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

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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. © 2004 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2004, 83, 561–576.


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 (paleo)ceanography 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).
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 \textit{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 \textit{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 \textit{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 \textit{P. minutus}. Thus, if present-day gene flow between Atlantic \textit{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 \textit{P. minutus}. Preliminary genetic studies of \textit{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

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Sampling sites of \textit{Pomatoschistus minutus} with main current patterns, compiled after Turrell (1992), Millot (1999) and Hansen & Østerhus (2000). Sampling sites are indicated with \textbullet. Abbreviations of sampling sites are listed in Table 1. NAC, North Atlantic Current; SEC, Shelf Edge Current.}
\end{figure}
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 region have been proposed (Garcia Marin 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>
<thead>
<tr>
<th>Oceanic basin / Sampling site</th>
<th>Code</th>
<th>Period</th>
<th>Latitude</th>
<th>Longitude</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atlantic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trondheim (Norwegian Sea, central Norway)</td>
<td>Tro</td>
<td>September 2000</td>
<td>63°24’N</td>
<td>10°24'E</td>
</tr>
<tr>
<td>Bergen (North Sea, western Norway)</td>
<td>Ber</td>
<td>September 1997</td>
<td>60°23’N</td>
<td>5°21'E</td>
</tr>
<tr>
<td>Tvärminne (Baltic Sea, Finland)</td>
<td>Tva</td>
<td>July 2001</td>
<td>59°50’N</td>
<td>23°15'E</td>
</tr>
<tr>
<td>Oban (Atlantic Ocean, western Scotland)</td>
<td>Ob</td>
<td>August 1999</td>
<td>56°24’N</td>
<td>5°28’W</td>
</tr>
<tr>
<td>Galway (Atlantic Ocean, western Ireland)</td>
<td>Ga</td>
<td>August 1999</td>
<td>53°16’N</td>
<td>9°03’W</td>
</tr>
<tr>
<td>Texel (Wadden Sea, the Netherlands)</td>
<td>Tex</td>
<td>December 1998</td>
<td>53°00’N</td>
<td>4°46’E</td>
</tr>
<tr>
<td>Doel (Schelde estuary, Belgium)</td>
<td>Do</td>
<td>October 1998</td>
<td>51°19’N</td>
<td>4°16’E</td>
</tr>
<tr>
<td>Stroombank (southern North Sea, Belgium)</td>
<td>Sb</td>
<td>February 1997</td>
<td>51°13’N</td>
<td>2°52’E</td>
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<tr>
<td>Plymouth (English Channel, UK)</td>
<td>Ply</td>
<td>November 1996</td>
<td>50°22’N</td>
<td>4°09’W</td>
</tr>
<tr>
<td>La Tremblade (Gulf of Biscay, France)</td>
<td>LT</td>
<td>October 2000</td>
<td>45°46’N</td>
<td>1°08’W</td>
</tr>
<tr>
<td>Mediterranean</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pérols (Gulf of Lions, France)</td>
<td>Per</td>
<td>January 1998</td>
<td>43°34’N</td>
<td>3°57’E</td>
</tr>
<tr>
<td>Venice (Adriatic Sea, Italy)</td>
<td>Ven</td>
<td>October 1999</td>
<td>45°26’N</td>
<td>12°19’E</td>
</tr>
</tbody>
</table>

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* (Kroyer, 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 AP082969 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: Goby-cytbF: 5’-CCC ATC AAA CAT TTC TGC-3’ and Goby-cytbR: 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 Higgins & 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 *α* 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 clad 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) \((\theta_0, \theta_1\text{ and } \tau)\) (Rogers & Harpending, 1992) were also calculated in the program ARLEQUIN. The parameter \(\tau = \mu T\), where \(\mu\) 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 \(\alpha\) 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 \((\pi)\), with standard deviation between parentheses and percentage of occurrence of the most common haplotype (MCH) | Site | \(N\) | \(n\) | \(nh\) | \(h\) | \(\pi\) | 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 \textit{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 \textit{P. minutus} were grouped with \textit{P. lozanoi} rather than with the other \textit{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 \textit{P. minutus} and \textit{P. lozanoi}. Pairwise genetic distances (not shown) between sequences varied between 0.007 and 0.018 for the Atlantic \textit{P. minutus} haplotypes, while those between \textit{P. lozanoi} and \textit{P. minutus} ranged from 0.087 to 0.095. The genetic distance between the Adriatic \textit{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 \textit{Pomatoschistus minutus} based on Tamura-Nei genetic distances with \textit{P. microps} and \textit{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.

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

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

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

<table>
<thead>
<tr>
<th>Atlantic</th>
<th>Mediterranean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tro</td>
<td>0.000</td>
</tr>
<tr>
<td>Ber</td>
<td>0.021</td>
</tr>
<tr>
<td>Ob</td>
<td>0.027</td>
</tr>
<tr>
<td>Tva</td>
<td>0.063</td>
</tr>
<tr>
<td>FrF</td>
<td>0.273</td>
</tr>
<tr>
<td>Tex</td>
<td>0.071</td>
</tr>
<tr>
<td>Do</td>
<td>0.327</td>
</tr>
<tr>
<td>Sb</td>
<td>0.471</td>
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<tr>
<td>Ply</td>
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</tr>
<tr>
<td>Per</td>
<td>0.234**</td>
</tr>
<tr>
<td>Ven</td>
<td>0.965**</td>
</tr>
<tr>
<td>Pl</td>
<td>0.984**</td>
</tr>
</tbody>
</table>

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.

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).

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

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>% of variation</th>
<th>F-statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among groups</td>
<td>27.83</td>
<td>FCT = 0.278*</td>
</tr>
<tr>
<td>Among populations</td>
<td>3.88</td>
<td>FSC = 0.054*</td>
</tr>
<tr>
<td>within groups</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Within populations</td>
<td>68.29</td>
<td>FST = 0.317*</td>
</tr>
</tbody>
</table>

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).

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

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>% of variation</th>
<th>F-statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among groups</td>
<td>27.83</td>
<td>FCT = 0.278*</td>
</tr>
<tr>
<td>Among populations</td>
<td>3.88</td>
<td>FSC = 0.054*</td>
</tr>
<tr>
<td>within groups</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Within populations</td>
<td>68.29</td>
<td>FST = 0.317*</td>
</tr>
</tbody>
</table>

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

**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.

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 clad distances vs. nested clad 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) clad 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, θ₀, and θ₁ were 0.731, 0.088 and 1669,
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 gobii 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-
Contemporary gene flow between Atlantic and Mediterranean P. minutus

Because of the physical oceanography at the Atlantic-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 (García-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 northeastern 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‰ (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-
associated 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 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.

**REFERENCES**


APPENDIX 1

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

| AN  | H  | 3  | 6  | 9  | 2  | 9  | 4  | 5  | 6  | 8  | 0  | 4  | 6  | 8  | 4  | 7  | 1  | 4  | 3  | 6  | 9  | 3  | 5  | 1  | 0  | 3  | 5  | 1  | 4  | 6  | 7  | 0  | 3  | 6  | 9  | 2  | 5  | 8  | 4  | 0  |
|-----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| AJ555096 | A | G | C | G | C | T | C | T | A | C | G | T | T | A | T | T | C | T | C | T | C | T | C | A | C | T | A | C | G | T | G | T | A | A | A | C | A | A | A | A | C |
| AJ555097 | B | A | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |…..
## APPENDIX 2

Haplotype distribution of *Pomatoschistus minutus*.

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