An increasing number of phylogeographic studies on marine species shows discordant patterns in the degree of population differentiation between nuclear and mitochondrial markers. To understand which factors have the potential to cause these patterns of discordance in marine organisms, a population genetic study was realized on the sand goby Pomatoschistus minutus (Pallas 1770; Gobiidae, Teleostei). Sand gobies from eight European locations were genotyped at eight microsatellite markers. Microsatellites confirmed the global phylogeographical pattern of P. minutus observed with mitochondrial DNA (mtDNA) markers and nuclear allozyme markers. Three groups consistent with the mitochondrial lineages were defined (the Mediterranean, Iberian and North Atlantic groups) and indications of a recent founder event in the northern Baltic Sea were found. Nevertheless, differences in the degree of population differentiation between the nuclear and mitochondrial markers were large (global \( F_{ST} \) values for microsatellites = 0.0121; for allozymes = 0.00831; for mtDNA = 0.4293). Selection, sex-biased dispersal, homoplasy and a high effective population size are generally accepted as explanations for this mitonuclear discrepancy in the degree of population differentiation. In this study, selection on mtDNA and microsatellites, male-biased dispersal and homoplasy on microsatellite markers are unlikely to be a main cause for this discrepancy. The most likely reason for the discordant pattern is a recent demographic expansion of the sand goby, resulting in high effective population sizes slowing down the differentiation of nuclear DNA.

Introduction

Traditionally, the genetic structure of marine organisms has been thought to be homogeneous because of the lack of obvious barriers to gene flow in the ‘open’ marine environment. In the last decade, however, an increasing number of population genetic studies have described distinct genetic structuring for several marine species on large and small geographical scales (Hauser and Carvalho, 2008). Those observed population genetic structures reflect both historical and contemporary processes (Bailoux and Lugon-Moulin, 2002). Geographical and climatic factors acting during the Pleistocene glaciations (1800–11.5 ka) are the major factors responsible for the present genetic structure of most extant marine species (Hewitt, 2000). Heterogeneity in the marine environment owing to the influence of climate, hydrodynamics and topography, together with biological traits, such as sex-dependent migration, phylopatry and assortative mating, which may counteract gene flow, enhance genetic structuring (Ruzzante et al., 1998).

These recent insights are mainly due to the increased popularity of polymorphic microsatellite markers in marine population and landscape genetics (Jørgensen et al., 2005). Microsatellites have proven for many species to be more powerful for resolving population structure than mitochondrial DNA (mtDNA) and allozyme markers (De Innocentiis et al., 2004; Nesbo et al., 2000). However, this is not applicable to all marine organisms (Lukoschek et al., 2008). There is almost certainly a publication bias for marine studies detecting microscale population structures with microsatellites (Hauser and Carvalho, 2008). An increasing number of studies have shown a large difference in the order of magnitude for the population divergence between nuclear and mitochondrial markers. Among those, mtDNA shows a higher sensitivity to resolve the phylogeographical and population genetic structure (Peijnenburg et al., 2006; Lukoschek et al., 2008). This discordance in resolution among markers may result from the differential effects of genetic drift, mutation and migration on a marker class, or may result from selection or sex-biased dispersal (Buonaccorsi et al., 2001). More research is required to study how common and important those factors are in the marine environment.

The sand goby Pomatoschistus minutus (Pallas 1770; Gobiidae, Teleostei) is a small marine demersal fish common in shallow waters along European coasts (Miller, 1986) (Figure 1). Although earlier analyses with allozyme markers showed very low population differentiation values for P. minutus (Stefanni et al., 2003), a recent study revealed that the sand goby is highly structured at the mtDNA cytochrome \( b \) (cyt \( b \)) locus
Materials and methods

Sampling and species/sex identification
A total of 696 *P. minutus* individuals were caught at eight locations along the European coast between September 2002 and January 2007 (Table 1 and Figure 1). Many of the samples have already been included in a previous mtDNA study (Larmuseau et al., 2009b). Locations BNS and VMS were sampled twice to perform an estimation of effective population size. All new samples were identified as *P. minutus* morphologically, based on the dermal head papillae (Miller, 1986) and pigmentation pattern (Hamerlynck, 1990), and genetically according to a molecular tool described by Larmuseau et al. (2008). The sex of each sand goby was determined by the shape of the urogenital papilla as drawn by Rodrigues et al. (2006).

Microsatellite genotyping
Genomic DNA was extracted from fin clips stored in 100% ethanol using the NucleoSpin Extraction Kit (Machery-Nagel GmbH, Düren, Germany). Each individual was genotyped at nine multiplexed microsatellite loci (*Pmin03*, *Pmin04*, *Pmin09*, *Pmin16-2*, *Pmin20*, *Pmin29*, *Pmin31*, *Pmin35* and *Pmin38*) (Larmuseau et al., 2007) on an ABI 3130 automated capillary DNA sequencer (Applied Biosystems, Foster City, CA, USA). Several methods were used to mitigate genotyping errors in the data set (Supplementary materials); marker *Pmin20* was excluded for further statistical analyses.

Genetic diversity
We used genotype and allele frequencies of the microsatellite loci to obtain standard estimates of genetic diversity within and between sample sites. Genetic variation in each population was measured by calculating the mean number of alleles per locus, the observed (*H*O) and unbiased expected (*H*E) heterozygosities and the *F*ST. The deviation from the Hardy–Weinberg equilibrium was assessed with GENETIX v.4.05 (Belkhir et al., 2004). Allelic richness, which corrects the number of alleles for sample size, was assessed using FSTAT v.2.9.3.2 (Goudet, 2001). Exact tests of linkage disequilibrium between pairs of loci were calculated at each location, each region and across all samples using GENEPOP v.3.4. (Raymond and Rousset, 1995).

Patterns of population subdivision
Different methods were used to reveal the population substructure of *P. minutus*. First, a factorial correspondence analysis (FCA) of individual multilocus genotypes was performed using GENETIX to reveal the portion of the hyperspace of all genotypes occupied by each group of individuals. Second, population differentiation was quantified in GENETIX using the standardized allelic variation *F*ST, estimated as *θ* (Weir and Cockerham, 1984), and in SPAGEDi 1.2.g (Hardy and Vekemans, 2002) using an analog of *F*ST for microsatellites *R*ST, estimated as *ñ* (Slatkin, 1995). *F*ST–linked pairwise genetic distances were calculated according to Cavalli-Sforza and Edwards (1967) (*D*CE) with GENETIX, and *R*ST–linked pairwise genetic distances calculated according to Goldstein et al. (1995) (*d*2) were obtained with SPAGEDi. *F*ST value and *D*CE value were tested for significance against 1000
random permutations of the data in GENETIX. The significance for $R_{ST}$ value and $d_{FST}$ value were tested in SPAGeDi also against $10^4$ random permutations. A sequential Bonferroni test (Rice, 1989) and the false discovery rate control (Verhoeven et al., 2005) were applied to correct significance levels for multiple testing. Standardized genetic differentiation measures were obtained by dividing $F_{ST}$ measures by the maximum values for $F_{ST}$ (Hedrick, 2005; Meirmans, 2006), calculated using the pragmatic recoding approach suggested by Meirmans (2006). To assess the influence of stepwise-like mutations versus drift on genetic differentiation, we performed a permutation test available in the software SPAGeDi. Allele size at each locus was randomly permuted among allelic states (2000 mutations) to simulate a distribution of uncorrelated allele frequency model with parameter $k$ set to 1 was used as the model assuming that the allele frequencies in each population are independent. This is the original model used in Pritchard et al. (2000). We selected the most likely number of clusters given the data by choosing the number of clusters where we observed the largest difference in log likelihoods ($\Delta K$) (Evanno et al., 2005). Finally, the overall pattern of population genetic structure among regions was assessed with a Bayesian approach developed by Ciofi et al. (1999) and implemented in the program 2MOD by MA Beaumont (http://www.rubic.rdg.ac.uk/~mab/software.html). Two models are evaluated: (1) the gene flow model, which assumes that gene frequencies within populations are caused by a balance between genetic drift and gene flow, and (2) the drift model, in which allele frequencies within populations are evolving purely through drift. The Markov chain Monte Carlo search was carried out twice using $10^5$ iterations. The first $10\%$ of the runs were discarded to remove the effects of initial starting parameters. In each case, the probability of a model was estimated from the number of times it appeared during the simulation.

Comparison between microsatellite and mitochondrial data
MitDNA cyt $b$ data were available for eight out of ten sand goby samples (TBS, WCS, RNS, BNS1, WIS, GOA, AAO and VMS1) (Larmuseau et al., 2009b). On the other hand, the allozyme analyses by Gysels (2003) and by Stefanni et al. (2003) were restricted to samples from other locations. Therefore, it was only possible to compare statistically the results of microsatellites and mtDNA cyt $b$ data. Two statistical methods were used to compare the degree of population differentiation between the types of genetic distances calculated for the microsatellite data ($D_{CE}$ and $d_{FST}$) and the genetic distances of Tamura and Nei (1993) calculated for the mtDNA data. Data on the mtDNA cyt $b$ were available for the populations. First, the pairwise $F_{ST}$ matrices were correlated by using simple Mantel procedures (Mantel, 1967) in the Vegan library in R (Oksanen et al., 2007). Permutations ($n = 10\,000$) were used to evaluate statistical significance. Then, two-dimensional multidimensional scaling analysis ordinations of the two types of genetic marker were compared by Procrustes analysis.
(Gower, 1975) using R software. Procrustes analysis is searching for the best match between two configurations of points in a multivariate Euclidean space using rotation, translation, reflection and dilation of one configuration. The criterion used to assess the best fit is the minimization of the sum of squares between the differences for each observation (m²). The significance of the result, an optimal superposition of one configuration on the other (reference), is obtained through a permutation test (PROTEST) (Jackson, 1995). PROTEST is using \( R = \sqrt{1 - m^2} \) as a test statistic, which can be interpreted as a correlation.

Impact of selection, sex-biased dispersal and effective population size on the degree of population divergence estimates

To verify if the variation on the microsatellites can be influenced by selection, two different approaches were considered. First, all microsatellite flanking regions were compared with sequences in GenBank by means of the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al., 1990) to verify if the microsatellites potentially are located within the described functional regions of the genome. Second, potential outlier microsatellites were identified by using the selection detection workbenchs LOSITAN (Antao et al., 2008) and BayeScan (Foll and Gaggiotti, 2008). Analyses are performed for LOSITAN with 10 000 simulations for IAM and SMM, both with the options ‘Neutral’ mean \( F_{IS} \) and force mean \( F_{ST} \). For BayeScan, 10 000 iterations were conducted with a thinning interval of 20 and with 10 pilot runs.

To detect differences in migration rates between females and males, deviation from the Hardy–Weinberg equilibrium (\( F_{IS} \)), differentiation among populations (\( F_{ST} \)), relatedness (r) (Queller and Goodnight, 1989), mean assignment index (mAlC) and variance of the assignment index (vAlC) were quantified separately for both sexes over all populations (Goudet et al., 2002). Statistical significance of differences in these within-population indices was determined with 10 000 permutations using the randomization method implemented in FSTAT (Goudet, 2001).

Effective population sizes were estimated using different point methods, which do not require samples spaced over at least one temporal interval, and temporal methods, where samples are taken from the same location at two or more points in time separated by a specified number of generations (one or more). Two different point estimation methods were estimated for the 10 samples. The program NeEstimator v.1.3 (Peel et al., 2004) was used for the Heterozygote Excess method, which examines the excess of heterozygotes in the sample compared with the proportion predicted under the Hardy–Weinberg equilibrium (Luikart and Cornuet, 1999). For the estimation of effective population size on the basis of linkage disequilibrium data, the program LDNe v.1.31 (Waples and Do, 2008) was used because it implements a recently developed bias correction (Waples, 2006). Finally, \( N_e \) was estimated using two temporal methods in NeEstimator v.1.3: (i) based on the Moment Approach (Waples, 1989) and (ii) on the pseudo-likelihood method (MLNE) developed by Wang and Whitlock (2003). These two methods require at least two temporally spaced samples, and therefore this analysis was only possible for VMS and BNS (Table 1).

Results

Genetic diversity

Mean allelic richness per location, corrected for sample size, varied between 12.088 (northern Baltic Sea) and 15.454 (MS) (mean = 13.622) (Table 2). Mean expected heterozygosity was relatively uniform among the different sampling sites with the lowest value in the northern Baltic Sea sample (0.740) and the highest in the MS samples (0.781) (mean = 0.756) (Table 2). Locations WCS and BNS2 showed a significant departure from the Hardy–Weinberg equilibrium (Table 2) (both multilocus \( F_{IS} = 0.050 \)). Pairwise comparisons between loci revealed no significant linkage disequilibrium after sequential Bonferroni corrections.

Patterns of population subdivision

First, the graphical distribution of populations from the FCA showed that the two Mediterranean samples (VMS1 and VMS2), as well as all Atlantic samples, except the one from the IB (AAO), clustered together (Figure 2). Disregarding the AAO sample, the sample of the Bay of Biscay (GOA) was the most aberrant among the Atlantic samples. The distribution of all individuals in a FCA plot (graph not shown) shows a small overlap between the Mediterranean and Atlantic samples as well as between AAO and the other Atlantic samples.

Second, the global \( \theta \) and \( \rho \)-values across all samples, excluding the temporal samples (BNS2 and VMS2), were 0.0121 and 0.0426, respectively (both highly significant, \( P < 0.001 \)). The global standardized multilocus \( F_{ST} \) was 0.0484. The pairwise \( F_{ST} \) values were significant after sequential Bonferroni correction and false discovery rate control for all population pairs including samples TBS, VMS and AAO, together with a limited number of population pairs between Atlantic populations (Table 3). Pairwise \( R_{ST} \) values were significant after sequential Bonferroni correction or false discovery rate control for several population pairs between VMS and Atlantic populations (Table 3). The pairwise \( F_{ST} \) and \( R_{ST} \) values between the two temporal samples were not statistically different (\( P > 0.05 \)) for BNS (0.0038 and 0.0005, respectively) and VMS (−0.0012 and −0.0025, respectively) (Table 3). The pairwise standardized \( F_{ST} \) values between the different samples are listed in Table 4. Jackknife

<table>
<thead>
<tr>
<th>Population</th>
<th>Sample size</th>
<th>( H_e ) n.b.</th>
<th>( H_O )</th>
<th>( F_{IS} )</th>
<th>Allelic richness</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBS</td>
<td>96</td>
<td>0.740</td>
<td>0.718</td>
<td>0.030</td>
<td>12.088</td>
</tr>
<tr>
<td>WCS</td>
<td>88</td>
<td>0.760</td>
<td>0.722</td>
<td>0.050</td>
<td>13.211</td>
</tr>
<tr>
<td>RNS</td>
<td>48</td>
<td>0.752</td>
<td>0.755</td>
<td>−0.003</td>
<td>12.725</td>
</tr>
<tr>
<td>BNS1</td>
<td>47</td>
<td>0.749</td>
<td>0.689</td>
<td>0.081</td>
<td>12.319</td>
</tr>
<tr>
<td>BNS2</td>
<td>95</td>
<td>0.762</td>
<td>0.724</td>
<td>0.050</td>
<td>13.404</td>
</tr>
<tr>
<td>WIS</td>
<td>45</td>
<td>0.754</td>
<td>0.724</td>
<td>0.040</td>
<td>13.534</td>
</tr>
<tr>
<td>GOA</td>
<td>40</td>
<td>0.752</td>
<td>0.732</td>
<td>0.027</td>
<td>14.087</td>
</tr>
<tr>
<td>AAO</td>
<td>95</td>
<td>0.752</td>
<td>0.714</td>
<td>0.051</td>
<td>14.015</td>
</tr>
<tr>
<td>VMS1</td>
<td>94</td>
<td>0.781</td>
<td>0.766</td>
<td>0.019</td>
<td>15.454</td>
</tr>
<tr>
<td>VMS2</td>
<td>48</td>
<td>0.766</td>
<td>0.723</td>
<td>0.057</td>
<td>15.575</td>
</tr>
</tbody>
</table>

\( H_e \) n.b. is the unbiased expected heterozygosity, \( H_O \) the observed heterozygosity and \( F_{IS} \) measures deviation from Hardy–Weinberg equilibrium. Statistically significant \( F_{IS} \) values are listed in bold. See Table 1 for sample codes.
analysis revealed that locus Pmin16-2 was responsible for the largest divergence as calculated with $R_{ST}$ but not with $F_{ST}$ (average pairwise $R_{ST} = 0.02974$ with locus Pmin16-2 and $R_{ST} = 0.00528$ without this locus). Random permutation of different allele sizes among allelic states at each locus revealed that estimates of $R_{ST}$ were significantly larger than the 95% CI range of the $pR_{ST}$ values at one single locus Pmin16-2 (Table 5), suggesting a mutational component to genetic differentiation. For the other microsatellites, neither loci allele size nor stepwise mutations strongly influence the population differentiation of sand gobies. Hardy et al. (2003) suggest that, in this situation, $F_{ST}$ should be preferred over $R_{ST}$ for estimating population differentiation.

Third, the classical multidimensional scaling analyses plots based on both types of genetic distances separated clearly the group of the two Mediterranean samples from the populations of the Atlantic (Figure 3). Both plots had a stress value below 0.20, suggesting interpretable information concerning intersite relationships.

Fourth, the global Mantel test revealed a significant isolation by distance pattern with $D_{CE}$ ($r = 0.713, P < 0.05$).

### Table 5
Mean single locus and multilocus pairwise estimates of $F_{ST}$, $R_{ST}$, $pR_{ST}$ (95% distribution of central values in parentheses) between the 10 samples of sand gobies following 2000 permutations of the microsatellite alleles (Hardy et al., 2003)

<table>
<thead>
<tr>
<th></th>
<th>TBS</th>
<th>WCS</th>
<th>RNS</th>
<th>BNS1</th>
<th>BNS2</th>
<th>WIS</th>
<th>GOA</th>
<th>AAO</th>
<th>VMS1</th>
<th>VMS2</th>
</tr>
</thead>
<tbody>
<tr>
<td>$F_{ST}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.17</td>
<td>0.04</td>
<td>0.018</td>
<td>0.118</td>
<td>0.118</td>
</tr>
<tr>
<td>$R_{ST}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$pR_{ST}$ (95% range)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Indicates statistically significant values ($P < 0.01$).

### Figure 2
Factorial correspondence analysis plot based on microsatellite data of the 10 samples of $P$. minutus. See Table 1 for sample codes.

### Table 3
Pairwise $F_{ST}$ (below diagonal) and $R_{ST}$ (above diagonal) values of the $P$. minutus samples based on eight microsatellite markers

<table>
<thead>
<tr>
<th></th>
<th>TBS</th>
<th>WCS</th>
<th>RNS</th>
<th>BNS1</th>
<th>BNS2</th>
<th>WIS</th>
<th>GOA</th>
<th>AAO</th>
<th>VMS1</th>
<th>VMS2</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBS</td>
<td>—</td>
<td>0.017</td>
<td>0.012</td>
<td>0.003</td>
<td>0.023</td>
<td>—</td>
<td>0.017</td>
<td>0.044</td>
<td>0.018</td>
<td>—</td>
</tr>
<tr>
<td>WCS</td>
<td>0.007*</td>
<td>—</td>
<td>—</td>
<td>0.002</td>
<td>—</td>
<td>0.003</td>
<td>0.006</td>
<td>0.004</td>
<td>0.006</td>
<td>—</td>
</tr>
<tr>
<td>RNS</td>
<td>0.009*</td>
<td>0.002</td>
<td>—</td>
<td>0.004</td>
<td>0.014</td>
<td>—</td>
<td>0.001</td>
<td>0.008</td>
<td>0.005</td>
<td>—</td>
</tr>
<tr>
<td>BNS1</td>
<td>0.010*</td>
<td>0.003</td>
<td>0.001</td>
<td>—</td>
<td>0.001</td>
<td>—</td>
<td>0.008</td>
<td>0.022</td>
<td>0.004</td>
<td>—</td>
</tr>
<tr>
<td>BNS2</td>
<td>0.007*</td>
<td>0.004*</td>
<td>—</td>
<td>0.004</td>
<td>—</td>
<td>—</td>
<td>0.001</td>
<td>0.061</td>
<td>0.002</td>
<td>0.081</td>
</tr>
<tr>
<td>WIS</td>
<td>0.013*</td>
<td>0.012*</td>
<td>0.007*</td>
<td>—</td>
<td>0.004</td>
<td>0.006</td>
<td>—</td>
<td>—</td>
<td>0.001</td>
<td>0.055*</td>
</tr>
<tr>
<td>GOA</td>
<td>0.013*</td>
<td>0.008*</td>
<td>0.010*</td>
<td>0.004</td>
<td>0.005*</td>
<td>—</td>
<td>0.009*</td>
<td>—</td>
<td>0.002</td>
<td>0.042*</td>
</tr>
<tr>
<td>AAO</td>
<td>0.012*</td>
<td>0.010*</td>
<td>0.009*</td>
<td>0.012*</td>
<td>0.008*</td>
<td>—</td>
<td>0.010*</td>
<td>0.013*</td>
<td>—</td>
<td>0.086</td>
</tr>
<tr>
<td>VMS1</td>
<td>0.025*</td>
<td>0.017*</td>
<td>0.019*</td>
<td>0.018*</td>
<td>0.017*</td>
<td>0.015*</td>
<td>0.015*</td>
<td>0.017*</td>
<td>—</td>
<td>0.003</td>
</tr>
<tr>
<td>VMS2</td>
<td>0.018*</td>
<td>0.011*</td>
<td>0.013*</td>
<td>0.013*</td>
<td>0.012*</td>
<td>0.014*</td>
<td>0.013*</td>
<td>0.012*</td>
<td>0.001</td>
<td>—</td>
</tr>
</tbody>
</table>

Statistically significant values are listed in bold after sequential Bonferroni correction and with * after the false discovery rate control. See Table 1 for sample codes.

### Table 4
Standardised pairwise $F_{ST}$ estimates (Hedrick, 2005) based on the microsatellite data (below diagonal) and the $F_{ST}$ estimated from mitochondrial cyt $b$ sequence data (above diagonal) between the eight locations for $P$. minutus

<table>
<thead>
<tr>
<th></th>
<th>TBS</th>
<th>WCS</th>
<th>RNS</th>
<th>BNS1</th>
<th>BNS2</th>
<th>WIS</th>
<th>GOA</th>
<th>AAO</th>
<th>VMS1</th>
<th>VMS2</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBS</td>
<td>—</td>
<td>0.165</td>
<td>0.196</td>
<td>0.243</td>
<td>—</td>
<td>0.384</td>
<td>0.257</td>
<td>0.659</td>
<td>0.688</td>
<td>—</td>
</tr>
<tr>
<td>WCS</td>
<td>0.027</td>
<td>—</td>
<td>0.093</td>
<td>0.133</td>
<td>—</td>
<td>0.036</td>
<td>0.015</td>
<td>0.616</td>
<td>0.650</td>
<td>—</td>
</tr>
<tr>
<td>RNS</td>
<td>0.024</td>
<td>0.001</td>
<td>—</td>
<td>0.049</td>
<td>—</td>
<td>0.245</td>
<td>0.866</td>
<td>0.556</td>
<td>0.645</td>
<td>—</td>
</tr>
<tr>
<td>BNS1</td>
<td>0.030</td>
<td>0.010</td>
<td>0.002</td>
<td>—</td>
<td>—</td>
<td>0.330</td>
<td>0.115</td>
<td>0.594</td>
<td>0.627</td>
<td>—</td>
</tr>
<tr>
<td>BNS2</td>
<td>0.024</td>
<td>0.020</td>
<td>0.002</td>
<td>—</td>
<td>—</td>
<td>0.019</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>WIS</td>
<td>0.032</td>
<td>0.047</td>
<td>0.018</td>
<td>0.019</td>
<td>0.021</td>
<td>—</td>
<td>0.064</td>
<td>0.709</td>
<td>0.724</td>
<td>—</td>
</tr>
<tr>
<td>GOA</td>
<td>0.050</td>
<td>0.042</td>
<td>0.031</td>
<td>0.014</td>
<td>0.030</td>
<td>0.034</td>
<td>—</td>
<td>0.579</td>
<td>0.661</td>
<td>—</td>
</tr>
<tr>
<td>AAO</td>
<td>0.045</td>
<td>0.043</td>
<td>0.042</td>
<td>0.054</td>
<td>0.037</td>
<td>0.038</td>
<td>0.055</td>
<td>—</td>
<td>—</td>
<td>0.756</td>
</tr>
<tr>
<td>VMS1</td>
<td>0.053</td>
<td>0.041</td>
<td>0.040</td>
<td>0.053</td>
<td>0.049</td>
<td>0.053</td>
<td>0.042</td>
<td>0.044</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>VMS2</td>
<td>0.036</td>
<td>0.025</td>
<td>0.029</td>
<td>0.043</td>
<td>0.037</td>
<td>0.055</td>
<td>0.036</td>
<td>0.029</td>
<td>—0.006</td>
<td>—</td>
</tr>
</tbody>
</table>

Mitochondrial divergence estimates were calculated as described by Larmuseau et al. (2009b).
but not with $d_m^2$ ($r = 0.166$, $P > 0.05$). However, no significant isolation by distance was found for both types of genetic distances when the MS and/or IB samples were excluded from the data set.

Fifth, testing the significance of the stepwise clustering procedure performed in STRUCTURE resulted in separation of the samples into three hypothetical clusters (highest $D_K$ for $K = 3$). The lowest proportion for a sample of membership to a particular cluster is 0.411 in cluster 1 for sample WCS and the highest proportion is 0.897 in cluster 2 for sample TBS (Table 6). All Atlantic samples (excluding TBS) had the highest assignment value for cluster 1; the northern Baltic sample (TBS) for cluster 2 and the two Mediterranean samples for cluster 3 (Table 6 and Figure 4).

Finally, the Bayesian approach used in 2MOD indicated that $P. minutus$ populations were at migration-drift equilibrium. All sampled iterations revealed that a migration-drift equilibrium model was more likely than the non-equilibrium drift model.

Comparison between types of genetic distances and mtDNA

$F_{ST}$-values are much lower for microsatellites (global $F_{ST} = 0.0121$ (CI = 0.0066–0.0174) and global standardized $F_{ST} = 0.0484$ (CI = 0.0264–0.0696), both without the temporal samples VMS2 and BNS2) than for mtDNA (global $F_{ST} = 0.4293$ (CI = 0.3231–0.5302)). The proportion of differences in $F_{ST}$ between mtDNA and nuclear microsatellites is not attributed to stochastic variability among loci as there were no notable differences between different microsatellite loci. The highest global $\theta$ was found for the locus Pmin 16-2 (0.0314); the lowest $\theta$-value was found for the locus Pmin 38 ($\theta = 0.0023$). Pairwise $F_{ST}$ values between all samples based on the mtDNA data are presented together with the standardized pairwise $F_{ST}$ estimates for microsatellites in Table 4. Each pairwise $F_{ST}$ value for mtDNA is on average 10 times higher than the value for microsatellites. The pairwise genetic distances of the microsatellite data correlated well with the estimates based on all microsatellites. The $D_{CE}$ pairwise genetic distances of the microsatellite data correlated well with Tamura and Nei (1993) genetic distances based on the mtDNA data (Procrustes $R = 0.746$, $P$-value < 0.001; Mantel $R = 0.6804$, $P$-value = 0.032). In contrast, the $d_m^2$ pairwise genetic distances of the microsatellite data did not correlate with each other (Procrustes $R = 0.8339$, $P$-value = 0.004; Mantel $R = 0.6804$, $P$-value = 0.032).

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**Table 6** Summary of the assignment analysis following STRUCTURE v. 2.2 based on microsatellite data of 10 $P. minutus$ samples

<table>
<thead>
<tr>
<th>Population</th>
<th>Inferred clusters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>TBS</td>
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</tr>
<tr>
<td>WCS</td>
<td>0.411</td>
</tr>
<tr>
<td>RNS</td>
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<td>BNS1</td>
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</tr>
<tr>
<td>BNS2</td>
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</tr>
<tr>
<td>WIS</td>
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</tr>
<tr>
<td>GOA</td>
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</tr>
<tr>
<td>AAO</td>
<td>0.492</td>
</tr>
<tr>
<td>VMS1</td>
<td>0.125</td>
</tr>
<tr>
<td>VMS2</td>
<td>0.160</td>
</tr>
</tbody>
</table>

The proportion of individuals assigned to the three hypothetical clusters is given. The highest assignment value for each population is listed in bold. See Table 1 for sample codes.

---

**Figure 3** Classical multidimensional scaling plots of pairwise genetic distance for the microsatellite data calculated according to (a) Cavalli-Sforza and Edwards (1967) ($D_{CE}$) and (b) Goldstein et al. (1995) ($d_m^2$) among the 10 samples of $P. minutus$. See Table 1 for sample codes.

**Figure 4** Results of the clustering analysis conducted in STRUCTURE 2.2 (Pritchard et al., 2000) based on the microsatellite data. In the bar plot, each of the 696 individuals is represented by a vertical bar indicating its estimated proportion of membership to the three clusters ($K = 3$). See Table 1 for sample codes.

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**Mito-nuclear discordance in sand goby**

MHD Larmuseau et al.
Impact of selection, sex-biased dispersal and effective population size on the degree of population divergence estimates

No indication for selection for some of the microsatellite markers was observed in the analysis. The results of Lositan and BayeScan were congruent with this pattern by revealing no outlier loci. Moreover, a blast search of the flanking regions of the microsatellite loci in the GenBank database revealed that they are not linked to a functional region already sequenced for any organism available in GenBank.

A total of 352 males and 344 females were genotyped and the population genetic parameters were estimated separately for each sex. No parameter was significantly different between the sexes (Table 7), suggesting no sex-biased dispersal.

Estimates from the various point and temporal methods to estimate the $N_e$ are not congruent with each other (Table 8). Both point methods cannot exclude the possibility that the population sizes are infinite. The two temporal methods are similar for the BNS population (~400 individuals) and for VMS (>1000 individuals).

Discussion

Phylogeography of the sand goby

The current study with microsatellites revealed a subtle but significant genetic structure within $P.\ minutus$. The highest divergence in the analysis was found between populations of the Atlantic, comprising all sand goby populations spanning from the IB to the Baltic Sea, and the MS, represented by individuals from the Vaccarès lagoon (Gulf of Lion) (Table 3 and Figures 2–4). The microsatellite marker Pmin16-2 shows a clear differentiation with a small overlap in allele size between the MS-Clade and AO-Clade, clearly indicating the historical divergence of both regions. In addition, the highest divergence noticed in the genetic analysis of allozyme markers were also found between Mediterranean and Atlantic sand gobies (Stefanni et al., 2003; Larmuseau et al., 2009b). The results are also congruent with the phylogenetic analysis based on mtDNA cyt b, which clearly showed two monophyletic clades within $P.\ minutus$, the Mediterranean Sea Clade (MS-Clade) and the AO-Clade (Larmuseau et al., 2009b). Divergence between the two sand goby clades is most likely the result of geographic isolation caused by one of the Middle Pleistocene sea-level drops (Larmuseau et al., 2009b). The Mediterranean populations showed the highest variability in the microsatellite markers (Table 2) despite the small distribution range along the northwestern MS coasts and lagoons (Miller, 1986) (Figure 1). Larmuseau et al. (2009b) noticed the highest diversity in mtDNA in the Mediterranean samples as well. Furthermore, the highest species diversity of the ‘sand goby’ group is observed within the MS (Huyse et al., 2004) supporting a Mediterranean origin of $P.\ minutus$, as suggested by Gyssels et al. (2004a). Huyse et al. (2004) estimated the origin of the species between 1.94 and 1.18 mya (early Pleistocene). The Pleistocene glaciations were the most significant historical events during the evolutionary lifespan of most Holarctic species and are believed to have accelerated the speciation process in the present day sister taxa (Avise, 2000).

Limited genetic differentiation was observed between the Atlantic samples with microsatellites, but the FCA and the pairwise $F_{ST}$ values suggest that the IB’s sample is different from those of all other Atlantic populations (Figure 2 and Table 3). These results are congruent with the two mitochondrial groups within the Atlantic samples: the Iberian (IB-Group) and the North Atlantic Group. This divergence is likely the result of the population decline within different refugia during glaciations in the Middle Pleistocene (Larmuseau et al., 2009b).
On the basis of the Bayesian assignment test and pairwise $F_{ST}$ values, the position for the sample of the northern Baltic Sea (TBS) was remarkable (Tables 3 and Figure 4). This is most likely the result of a founder event, based on the low variation on the microsatellite loci in this population (Table 2) and as suggested by Larmuseau et al. (2009b) based on the mtDNA data. The difference in the cyt $b$ haplotype network between the southern and northern Baltic Sea samples suggested that the Baltic Sea has been colonized in two phases over a period of 8000 years, with a stronger founder effect in the north. The presence of only two common cyt $b$ haplotypes in the northern samples HBS and TBS (haplotypes NA01 and NA28) with their many uniquely derived haplotypes suggested that only a few individuals founded the northern Baltic Sea (Johannesson and André, 2006). A recent study has already shown that sand gobies are locally adapted to the specific light environment of the northern Baltic Sea in comparison with the sand gobies of the North Sea and the ÅO (Larmuseau et al., 2009a). If only a few sand gobies colonized the northern Baltic, the populations in this region will be genetically diverged from the founding population, mainly through a shift in allele frequencies. Such shifts are detectable with pairwise $F_{ST}$ values and the STRUCTURE analysis (Table 3 and Figure 4). In contrast, individual multilocus genotypes are less sensitive for shifts in allele frequencies, and analyses based on multilocus genotypes, such as the FCA plot (Figure 2), will not be able to distinguish between different groups after a founding event.

The Mantel test and Procrustes analysis illustrated that the microsatellite results are congruent with the mtDNA phylogeographic pattern. However, those tests were not significant when the $d_{R}^2$ genetic distance was used for microsatellites. This suggests that, although both measures are correlated, $F_{ST}$ correlates better with mtDNA than $R_{ST}$. In theory, $F_{ST}$ is more sensitive than $R_{ST}$ for recent intraspecific divergence (Gaggiotti et al., 1999; Balloux and Lugon-Moulin, 2002). Moreover, $R_{ST}$ can be less accurate in reflecting population differentiation because of its higher associated variances (Balloux and Lugon-Moulin, 2002). Therefore, the number of loci screened has to increase before a consistent pattern is reached (Gaggiotti et al., 1999; Balloux and Goudet, 2002).

Overall, the results confirm the first hypothesis of this study that stated that microsatellite markers reveal a phylogeographic pattern congruent with the patterns based on mtDNA and allozyme data.

First, selection may have differential effects on genetic markers. It has been suggested that balancing selection may significantly influence the distribution of allozyme diversity (De Innocentiis et al., 2001). Loci experiencing balancing selection will have allele frequencies more similar than expected under neutrality, reducing the $F_{ST}$ estimates. Allendorf and Seeb (2000) concluded that estimates of population structure produced by allozymes were generally comparable to those obtained with other nuclear markers, including microsatellites. They noted that when differences between marker classes did occur, they were usually due to one or a few exceptional loci and not all of them. Each used microsatellite marker was tested for positive or balanced selection in comparison with the other markers in the Lositan and BayeScan analysis, but no marker seemed to be an outlier. Similar to microsatellites, it is also not safe to assume a priori that mtDNA evolves as a strictly neutral marker (Ballard and Whitlock, 2004). Selection on mtDNA may accelerate the coalescence of lineages, and thus increase the levels of differentiation observed between populations (Peijnenburg et al., 2006). The observation of very low nuclear differentiation in sand gobies while mtDNA data revealed no gene flow between the three isolated Middle Pleistocene lineages cannot be explained by selective evolution for mtDNA. The rejection of the null hypothesis in different neutrality tests for the mtDNA data of $P$. minutus was assigned to demographical expansions instead of selection (Larmuseau et al., 2009b). Various demographic analyses on the mtDNA data showed that the intra-assemblage genetic structure of $P$. minutus contains signatures of demographic expansion events.

Second, studies documenting a weaker population subdivision for nuclear than maternally inherited genetic markers often attribute these discrepancies to male-biased dispersal. Sex-biased dispersal is common in nature (Cano et al., 2008); however, it has only been described for a limited number of marine fishes (Consegra and de Leaniz, 2007). No indications for sex-biased dispersal were found for $P$. minutus with microsatellite markers (Table 7). Contemporary gene flow between the Mediterranean and Atlantic basins is also unlikely because of the discontinuous distribution of the sand goby (Miller, 1986) (Figure 1). Moreover, only males have to migrate successfully to explain the pattern. However, females are expected to be the most mobile sex, especially during the spawning period when males are guarding their nest (Lindström et al., 2006).

Third, various technical problems, including homoplasy, may have reduced the signal of differentiation detected by the microsatellite markers. Homoplasy occurs when different copies of a locus are identical in state, although not identical by descent. The situations where size homoplasy is most prevalent involve high mutation rates and large population sizes together with strong allele size constraints (Estoup et al., 2002). Therefore, effects of homoplasy are expected to be common for microsatellites in marine fishes (O’Reilly et al., 2004), which has implications for the identification of genetic structuring (Carreras-Carbonell et al., 2006). Microsatellites probably suffered from higher levels of homoplasy than mtDNA because of higher mutation rates and larger effective population sizes (Balloux et al., 2000). The various microsatellite markers of $P$. minutus indicate homoplasy because of the high allele numbers and the
limited size range of all markers (on average 1 allele per 2,053 bp). Single-nucleotide polymorphisms and microsatellite markers with less alleles covering a limited size range might be useful to understand the power of homoplasy in the analysis. However, simulation studies suggest that size homoplasy will have much less effect on estimates of population differentiation than gene migration or genetic drift (Estoup et al., 2002). Therefore, it is unlikely that homoplasy is the main cause of the observed differences.

Finally, mitochondrial markers can be more sensitive in detecting differentiation because of a lower effective population size than nuclear markers (Shaw et al., 2004). Genetic drift effects are linked to effective population size \( (N_e) \), and therefore it is possible that an ecologically relevant population structure remains undetectable by using neutral markers when the \( N_e \) is high (Bentzen, 1998). Marine fish have the potential to have high \( N_e \) and therefore recently separated large populations may appear genetically homogeneous even in the complete absence of contemporary gene flow (Hauser and Carvalho, 2008). Simulations in the study by Buonaccorsi et al. (2001) showed that differences in the magnitude of estimated population subdivision from nuclear and mitochondrial markers could be accounted for entirely by differences in effective population sizes and polymorphisms on \( F_{ST} \) estimates. The haploid and maternal-only inheritance of mtDNA has an effective population size of one-quarter that of nuclear DNA, making it more susceptible to effects of genetic drift (Shaw et al., 2004). This explanation has been invoked for the discordant patterns in population differentiation between nuclear and mitochondrial markers for marine organisms, such as the blue marlin Makaira nigricans (Buonaccorsi et al., 2001), Patagonian toothfish Dissostichus eleginoides (Appleyard et al., 2002; Shaw et al., 2004) and the olive sea snake Aipysurus laevis (Lukoschek et al., 2008). Simulations in EASYPOP v.1.7 (Balloux, 2001) with specific biological information about the sand goby showed no difference with the simulations of the study by Buonaccorsi et al. (2001) (results not shown). Moreover, \( P. \ minutus \) is known to be one of the most abundant fish species across almost its full range (Pasquaud et al., 2004; Ehrenberg et al., 2005; Maes et al., 2005). Therefore, high \( N_e \) most likely explains the discordant patterns between nuclear and mitochondrial data for the sand goby. Point methods to estimate the \( N_e \) of the present sampled populations cannot invalidate the null hypothesis of an infinite population size for \( P. \ minutus \) (Table 8). Nevertheless, point methods are not always reliable and are biased (Wang and Whitlock, 2003), especially when the sample size is small (<100 individuals) and below the true \( N_e \) (England et al., 2006). Both temporal \( N_e \) estimates show a limited population size, especially for the BNS location (100–1000 individuals) (Table 8). Still, there are crucial differences between the two temporal methods and the assumption of a closed system without migration could not be fulfilled. Migration inside the marine system can therefore cause a strong underestimation of the \( N_e \) (Wang and Whitlock, 2003). On the other hand, the high number of alleles for microsatellites confirms the hypothesis of a high effective population size of more than one thousand individuals to maintain the high genetic variation (Ewens, 1972; Poulsen et al., 2006). The \( P_{min20} \) locus was excluded from the analysis because, after genotyping more than 90 individuals, the number of alleles almost matched the number of fish genotyped. The studies of Jones et al. (2001) and Pampoulie et al. (2004) also observed microsatellite markers with a very high number of alleles. By organizing a better sampling strategy and using straightforward \( N_e \)-estimation methods incorporating migration (Wang and Whitlock, 2003), better estimates of the \( N_e \) for \( P. \ minutus \) might confirm the expected high \( N_e \).

Conclusion

Marine organisms have a high potential for gene flow and population size. However, it is not known whether the observed low genetic differentiation for so many marine species reflects high effective population sizes and low gene flow, high effective population sizes and high rates of gene flow, or low effective population sizes and high rates of gene flow (Hauser and Carvalho, 2008). In this study on \( P. \ minutus \), the scenario of high effective population sizes and low gene flow best explains the observed genetic pattern. However, this conclusion could only be reached with more than one genetic marker. Therefore, one has to be cautious when interpreting the present-day genetic structure in terms of gene flow while using one type of marker or statistical method.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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