208	0.000							
Tex	0.071	0.106	0.133	0.039	0.028	0.000						
Do	0.337	0.341**	0.455	0.301	–0.039	0.078	0.000					
Sb	0.471	0.451**	0.614	0.415	0.019	0.135	–0.007	0.000				
Ply	0.008	0.062	0.044	0.002	0.189	0.026	0.257	0.345	0.000			
Per	0.234**	0.252**	0.342**	0.185	0.267**	0.243**	0.338**	0.353**	0.229**	0.000		
Ven	0.965**	0.964**	0.982**	0.953**	0.948**	0.956**	0.960**	0.962**	0.960**	0.920**	0.000	
Pl.	0.984**	0.979**	0.993**	0.976**	0.960**	0.963**	0.969**	0.974**	0.973**	0.920**	0.934**	0.000

Values indicated with an ** are significant at the 0.01 level, after sequential Bonferroni correction. A population of *P. lozanoi* (Pl) is added for a comparison. For sample abbreviations see Table 1.

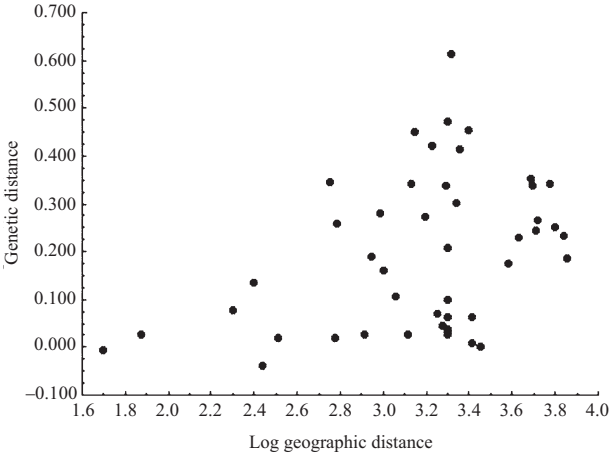

Figure 4. Plot of log-transformed geographical distances against genetic distances for all samples of *Pomatoschistus minutus*. The Venetian sample is excluded.

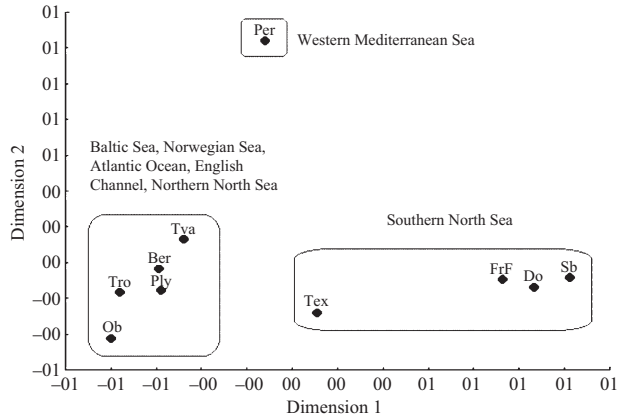

Figure 5. First and second dimensions of the Multidimensional Scaling Analysis of *Pomatoschistus minutus*. The Venetian sample is excluded to avoid distortion. Sample abbreviations are listed in Table 1.

Table 4. Hierarchical analysis of variance (AMOVA) based on mtDNA haplotypes scored in the Atlantic samples of *Pomatoschistus minutus*

Source of variation	% of variation	F-statistics
Among groups	27.83	FCT = 0.278*
Among populations within groups	3.88	FSC = 0.054*
Within populations	68.29	FST = 0.317*

Group 1 = southern North Sea; Group 2 = Baltic Sea, northern North Sea and Atlantic Ocean. *$P < 0.05$.

P. minutus. In order to ascertain whether the structure was significant, we carried out an AMOVA on the Atlantic samples. Samples were divided into two groups: (1) the southern North Sea (Doel, Stroombank, Frisian Front and Texel), and (2) all the others (Baltic Sea, Oban, Bergen and Trondheim). The AMOVA showed that most variation was found within populations (68%), while 27% was due to variation between the two geographical groups (southern North Sea–all other sites). Four percent was explained by variation among samples. All values were highly significant (Table 4).

PHYLOGEOGRAPHICAL ANALYSIS

Nested clade analysis

Intraspecific relationships between haplotypes are often better represented by a network than by a bifurcating tree because they are not hierarchical; a natural population consists of a combination of ancestral haplotypes and new variants (Posada & Crandall, 2001). The minimum spanning tree (Fig. 6) grouped the haplotypes of *P. minutus* in two geographically distinct clusters: Clade III-1, comprising the unique western Mediterranean haplotypes, and Clade III-2, containing the Atlantic haplotypes (Table 5). Clades III-1 and III-2 were connected through haplotype K, which was present in both basins. The topology of the clusters was clearly different: the Atlantic clade showed a star-like pattern with one central and common haplotype A and a number of rare variants radiating from it, generally separated by only one mutational step from haplotype A. The Mediterranean cluster revealed more differences between the various haplotypes and no clear star-like pattern was observed. Permutation analysis of clade distances vs. nested clade distances revealed that the null hypothesis of no geographical association of haplotypes could be rejected at several levels in the NCA. At the lowest level (one-step) clade containing haplotype A (Clade 1–11), restricted gene flow and isolation-by-distance was suggested. At the two-step level a contiguous range expansion was suggested for Clade 2–5 (containing haplotype F), which was the dominating haplotype in the Southern Bight. Restricted gene flow and isolation by distance was suggested for Clade 2–4. Due to the uncertain tip/interior status of Clades III-1 and III-2, no analysis of the total cladogram could be performed.

Mismatch distribution

As the star-like phylogeny of the Atlantic clade pointed to a population expansion, the mismatch distribution was calculated for assessing the time of expansion of the Atlantic haplotypes. Tests of the goodness-of-fit revealed that the model of a sudden population expansion could not be rejected ($P < 0.0001$). The values of the parameters τ, θ_0 and θ_1 were 0.731, 0.088 and 1669,

Table 5. Phylogeographical inferences from the nested clade analysis (Templeton, 1998) of *Pomatoschistus minutus*

Clade	Dominant haplotype/geographical distribution	Inference
1–11	A/all sites	Restricted gene flow and isolation-by-distance
2–4	K/western Mediterranean Sea, Atlantic Ocean	Restricted gene flow and isolation-by-distance
2–5	F/Southern Bight	Contiguous range expansion

Only clades where the null hypothesis could be rejected are represented. For the nested design, see Figure 6.

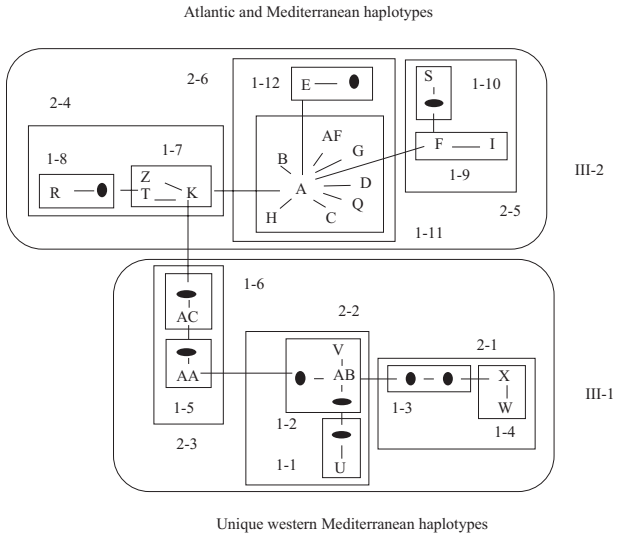

Figure 6. Nested design for *Pomatoschistus minutus* following Templeton *et al*. (1992). Black ovals represent missing haplotypes. Each branch represents one mutational step and the branch length is not proportional to the distance between the haplotypes.

respectively. Tajima's D-statistic amounted to −1.83 and was significantly negative ($P = 0.019$), further supporting the model of a sudden expansion. Assuming a mutation rate of 2% per Myr, this corresponds to a time of expansion of 130 kyr BP.

DISCUSSION

The results point to: (1) the complete isolation of northern Adriatic *P. minutus*, (2) significant differentiation between *P. minutus* of the western Mediterranean Sea and those of the Atlantic Ocean, and (3) limited genetic differentiation between *P. minutus* within the Atlantic and Baltic basins with a weak pattern of isolation-by-distance, but with a separation into two groups.

The Mediterranean Sea

Three striking patterns were recorded for *P. minutus* in the Mediterranean basin: (1) the complete isolation of the northern Adriatic *P. minutus*, (2) the high level of endemism of the Mediterranean haplotypes, and (3) the presence of the Atlantic haplotype A in an appreciable frequency.

Cryptic speciation of P. minutus in the northern Adriatic Sea

Our results support the findings of Stefanni *et al*. (2003), who reported that the genetic distance as observed with allozymes between Adriatic and other *P. minutus* is similar to the distance between *P. minutus* and *P. lozanoi*. Wallis & Beardmore (1983) recorded distinct alleles at the loci *LDH-A** and *LDH-C** in the Adriatic as opposed to Atlantic *P. minutus*. When recalculating our distances using the Kimura-2 parameter model we obtained values ranging from 0.092 to 0.113 for the difference between *P. minutus* and *P. lozanoi*, and a distance from 0.122 to 0.147 between Adriatic and other *P. minutus*. This falls within the range reported by Johns & Avise (1998) for interspecific differences. These data suggest that the Adriatic population must have been subject to an extended period of isolation. This contrasts with the suggestion of Stefanni & Thorley (2003), who date the split between the Adriatic and other Mediterranean *P. minutus* between 5 and 10 kyr BP based on D-loop sequences. They suggest that *P. minutus* in the Mediterranean Sea migrated northwards in reaction to rising temperatures after the last glacial period and became trapped in the cul-de-sac of the Adriatic Sea. The present isolation of the northern Adriatic *P. minutus* would be maintained by a temperature barrier in the southern Adriatic, where the summer sea surface temperature may be as high as 24 °C. (P. D. Simonovic, pers. comm.). As *P. minutus* tends to avoid water temperatures above 19 °C (Hesthagen, 1977), the species may not be able to cross the warm waters of the southern Adriatic Sea, remaining isolated in the northern part of the sea.

An extensive morphometric study did not reveal sufficient differentiation for discrimination between the Venetian and other *P. minutus* on morphological grounds (Stefanni, 2000a). However, a lack of congru-

ence between results from morphological and genetic markers is often observed. Fish are subject to phenotypic plasticity, suggesting that morphological criteria might not be appropriate for delineating species boundaries. Indeed, sequence analysis of the 12S and 16S rRNA genes (Huyse, 2002) confirms the genuine reproductive isolation of the Venetian *P. minutus*. Our data show also that the Adriatic *P. minutus* fall completely outside the group of the other (genuine) *P. minutus* (western Mediterranean Sea and Atlantic Ocean). This is in line with the results of Huyse, Van Houdt & Volckaert (2004), suggesting that the Adriatic *P. minutus* may have split off before the speciation of *P. lozanoi*, *P. minutus* and *P. norvegicus* within the *P. minutus* complex. Whether *P. lozanoi* and the Adriatic *P. minutus* indeed share a common ancestor, distinct from the other *P. minutus*, as suggested in the haplotype dendrogram, remains unsure, given the low bootstrap value. Speciation within this group apparently occurred very rapidly over a small time frame, and may even have been simultaneous (Huyse *et al.*, 2004).

Assuming a molecular clock of between 1% (slow) and 2% (fast) per 10^6 years (Myr) for cytochrome *b*, this would mean that the Venetian *P. minutus*, which differed by 9.19% from the other *P. minutus*, must have become isolated between 4.5 and 9 Mya. It has been suggested that the Messinian salinity crisis (Hsü *et al.*, 1977), which occurred between 5.96 and 5.33 Mya, played a major role in the speciation of Mediterranean gobies (Miller, 1990). During this period, the Mediterranean basin was completely isolated from the Atlantic Ocean and desiccated, resulting in the formation of a series of hypo- and hypersaline lakes and the extinction of most of the existing marine fauna. *P. minutus* is adapted to brackish-water conditions and hence might have been able to survive in river systems in the northern Adriatic basin. However, this time frame conflicts with the view of Huyse (2002), who suggests a more recent origin of the Adriatic *P. minutus*, of about 1.73–1.13 Mya. In this interpretation, sea-level changes during glaciations might have isolated the Adriatic basin and induced allopatric speciation of a relict population of proto-*P. minutus*. The small fragment that we sequenced of cytochrome *b* (283 bp), which may have been hypervariable and has a limited resolution for addressing phylogenetic issues, might have biased our estimation. Penzo *et al*. (1998) also found no concordance between the timing of speciation of Mediterranean freshwater gobies and the Messinian salinity crisis. Nevertheless, the estimates of allozymes, cytochrome *b*, 12S and 16S show that the split between the Adriatic and the other *P. minutus* must have a much older origin than the 5–10 kyr suggested by Stefanni & Thorley (2003).

The western Mediterranean Sea

The origin of the Atlanto-Mediterranean gobies lies probably within the Mediterranean basin, where the oldest known fossil goby remains were found (Simonovic, 1999). It is hypothesized that the Atlanto-Mediterranean goby group evolved in the Mediterranean Sea independently from the Indo-Pacific gobies after the closing of the Thetys Sea from the Indian Ocean (McKay & Miller, 1997). This is supported by the fact that the closest relatives of the Atlanto-Mediterranean gobies belong to the Indo-Pacific gobiid fauna (McKay & Miller, 1997; Huyse *et al.*, 2004).

Ancestors of the present goby fauna probably migrated into the Atlantic Ocean and evolved in allopatry during the Messinian salinity crisis. After reflooding of the Mediterranean basin at 5.3 Mya they re-invaded the Mediterranean basin through the Strait of Gibraltar (Simonovic, 1999). Thus, assuming that the origin of the *P. minutus* complex took place in the Mediterranean Sea, we suggest that a population of proto-*P. minutus* might have become isolated in the Adriatic Sea. Another population of this ancestor might have given rise to the three species of the *P. minutus* complex elsewhere in the Mediterranean Sea. This may have happened during Pliocene or Pleistocene sea-level drops when populations of various basins became isolated. When the connection with the Atlantic Ocean was re-established, populations of the three *P. minutus*-complex species may have dispersed into the Atlantic Ocean. Although *P. lozanoi* has never been recorded from the Mediterranean Sea, this species has long been considered a subspecies of *P. minutus* and thus its presence may have been overlooked. Alternatively, the stenotopic *P. lozanoi* might have died out in the Mediterranean Sea because of unfavourable ecological conditions. *P. norvegicus*, on the contrary, has been recorded at several locations in the Mediterranean Sea (Stefanni, 2000b and references therein). Support for this hypothesis is provided by the haplotype distribution and diversity of *P. minutus*. The minimum spanning tree showed the endemic Mediterranean haplotypes as a separate cluster. A genetic architecture characterized by high nucleotide and haplotype diversity may be attributed to secondary contact of different mitochondrial lineages, which evolved in allopatry (Bowen & Grant, 1997). Magoulas, Tsimenides & Zouros (1996) suggested such a scenario for explaining the haplotype distribution of the European anchovy.

Hence, we propose that the most common haplotype A and its descendants originated more recently in the Atlantic Ocean from a Mediterranean ancestor, invaded the Mediterranean Sea and came into secondary contact with the resident *P. minutus*. Alvarado-Bremer *et al*. (1995) found a similar pattern of differ-

entiation between Atlantic and Mediterranean populations of the swordfish *Xiphias gladius*, suggesting both historical separation and ongoing gene flow.

Contemporary gene flow between Atlantic and Mediterranean P. minutus

Because of the physical oceanography at the Atlanto-Mediterranean boundary (Krijgsman, 2002), any gene flow at present is likely to be unidirectional from the Atlantic into the Mediterranean. The pattern of isolation-by-distance for haplotype A at the one-step level in the NCA suggests at least some ongoing gene flow across the Almeria-Oran front. Yet, a significant degree of differentiation between Atlantic and western Mediterranean *P. minutus* is observed. These findings contrast with other studies on *P. minutus*, where limited or no differentiation between western Mediterranean and North Sea samples of *P. minutus* was found (Huyse, 2002; Stefanni *et al*., 2003). Stefanni *et al*. (2003) employed allozymes, which in general have a lower resolution compared with mtDNA for detecting population structure. Huyse (2002) did not record any differentiation between gobies from the western Mediterranean Sea and the Belgian coast based on the conservative 12S and 16S rRNA loci. The nature of these markers might explain the failure to detect recent population divergence.

Phylogeographical patterns of *P. minutus* in the north-eastern Atlantic Ocean

The mismatch analysis, the negative value of Tajima's D-statistic and the star-like pattern in the minimum spanning tree point to a population expansion of *P. minutus* in the Atlantic Ocean. The value for τ suggests that this happened about 130 kyr BP, which coincides with the onset of the Eemian, the interglacial before the last glaciation event (Lowe & Walker, 1997). At the same time *P. minutus* must have experienced a range expansion into northern areas, before it was pushed south by the last glacial event, the Weichselian (110–10 kyr BP). During the glacials the refugia of marine fishes must have shifted north–south to become located between the Bay of Biscay and the Senegal coast (the southern border of cold temperate water masses). A glacial refugium along the Iberian peninsula has been proposed for various fish species such as Atlantic salmon (Consuegra *et al*., 2002), brown trout (Garciá-Marin *et al*., 1999) and the common goby *P. microps* (Gysels *et al*., 2004). Although we have no *P. minutus* samples from the Iberian Atlantic coast, the presence of haplotype A both in the western Mediterranean Sea and along the north-eastern Atlantic coasts up to central Norway suggests that this haplotype might have survived the Weichselian in this area, carrying out a range expansion eastward into the Mediterranean Sea and northward along the European coasts.

Loss of genetic variation at higher latitudes is common in populations inhabiting formerly glaciated areas, and is usually attributed to founder events during range expansion following deglaciation (Hewitt, 2000). According to this hypothesis the low level of diversity in the British samples (Plymouth, Galway and Oban) is probably best explained by a founder event during postglacial range expansion by a small and/or genetically homogeneous population of *P. minutus* dominated by haplotype A, as suggested by the two-step level in the NCA. Alternatively, selection on mtDNA haplotypes or lineage sorting may yield a similar pattern. A similar loss of variation in British compared with continental populations has been reported for the related common goby *P. microps* (Gysels *et al*., 2004).

While haplotype A was common in the *P. minutus* from the southern North Sea as well, these samples were dominated by haplotype F. Considering that haplotype F seems to be unique to this area, a glacial refugium for *P. minutus* north of the English Channel may explain this distribution. This is supported by the NCA, suggesting a contiguous range expansion of this clade in the southern North Sea. Verspoor *et al*. (1999) proposed a refugium for salmon in the glacial lake that existed in the southern North Sea. As *P. minutus* is able to survive temperatures as low as –1 °C (Fonds, 1973), the species should have been able to withstand the temperatures in this glacial marine lake, which must have had winter temperatures of around 1 °C (H. Weerts, pers. comm.). Moreover, the species is adapted to the brackish-water environment, tolerating salinity levels as low as 8°/oo (Fonds, 1973). Thus, in our view, the Southern Bight would constitute a zone of secondary contact between: (1) haplotype A and its descendants, expanding northward from a southern refugium, and (2) a resident population dominated by haplotype F.

The Baltic Sea

Studies on salmon and trout revealed phylogenetically different lineages within the Baltic Sea, suggesting distinct postglacial recolonization events from the adjacent North Sea as well as from refugia in glacial lakes east of the Baltic Sea (Osinov & Bernatchez, 1996; Koljonen *et al*., 1999). The fact that most Baltic *P. minutus* belonged to the Atlantic haplotype A, despite the samples being caught in the eastern part of the Baltic Sea (Finland), suggests an invasion from the North Sea by haplotype A via a contiguous eastward range expansion. The low degree of haplotype diversity compared with the North Sea samples might be attributed to founder events similar to those asso-

ciated with the samples from the British Isles. No significant differentiation from the Atlantic *P. minutus* is recorded. A similar lack of differentiation between Baltic and Atlantic populations was recorded for flounder (Borsa *et al*., 1997). However, water exchange with the North Sea is severely restricted. As such, it is not unlikely to assume limited gene flow between the Baltic and the North Sea in species depending on a planktonic larval stage for dispersal. Considering the fact that invasion of the Baltic Sea by *P. minutus* was less than 8000 years ago, populations might still not be at equilibrium. Alternatively, the resolution of mtDNA might not have been sufficient for detecting processes of ongoing and recent population divergence. For example, microsatellite analysis showed clear genetic differences between Atlantic and Baltic cod (Nielsen *et al*., 2001) and a hybrid zone in between (Nielsen *et al*., 2003). Microsatellite screening of *P. minutus* populations in the Southern Bight yielded evidence for distinct breeding units on a small scale (Pampoulie *et al*., 2004). Thus, for more final conclusions regarding the ongoing gene flow of *P. minutus* between the Atlantic Ocean and the Baltic Sea, the use of more sensitive markers is warranted.

CONCLUSIONS

The genetic structure of *P. minutus* is best explained by a combination of present and historic factors. Due to its pelagic larval stage the species has a high potential for dispersal via oceanic currents. This is reflected in the weak genetic differentiation and the pattern of isolation-by-distance within the Atlantic and the Baltic basins. A glacial refugium for *P. minutus* in the southern North Sea is proposed as the distribution of haplotypes suggests secondary contact after a period of isolation. Alternatively, we cannot exclude the effect of lineage sorting. The occurrence of the most common Atlantic haplotype in the western Mediterranean Sea in an appreciable frequency indicates ongoing gene flow across the Almería-Oran front. Yet, a high proportion of unique haplotypes was found in the population from the western Mediterranean Sea. This points to historical isolation followed by secondary contact between *P. minutus* invading the Mediterranean Sea from the Atlantic Ocean and resident *P. minutus*.

The large genetic distance between the Venetian *P. minutus* and all the others supports the hypothesis of allopatric speciation in the northern Adriatic Sea. This may be linked to isolation of the Adriatic basin during the Pleistocene or the Pliocene. Regarding the exact timing of this speciation event in the late Pliocene or early Pleistocene, results of the various genetic markers are in conflict. The screening of additional genes would be warranted for more final conclusions regarding the speciation processes of Mediterranean *P. minutus*.

ACKNOWLEDGEMENTS

We would like to thank the following people for providing samples: M. Fonds, S. Stefanni, P. Boudry, J. Dunne, R. Gibson, P. Torricelli and J. Mork. D. Klittegaard-Kristensen and H. Weerts are acknowledged for providing valuable information on the quaternary geology of the north-eastern Atlantic Ocean. A. Dewicke provided data on the dispersal of *Pomatoschistus* sp. larvae. E. Penzo is acknowledged for help with the sequencing and the SSCP analysis. We are grateful to G. Nævdal and J.M. Pujolar for valuable comments on the manuscript and to J.K. Van Houdt and T. Huyse for inspiring discussions. E.G. was supported by the Institute for Scientific and Technological Research (IWT–Belgium) and by a Marie Curie Fellowship of the European Community, contract nr. EVK3-CT-2000–57129, at Bergen Advanced Training Site in Marine Ecology (BATMARE), University of Bergen, Norway. Research was funded by the Belgian Federal Services of Scientific and Technological Affairs (DWTC contract nr. MN/DD/42) and by the Training and Mobility of Researchers–Access to Large-Scale Facilities Program of the European Commission (LSF-Trondheim).

REFERENCES

Alvarado-Bremer J, Baker AJ, Mejuto J. 1995. Mitochondrial DNA control region sequences indicate extensive mixing of swordfish (*Xiphias gladius*) populations in the Atlantic Ocean. *Canadian Journal of Fisheries and Aquatic Sciences* **52:** 1720–1732.

Balson PS, Laban C, Frantsen PJ, Parker N, Henriet JP, de Batist M, Mostaert F. 1991. Ostend: sheet 52°N/02°E. Solid Geology, 1: 250 000 Series British Geological Survey. *Geological Survey of The Netherlands & Belgian Geological Survey* Southampton: Ordnance Survey for the British Geological Survey.

Bernatchez L, Wilson CC. 1998. Comparative phylogeography of Nearctic and Palearctic fishes. *Molecular Ecology* **7:** 431–452.

Bianchi CN, Morri C. 2000. Marine biodiversity of the Mediterranean Sea: Situation, problems and prospects for future research. *Marine Pollution Bulletin* **40:** 367–376.

Borsa P, Blanquer A, Berrebi P. 1997. Genetic structure of the flounders *Platichtys flesus* and *P. stellatus* at different geographic scales. *Marine Biology* **129:** 233–246.

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.

Claridge PN, Hardisty MW, Potter IC, Williams CW. 1985. Abundance, life history and ligulosis in the gobies (Teleostei) of the inner Severn estuary. *Journal of the Marine Biological Association of the United Kingdom* **65:** 951–968.

Consuegra S, García de Leániz C, Serdio A, González Morales M, Straus LG, Knox D, Verspoor E. 2002. Mitochondrial DNA variation in Pleistocene and modern Atlantic salmon from the Iberian glacial refugium. *Molecular Ecology* **11:** 2037–2048.

Excoffier L, Smouse PE, Quattro JM. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* **131:** 479–491.

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39:** 783–791.

Fonds M. 1973. Sand gobies in the Dutch Wadden Sea (Pomatoschistus, Gobiidae, Pisces). *Netherlands Journal of Sea Research* **6:** 417–473.

García-Marín JL, Utter FM, Pla C. 1999. Postglacial colonization of brown trout in Europe based on distribution of allozyme variants. *Heredity* **82:** 46–56.

Gysels E. 2003. Genetic structure and evolutionary patterns in *Pomatoschistus* gobies on the European Continental Shelf and in the Mediterranean Sea. PhD Thesis. KU Leuven, Belgium.

Gysels ES, Hellemans B, Pamapoulie C, Volckaert F. 2004. Phylogeography of the common goby, *Pomatoschistus microps*, with particular emphasis on the colonization of the Mediterranean and the North Sea. *Molecular Ecology* **13:** 403–417.

Hansen B, Østerhus S. 2000. North Atlantic-Nordic Seas exchange. *Progress in Oceanography* **45:** 109–208.

Healey MC. 1971. The distribution and abundance of sand gobies, *Gobius minutus*, in the Ythan estuary. *Journal of Zoology* **163:** 177–229.

Hesthagen IH. 1977. Migrations, breeding and growth in *Pomatoschistus minutus* (Pallas) (Pisces, Gobiidae) in Oslofjorden. *Norway Sarsia* **63:** 17–26.

Hewitt G. 2000. The genetic legacy of the Quaternary ice ages. *Nature* **405:** 907–913.

Hsü K, Montadert L, Bernouilli D, Cita MB, Erickson A, Garrison RE, Kidd RB, Mèliereés F, Müller C, Wright R. 1977. History of the Messinian salinity crisis. *Nature* **267:** 399–403.

Huyse T. 2002. Evolutionary associations between *Gyrodactylus* and its goby host: bound forever? PhD Thesis. KU Leuven, Belgium.

Huyse T, Van Houdt J, Volckaert FAM. 2004. Paleoclimatic history and vicariant speciation in the 'sand goby' group (Gobiidae, Teleostei). *Molecular Phylogenetics and Evolution* **32:** 324–336.

Johns GC, Avise JC. 1998. A comparative summary of genetic distances in the vertebrates from the mitochondrial cytochrome *b* gene. *Molecular Biology and Evolution* **15:** 1481–1490.

Kocher TD, Thomas WK, Meyer A, Edwards SV, Pabbo S, Villablanca FX, Wilson AC. 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences, USA* **86:** 6196–6200.

Koljonen MJ, Jansson H, Paaver T, Vasin O, Koskiniemi J. 1999. Phylogeographic lineages and differentiation pattern of Atlantic salmon (*Salmo salar*) in the Baltic Sea with management implications. *Canadian Journal of Fisheries and Aquatic Sciences* **56:** 1766–1780.

Krijgsman W. 2002. The Mediterranean: mare nostrum of earth sciences. *Earth and Planetary Science Letters* **205:** 1–12.

Kumar S, Tamura K, Jakobsen IB, Nei M. 2001. *MEGA: molecular evolutionary genetic analysis*, Version 2.1 Pennsylvania: The Pennsylvania State University Park, PA.

Lessios HA, Kessing BD, Robertson DR. 1999. Massive gene flow across the world's most potent marine biogeographic barrier. *Proceedings of the Royal Society of London B* **265:** 583–588.

Lowe JJ, Walker MCJ. 1997. *Reconstructing quaternary environments*, 2nd edn. Essex: Pearson Education Ltd.

Magoulas A, Tsimenides N, Zouros E. 1996. Mitochondrial DNA phylogeny and the reconstruction of the population history of a species, the case of the European anchovy (*Engraulis encrasicolus*) *Molecular Biology and Evolution* **13:** 178–190.

Mantel N. 1967. The detection of disease clustering and a generalized regression approach. *Cancer Research* **27:** 209–220.

McKay SI, Miller PJ. 1997. The affinities of European sand gobies. *Journal of Natural History* **31:** 1457–1482.

Miller PJ. 1986. Gobiidae. In: Whitehead PJP, Bauchot M-L, Hureau JC, Nielson J, Tortonese E, eds. *Fishes of the north-eastern Atlantic and Mediterranean*, 1019–1085.

Miller PJ. 1990. The endurance of endemism – the Mediterranean fresh-water gobies and their prospects for survival. *Journal of Fish Biology* **37:** 145–156.

Millot C. 1999. Circulation in the Western Mediterranean Sea. *Journal of Marine Systems* **20:** 423–442.

Nei M. 1987. *Molecular evolutionary genetics*. New York: Columbia University Press.

Nesbø CL, Rueness EK, Iversen SA, Skagen DW, Jakobsen KS. 1999. Phylogeography and population history of Atlantic mackerel (*Scomber scombrus* L.): a genealogical approach reveals genetic structuring among. the Eastern Atlantic stocks. *Proceedings of the Royal Society of London B* **267:** 281–292.

Nielsen EE, Hansen MM, Ruzzante DE, Meldrup D, Grønkjær P. 2003. Evidence of a hybrid zone in Atlantic cod (*Gadus morhua*) in the Baltic and the Danish Belt revealed by individual admixture analysis. *Molecular Ecology* **12:** 1497–1508.

Nielsen EE, Hansen MM, Schmidt C, Meldrup D, Grønkjær P. 2001. Population origin of Atlantic cod. *Nature* **413:** 272.

Orita M, Iwahana H, Kanazawa H, Hayashi K, Sekiya T. 1989. Detection of polymorphisms of human DNA by gel electrophoresis as single strand conformation polymorphism. *Proceedings of the National Academy of Sciences, USA* **86:** 2766–2770.

Osinov AG, Bernatchez L. 1996. 'Atlantic' and 'Danubian' phylogenetic groupings of brown trout *Salmo trutta* complex: genetic divergence, evolution and conservation. *Journal of Ichthyology* **36:** 723–746.

Pampoulie C, Gysels E, Hellemans B, Maes GE, Leentjes V, Jones AG, Volckaert FAM. 2004. Evidence for fine scale genetic structure and estuarine colonisation in a high gene flow marine goby (*Pomatoschistus minutus*). *Heredity* **92:** 434–445.

Penzo E, Gandolfi G, Bargelloni L, Colombo L, Patarnello T. 1998. Messinian salinity crisis and the origin of freshwater lifestyle in Western Mediterranean gobies. *Molecular Biology and Evolution* **15:** 1472–1480.

Posada D, Crandall KA. 2001. Intraspecific gene genealogies: trees grafting into networks. *Trends in Ecology and Evolution* **16:** 37–45.

Posada D, Crandall KA, Templeton AR. 2000. GeoDis: a program for the cladistic nested analysis of the geographical distribution of genetic haplotypes. *Molecular Ecology* **9:** 487–488.

Rice W. 1989. Analyzing tables of statistical tests. *Evolution* **43:** 223–225.

Ríos C, Sanz S, Saavedra C, Peña JB. 2002. Allozyme variation in populations of scallops, *Pecten jacobeus* (L.) and *P. maximus* (L.) (Bivalvia: Pectinidae), across the Almería-Oran front. *Journal of Experimental Marine Biology and Ecology* **267:** 223–244.

Rogers AR, Harpending H. 1992. Population growth makes waves in the distribution of pairwise genetic differences. *Molecular Biology and Evolution* **9:** 552–569.

Rozas J, Rozas R. 1999. DNASP, Version 3: an integrated program for molecular population genetics and molecular evolution analysis. *Bioinformatics* **15:** 174–175.

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

Schmidt HA, Strimmer K, Vingron M, von Haeseler A. 2002. *TREE-PUZZLE*, Maximum likelihood analysis using quartets and parallel computing. *Bioinformatics* **18:** 502–504.

Schneider S, Roessli D, Excoffier L. 2000. *ARLEQUIN: a software for population genetic analysis*. Geneva: University of Geneva.

Simonovic PD. 1999. Phylogenetic relationships of Ponto-Caspian gobies and their relationship to the Atlantic-Mediterranean Gobiinae. *Journal of Fish Biology* **54:** 533–555.

Stefanni S. 2000a. A multidisciplinary investigation of intraspecific variation in the European Sand Goby, *Pomatoschistus minutus* (Pallas) (Teleostei, Gobiidae). PhD Thesis, University of Bristol, Bristol, UK.

Stefanni S. 2000b. First record of the Norway goby in the Adriatic Sea. *Journal of Fish Biology* **57:** 828–830.

Stefanni S, Gysels ES, Volckaert FAM, Miller PJ. 2003. Allozyme variation and genetic divergence in the sand goby, *Pomatoschistus minutus* (Teleostei: Gobiidae). *Journal of the Marine Biological Association of the United Kingdom* **83:** 1143–1149.

Stefanni S, Thorley JL. 2003. Mitochondrial DNA phylogeny reveals the existence of an Evolutionary Significant Unit of the sand goby *Pomatoschistus minutus* in the Adriatic (Eastern Mediterranean). *Molecular Phylogenetics and Evolution* **28:** 601–609.

Tamura K, Nei M. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution* **10:** 512–526.

Templeton AR. 1998. Nested clade analyses of phylogeographic data: testing hypotheses about gene flow and population history. *Molecular Ecology* **7:** 381–397.

Templeton AR, Crandall KA, Singh CF. 1992. A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. *Genetics* **132:** 619–633.

Templeton AR, Routman E, Phillips CA. 1995. Separating population structure from population history: a cladistic analysis of the geographical distribution of mitochondrial DNA haplotypes in the Tiger Salamander, *Ambystoma tigrinum*. *Genetics* **140:** 767–782.

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22:** 4673–4680.

Tintore J, La Violette PE, Blade I, Cruzado A. 1988. A study of an intense density front in the eastern Alboran Sea: the Almeria-Oran front. *Journal of Physical Oceanography* **18:** 1384–1397.

Turrell WR. 1992. New hypotheses concerning the circulation of the northern North Sea and its relation to North Sea fish stock recruitment. *ICES Journal of Marine Science* **49:** 107–123.

Van der Molen J, de Swart HE. 2001. Holocene tidal conditions and tide-induced sand transport in the southern North Sea. *Journal of Geophysical Research* **106:** 9339–9362.

Verspoor E, McCarthy EM, Knox D. 1999. The phylogeography of European Atlantic salmon (*Salmo salar* L.) based on RFLP analysis of the ND1/ 16S RNA region of the mtDNA. *Biological Journal of the Linnean Society* **68:** 129–146.

Wallis GP, Beardmore JA. 1983. Genetic differentiation between populations of *Pomatoschistus minutus* from the Bristol Channel and the Adriatic. *Genetica* **62:** 75–80.

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

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.

Zane L, Ostellari L, Maccatrozzo L, Bargelloni L, Cuzin-Roudy J, Buchholz F, Patarnello T. 2000. Genetic differentiation in a pelagic crustacean (*Meganyctiphanes norvegica*: Euphausiacea) from the North East Atlantic and the Mediterranean Sea. *Marine Biology* **136:** 191–199.

APPENDIX 1

Partial cytochrome *b* sequences of the various haplotypes of *Pomatoschistus minutus* with EMBL accession numbers (AN)

AN	H	013	016	019	022	029	034	035	046	058	060	064	076	088	094	097	111	124	133	136	139	163	169	184	187	202	205	211	220	223	235	241	244	246	247	250	253	256	259	262	265	268	274	280	283
AJ555096	A	G	C	G	C	A	T	C	T	A	C	A	G	T	T	A	T	T	C	A	C	C	T	C	T	C	C	A	C	T	A	C	G	T	G	T	A	A	A	C	A	A	A	A	C
AJ555097	B	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AJ555098	C	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AJ555099	D	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AJ555100	E	.	T	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AJ555101	F	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AJ550102	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.
AJ555103	H	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AJ555118	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.
AJ555104	K	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AJ555105	L	A	G	T	C	.	C	.	.	G	.	G	A	A	C	.	G	C	G	.	.	T	.	T	C	.	A	G	.	C	.	T	.	.	T	C	.	.	.	.	G	G	G	T
AJ555106	M	A	G	T	C	.	C	.	.	G	.	G	.	A	C	.	.	C	G	G	.	T	C	T	C	.	A	G	.	C	.	T	.	.	C	C	.	.	.	.	G	G	G	T
AJ555107	N	A	G	T	C	.	C	.	.	G	.	G	.	A	C	.	.	C	G	G	.	T	.	T	C	.	A	G	.	C	.	T	.	.	T	C	.	.	G	.	.	G	G	G	T
AJ555108	O	A	G	T	C	.	C	.	.	G	.	G	.	A	C	.	G	C	G	.	.	T	.	T	C	.	A	G	.	C	.	T	.	.	T	C	.	.	.	.	G	G	G	T
AJ555109	P	A	G	T	C	.	C	.	.	G	.	G	.	A	C	.	G	C	G	G	T	T	.	T	C	.	A	G	.	C	.	T	.	.	T	C	.	.	.	.	G	G	G	T
AJ555110	Q	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AJ555111	R	.	.	A	A	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AJ555112	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	G	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.
AJ555113	T	.	.	A	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AJ555114	U	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	..	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	C	.	.	G	G	.	.	.	.
AJ555115	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	C	.	.	.	.	.	.	.	.
AJ555116	W	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	G	.	C	.	.	.	.	.	.	.	T
AJ555117	X	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	C	.	.	.	.	.	.	.	T
AJ555119	Z	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.
AJ555120	AA	.	.	.	.	.	.	A	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	C	.	.	.	.	.	.	.	.
AJ555121	AB	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	C	.	.	.	.	.	.	.	.
AJ555122	AC	.	.	.	A	.	.	A	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AJ555123	AF	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.

Only variable sites and their positions are represented. H refers to the various haplotypes. Haplotype A is the reference.

APPENDIX 2

Haplotype distribution of *Pomatoschistus minutus*.

H	Tro (Atl)	Ber (Atl)	Tva (Atl)	Ob (Atl)	Ga (Atl)	FrF (Atl)	Tex (Atl)	Do (Atl)	Sb (Atl)	Ply (Atl)	LT (Atl)	Per (MS)	Ven (MS)	Total
A	15	15	8	36	4	3	13	6	5	18	2	8		133
B	1	4												5
C		1												1
D	1													1
E							1							1
F						6	6	14	13					39
G						4	2	6	2					14
H												1		1
I						1								1
K			2			1						1		4
L													1	1
M													1	1
N													4	4
O													2	2
P													2	2
Q			1											1
R										1				1
S										1				1
T							1							1
U												1		1
V												1		1
W												2		2
X												2		2
Z							1							1
AA												1		1
AB												4		4
AC												1		1
AF				1										1
Total	17	20	11	37	4	15	24	26	20	20	2	22	10	228

Atl, Atlantic basin; MS, Mediterranean Sea; H, haplotype. For sample abbreviations see Table 1.