Journal of Biogeography (*J. Biogeogr.*) (2009) **36**, 1138–1151

ORIGINAL ARTICLE

Distributional and demographic consequences of Pleistocene climate fluctuations for a marine demersal fish in the north-eastern Atlantic

Maarten H. D. Larmuseau[1]*, Jeroen K. J. Van Houdt[2], Jef Guelinckx[3], Bart Hellemans[1] and Filip A. M. Volckaert[1]

[1]*Laboratory of Animal Diversity and Systematics, Katholieke Universiteit Leuven, Leuven,* [2]*Royal Museum of Central Africa, Tervuren and* [3]*Laboratory of Aquatic Ecology and Evolutionary Biology, Katholieke Universiteit Leuven, Leuven, Belgium*

*Correspondence: Maarten H. D. Larmuseau, Laboratory of Animal Diversity and Systematics, Katholieke Universiteit Leuven, Deberiotstraat 32, B-3000 Leuven, Belgium.
E-mail: maarten.larmuseau@bio.kuleuven.be

ABSTRACT

Aim The Pleistocene glaciations were the most significant historical event during the evolutionary life span of most extant species. However, little is known about the consequences of these climate changes for the distribution and demography of marine animals of the north-eastern Atlantic. The present study focuses on the phylogeographic and demographic patterns of the sand goby, *Pomatoschistus minutus* (Teleostei: Gobiidae), a small marine demersal fish.

Location North-eastern Atlantic, Mediterranean, Irish, North and Baltic seas.

Methods Analysis was carried out by sequencing the mtDNA cytochrome *b* gene of sand gobies from 12 localities throughout the species' range, and using this information in combination with published data of allozyme markers and mtDNA control region sequences. Several phylogenetic methods and a network analysis were used to explore the phylogeographic pattern. The historical demography of *P. minutus* was studied through a mismatch analysis and a Bayesian skyline plot.

Results Reciprocal monophyly was found between a Mediterranean Sea (MS) clade and an Atlantic Ocean (AO) clade, both with a Middle Pleistocene origin. The AO Clade contains two evolutionary significant units (ESUs): the Iberian Peninsula (IB) Group and the North Atlantic (NA) Group. These two groups diverged during Middle Pleistocene glacial cycles. For the NA Group there is evidence for geographic sorting of the ancestral haplotypes with recent radiations in the Baltic Sea, Irish Sea, North Sea and Bay of Biscay. The demographic histories of the Mediterranean Clade and the two Atlantic ESUs were influenced mainly by expansions dated as occurring during the Middle Pleistocene glaciations and post-Eem, respectively.

Main conclusions The pre-LGM (Last Glacial Maximum) subdivision signals were not erased for *P. minutus* during the LGM. Middle Pleistocene glaciations yielded isolated and differently evolving sets of populations. In contrast to the case for most other taxa, only the northern Atlantic group contributed to the post-glacial recolonization. The historical demography of Mediterranean sand gobies was influenced mainly by Middle Pleistocene glaciations, in contrast to that of the Atlantic populations, which was shaped by Late Pleistocene expansions.

Keywords

Bayesian skyline plot, glaciations, Gobiidae, mismatch analysis, mtDNA, north-eastern Atlantic, phylogeography, *Pomatoschistus minutus*, radiation, sand goby.

www.blackwellpublishing.com/jbi
doi:10.1111/j.1365-2699.2008.02072.x

INTRODUCTION

Organisms live in a constantly changing environment, and the genetic patterns we observe today are the result of both contemporary and historical factors (Avise, 2000). The glaciation cycles during the Pleistocene (1800–11.5 ka) were arguably the most important climatological events during the evolutionary life span of most extant species (Hewitt, 2000). Although the impact of the Pleistocene climate fluctuations on the distribution and demography of continental species is firmly established in the literature (Bernatchez & Wilson, 1998; Taberlet *et al.*, 1998; Hewitt, 2004), little is known about the impact of the glaciation cycles on the distribution and demography of north-eastern Atlantic marine species (Wilson, 2006).

During glaciation events, ice-covered areas to the north were unsuitable habitats for near-shore marine species, forcing their extinction or a southward migration into one or more refugial areas. Although it is difficult to identify potential refugia from genetic data, several southern refugia have been suggested for north-eastern Atlantic marine species: around the Iberian Peninsula (Gysels *et al.*, 2004a; Hoarau *et al.*, 2007), Macaronesia (Domingues *et al.*, 2005, 2007) and the Mediterranean Sea (Olsen *et al.*, 2004; Sa-Pinto *et al.*, 2005). In addition to the large southern glacial refugia, small northern peri-glacial refugia have been proposed, such as the Bay of Biscay (Nesbø *et al.*, 2000), the south-western coast of Ireland (Jolly *et al.*, 2006; Hoarau *et al.*, 2007) and Hurd Deep in the English Channel, a 150-km-long depression about 100 m deeper than the adjoining sea floor (Coyer *et al.*, 2003; Provan *et al.*, 2005). When temperatures increased during the interglacials, populations from these refugia recolonized previously ice-covered areas in the north. These recolonization patterns remain poorly understood and are likely to be species-specific (Chevolot *et al.*, 2006). Moreover, the role of the Mediterranean Sea for Atlantic species during the Pleistocene glaciation cycles is still controversial. During the glaciations, the decrease in sea level isolated the Mediterranean and Atlantic basins. Within the Mediterranean basin, extinction and recolonization played a major role during the glacial cycles, as organisms were isolated and could not follow favourable isothermals. No clear relationship is apparent between biological traits and observed patterns of partial or complete genetic isolation of the Atlantic and Mediterranean populations (Patarnello *et al.*, 2007).

Owing to the open character of the sea, it is difficult to study the historical distribution and demography of species. The absence of obvious barriers to gene flow in the marine realm seems to facilitate extensive gene flow among marine populations, dimming the influence of historical events (Palumbi, 1994). Most studies on marine species of the north-eastern Atlantic have focused on the genetic patterns of fishes and invertebrates with high dispersal potential. It is reasonable to assume that species showing reduced levels of contemporary gene flow and a fast evolutionary rate are better suited for elucidating phylogeographic patterns. Therefore, a marine demersal fish, the sand goby, *Pomatoschistus minutus* (Pallas, 1770) (Teleostei: Gobiidae), was selected for the present study on the influence of the Pleistocene climate changes on the distribution and demography of marine demersal fishes. Sand gobies reach high densities along their geographical range: the Atlantic coasts from Norway to Spain, the North Sea, the Baltic Sea and the Irish Sea. The distribution pattern is more fragmented in the Mediterranean Sea and on the west coast of the Black Sea (Miller, 1986; Fig. 1). In connection with its ecological significance (Pasquaud *et al.*, 2004; Ehrenberg *et al.*, 2005) and use as a model organism in ecology (Jones *et al.*, 2001; Singer *et al.*, 2006; Guelinckx *et al.*, 2008), various phylogeographic and population genetic studies have been carried out on this species (Stefanni & Thorley, 2003; Stefanni *et al.*, 2003; Gysels *et al.*, 2004b).

The main objective of the present study was to analyse how the Pleistocene climatic fluctuations shaped the regional distribution and demography of *P. minutus* along north-eastern Atlantic shores and in the Mediterranean basin. Three

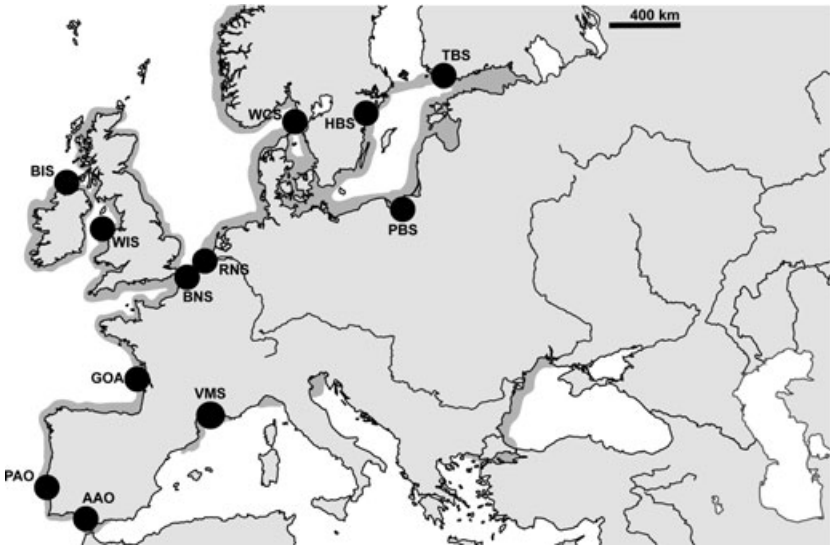

Figure 1 Geographical distribution of the 12 sampling locations in eight European marine systems for the sand goby, *Pomatoschistus minutus*. The shaded area represents the distribution range of *P. minutus* conforming to Miller (1986). See Table 1 for definitions of the sampling locations.

hypotheses based on former studies on the sand goby (Gysels *et al.*, 2004b; Stefanni & Thorley, 2003) were critically evaluated: (1) the Mediterranean Sea was recolonized by Atlantic sand gobies after the Last Glacial Maximum (LGM; 20 ka) – earlier studies show conflicting results for the present relationship between Mediterranean and Atlantic populations; (2) there is no population differentiation within the Atlantic sand gobies – earlier studies revealed no significant genetic differentiation between sand goby populations in the Atlantic basin, although the species is expected to show reduced levels of contemporary gene flow owing to its life style; and (3) a population expansion of *P. minutus* in the North Atlantic occurred after the LGM – this was hypothesized despite a lack of demographic analyses for the sand goby.

Compared with a previous study on the phylogeography of *P. minutus* by Gysels *et al.* (2004b), we sequenced a much larger fragment of the cytochrome *b* (cyt *b*) gene from a higher number of sand gobies and we collected at more sampling sites. These new data were pooled with D-loop (Stefanni & Thorley, 2003) and allozyme (Stefanni *et al.*, 2003; Gysels *et al.*, 2004c) data for statistical analyses.

MATERIALS AND METHODS

Sampling and mtDNA sequencing

A total of 263 *Pomatoschistus minutus* individuals were caught at 12 locations along European coasts between October 2005 and February 2007 (Table 1, Fig. 1). Samples were taken by fyke, hand net or beam trawling. Three individuals of *Pomatoschistus lozanoi* were caught as an outlier group for the phylogenetic analyses, one in the Gironde estuary, France (45°36′ N, 01°01′ W) (LO1), and two in Ostend, Belgium (51°17′ N, 02°51′ E) (LO2 and LO3). *Pomatoschistus* species were identified morphologically, based on the dermal head papillae (Miller, 1986) and on pigmentation patterns (Hamerlynck, 1990), and genetically according to Larmuseau *et al.* (2008). For each sample, an 850-bp fragment of the mitochondrial DNA (mtDNA) cyt *b* was sequenced as detailed in Appendix S1 (see Supporting Information). All sequences were deposited in the GenBank database (EU736948–EU737080). To verify the advantage of the longer sequenced gene fragment, the 850-bp sequences of the cyt *b* gene were compared with the 283-bp sequences of the same gene (AJ555096–AJ555123) determined by Gysels *et al.* (2004b). A sliding-window analysis of 100-bp and 10-bp steps was performed to illustrate nucleotide diversity variation along the whole cyt *b* gene with DnaSP ver. 4.10.9 (Rozas *et al.*, 2003). For the phylogenetic and phylogeographical analyses only the long fragments of cyt *b* were used. The D-loop sequences of Stefanni & Thorley (2003) were recovered from GenBank (AY033004–AY033053).

Phylogenetic and network analyses

Phylogenetic reconstruction was conducted using neighbour-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) analyses. The trees were rooted with the sister species *P. lozanoi* (Huyse *et al.*, 2004). NJ trees were constructed in MEGA ver. 4.0 (Tamura *et al.*, 2007). Bootstrap analyses were performed with 10,000 replicates. MP was performed using PAUP* ver. 4.0b10 (Swofford, 2002) with the following heuristic search settings: 10^5 random taxon addition replicates followed by tree bisection–reconnection (TBR) branch swapping and 1000 bootstrap replicates. According to the Akaike information criterion (AIC), MODELTEST ver. 3.7 (Posada & Crandall, 1998) selected the TrN + I + Γ model as the most suitable model for further analysis. The ML analysis with 100 bootstrap replicates was performed using PhyML ver. 2.4.4 (Guindon & Gascuel, 2003), via a web server (Guindon *et al.*, 2005). TCS ver. 1.3 (Clement *et al.*, 2000) was used to construct a statistical parsimony network, and a median-joining network analysis was performed using the software NETWORK ver. 4.5.0.1 (http://www.fluxus-engineering.com).

The times to the most recent common ancestor (tMRCA) of *P. minutus* and the major sand goby mtDNA lineages were estimated using Bayesian inference as implemented by the

Table 1 Overview of the *Pomatoschistus minutus* samples collected from 12 sampling sites in the north-eastern Atlantic.

Code	Area	Country	Location	Date	Coordinates	*N*	*R*
TBS	Northern Baltic Sea	Finland	Tvärminne	July/2006	59°50′ N–23°12′ E	29	11
HBS	Northern Baltic Sea	Sweden	Värtan	November/2005	58°59′ N–17°27′ E	24	12
PBS	Southern Baltic Sea	Poland	Sopot, Gulf of Gdańsk	February/2007	54°27′ N–18°35′ E	12	9
WCS	Skagerrak	Sweden	Bökevik Bay, Skaftö island	June/2006	58°14′ N–11°26′ E	17	12
RNS	North Sea	Netherlands	Renesse	November/2005	51°44′ N–03°47′ E	22	14
BNS	North Sea	Belgium	Oostduinkerke	November/2006	51°08′ N–02°40′ E	12	5
BIS	Irish Sea	North Ireland (UK)	River Bann estuary	September/2006	55°09′ N–06°45′ W	9	5
WIS	Irish Sea	Wales (UK)	Llanfairfechan	November/2006	53°59′ N–03°59′ W	30	13
GOA	Bay of Biscay	France	Gironde estuary	August/2006	45°36′ N–01°01′ W	30	18
PAO	Iberian Peninsula	Portugal	Alcochete, Tagus estuary	October/2005	38°45′ N–08°57′ W	10	5
AAO	Iberian Peninsula	Spain	Guadalquivir river estuary	November/2006	36°58′ N–06°10′ W	35	23
VMS1	Mediterranean Sea	France	Vaccarès lagoon	January/2006	43°32′ N–04°35′ E	25	24
VMS2	Mediterranean Sea	France	Vaccarès lagoon	January/2007	43°32′ N–04°35′ E	8	8

N, sample size; *R*, number of unique cyt *b* haplotypes.

software program BEAST ver. 1.4.6 (Drummond & Rambaut, 2006). Because of limitations in the BEAST program, all analyses were performed using the GTR model as best alternative for the TrN model. The rate variation among sites was modelled using a gamma distribution with 10 rate categories. The divergence times and their credibility intervals were estimated under the coalescent model with constant population size and with expansion growth, using the strict molecular clock. The posterior distributions of the dates being estimated were approximated by sampling parameter values at every 500th generation over 10^7 Markov chain Monte Carlo (MCMC) steps, after discarding 10^6 burn-in steps. Convergence of the sampled parameters was verified using the program TRACER ver. 1.4 (Rambaut & Drummond, 2007). The effective sample size for each parameter was found to exceed 100 individuals, which is the minimum recommended effective sample size (Weinstock *et al.*, 2005). Dates of divergences between the clades were calculated using a conventional clock for the mitochondrial cyt *b* gene in bony fishes, namely 2% sequence divergence per million years (Bowen *et al.*, 2001; Domingues *et al.*, 2005). As the mtDNA of *Pomatoschistus* gobies has a much higher rate of evolution than that of other teleosts (Gysels *et al.*, 2004a; Huyse *et al.*, 2004), divergence times were also estimated using higher rates of 4% and 6% for the cyt *b* locus. Then, the specific molecular clock for the cyt *b* locus of *Pomatoschistus* gobies was calibrated based on the divergence of the Indo-Pacific and the Atlantic–Mediterranean gobies through the closure of the Atlantic–Mediterranean part of the Tethys Sea *c.* 15–14 Ma (McKay & Miller, 1997). Therefore, the tMRCA was estimated with the various selected clocks between the two *Pomatoschistus* species and two Indo-Pacific gobies, *Tridentiger bifasciatus* (Acc. no. AB021254) and *Tridentiger obscurus* (Acc. no. AB021255).

After analyses on the cyt *b* sequences, phylogenetic and network analyses were conducted on the reconstructed data set of all available D-loop sequences and compared with the mtDNA-based results of Stefanni & Thorley (2003). The divergence times of the sand goby lineages were estimated using various rates (2%, 4%, 6% and 8% sequence divergence per million years) to calibrate the specific molecular clock for the D-loop of the *Pomatoschistus* species.

The genetic diversity and population differentiation in *P. minutus* were analysed based on the cyt *b* and D-loop data using several common indices (π, h and F_{ST}) and non-metric multi-dimensional scaling (NMDS) analyses (for a detailed methodology see Appendix S1). In addition, these statistical analyses were also performed on two original allozyme data sets of Stefanni *et al.* (2003) and Gysels *et al.* (2004c), which were obtained under the same laboratory conditions and for which high sample quality was guaranteed (Gysels, 2003; detailed in Appendix S1).

Demographic analyses

Demographic expansions were first investigated on the cyt *b* and the published control region sequences by means of Tajima's (1989) *D*-test and Fu's (1997) F_s-test of neutrality using ARLEQUIN ver. 3.11 (Excoffier *et al.*, 2005). For neutral markers, significant negative values can be expected in cases of population expansion (Tajima, 1989; Fu, 1997). Furthermore, the demographic history was examined using the frequency distribution of pairwise differences among sequences (Mismatch distribution; Harpending, 1994). Because the sand goby probably experienced demographic as well as range expansions and contractions during successive cycles of glaciations, both demographic and spatial mismatch analyses (Excoffier, 2004; Excoffier *et al.*, 2005) were conducted using ARLEQUIN. Past population demographics of *P. minutus* were also inferred for the cyt *b* and the D-loop data using the coalescent Bayesian skyline plot (BSP) model (Drummond *et al.*, 2005) as implemented in BEAST and visualized in TRACER. Final analyses were run for 3×10^7 generations, sampling every 1000th generation, and a burn-in of 3×10^6 generations (after a pre-burn-in of 3×10^5 generations). The analyses were repeated using different values for the number of grouped intervals (m = 5, 10, 20) and different clock models (strict clock and relaxed clock) with uncorrelated rates drawn from a lognormal distribution, conducting two independent MCMC runs for each parameter combination.

RESULTS

MtDNA haplotypes

The alignment of sequences was straightforward as there were no gaps and translation into amino acids did not indicate nonsense or stop codons. The sequence characteristics matched the general properties of the *P. minutus* cyt *b* gene (Gysels *et al.*, 2004b; Keith *et al.*, 2005), suggesting a functional mtDNA cyt *b* gene and not a nuclear pseudogene (Zhang & Hewitt, 1996). The alignment of all cyt *b* gene sequences revealed 130 unique haplotypes within the 263 sand goby individuals. A total of 130 variable sites (15.3% of the total) were detected, of which 70 positions (8.2% of the total) were parsimony-informative. Of the 130 variable sites, 18 (13.8%) were in first codon position, four (3.1%) were in second codon position, and 108 (83.1%) were in third codon position. Translation using the vertebrate mitochondrial genetic code indicated that 17 out of 282 amino acid residues were polymorphic.

The first 158 bp of our sequences match with the short fragment of cyt *b* sequenced by Gysels *et al.* (2004b) (see Table S1 in Appendix S2). Sliding-window analysis showed a low variability in this first part of the gene. Moreover, the original sequence data of Gysels *et al.* (2004b) often revealed low quality at the end of the sequence, in particular for the Mediterranean Sea samples. Because of conservative scoring, some mutations characteristic for the Mediterranean population were not detected by Gysels *et al.* (2004b). The high precision of the capillary DNA sequencer, the SeqScape software and the negative tests for detecting contamination guarantee the high quality of the sequences in the present study.

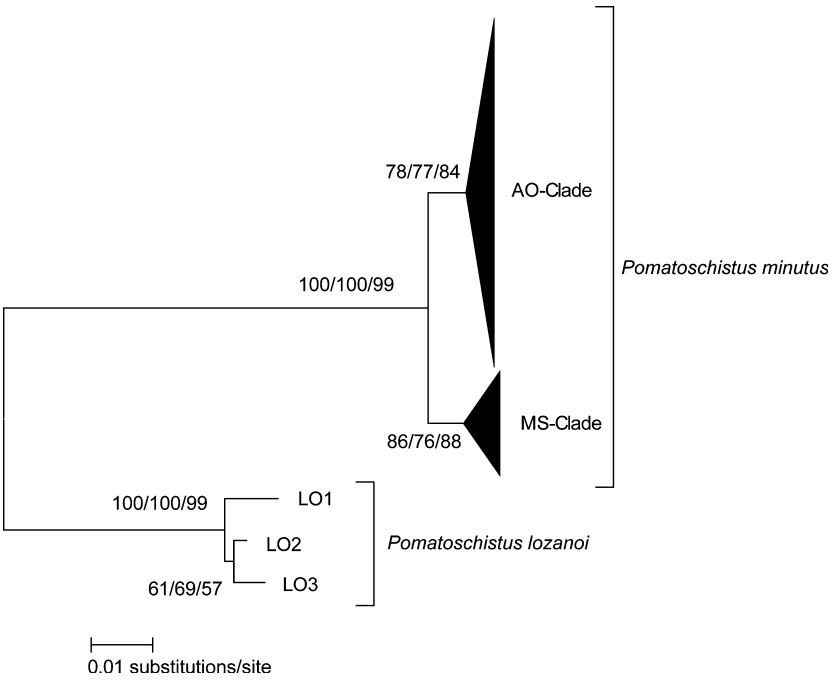

Figure 2 Maximum likelihood tree of all *Pomatoschistus minutus* cyt *b* haplotypes and three haplotypes of *Pomatoschistus lozanoi* as the genetic outgroup. Bootstrap values are indicated for statistically supported groupings (≥ 50%) for maximum likelihood (ML), maximum parsimony (MP) and neighbour joining (NJ) (ML/MP/NJ). Because of the high number of haplotypes, the clades for which no supported groupings were detected are compressed. MS Clade, Mediterranean Sea Clade; AO Clade, Atlantic Ocean Clade.

Phylogenetic and network analyses

Phylogenetic analyses of the data set, including all sequences of *P. minutus* and the outgroup taxon, revealed consistent results for the tree methods (Fig. 2). MP analysis generated a consensus tree of 339 steps (consistency index = 0.6012, retention index = 0.8746, rescaled consistency index = 0.5258). A similar tree was obtained with the NJ and ML algorithms ($-\ln L = 3219.065$). In all phylogenetic trees, two main distinct mitochondrial lineages were identified within the ingroup: the Mediterranean Clade (MS Clade), with 30 haplotypes from the sampling location in the Mediterranean Sea (VMS1 and VMS2), and the Atlantic Clade (AO Clade), with 100 haplotypes from the Iberian Peninsula, the Bay of Biscay, Irish Sea, North Sea, Skagerrak and Baltic Sea (Fig. 2; Table S2 in Appendix S2).

The network of the MS Clade based on cyt *b* displays a star-like pattern, with a high number of haplotypes compared with the number of sequenced individuals (Fig. 3). The network analysis of the AO Clade reveals two separate clusters of haplotypes, the Iberian Peninsular Group (IB Group), including all individuals caught in the Guadalquivir estuary (sample AAO) and in the Tagus estuary (sample PAO), and the North Atlantic Group (NA Group), comprising all sampling locations from the Bay of Biscay to the Baltic Sea (Fig. 4). The IB Group reveals a star-like pattern with one central haplotype (IB02), which was found with high frequency in both sampled locations (AAO and PAO). Network analysis for all haplotypes of the NA Group displays a pattern with several highly frequent haplotypes instead of a star-like pattern with one central haplotype. The network shows one main ancestral haplotype (haplotype NA01), which occurred in each North Atlantic location, and a few highly frequent haplotypes, for example NA28 and NA09, which are very common in the northern Baltic Sea and the southern North Sea, respectively. Many haplotypes are connected with single mutation steps to those ancestral haplotypes. Such radiations were found in each marine system (Fig. 4). In the network of the NA Group, the clear geographic association of the haplotypes suggests limited contemporary gene flow between marine systems. The network analyses gave congruent results between the different methods. The phylogenetic and network analyses conducted on the reconstructed data set of all control region (D-loop) sequences were comparable with the initial analysis of Stefanni & Thorley (2003).

For dating the various historical events, the molecular clock was calibrated by calculating the tMRCA of the two *Pomatoschistus* species and two Indo-Pacific gobies (*T. bifasciatus* and *T. obscurus*) with the cyt *b* locus. The tMRCA was estimated at 49.93, 24.95 and 16.63 Ma with the 2%, 4% and 6% molecular clocks, respectively. The tMRCA of the data set including all cyt *b* haplotypes of *P. minutus* and *P. lozanoi* was dated at *c.* 6.37, 3.23 and 2.14 Ma. Based on the D-loop data, the tMRCA of *P. minutus* and *P. lozanoi* was dated at *c.* 13.6, 6.8, 4.5 and 3.4 Ma with the 2%, 4%, 6% and 8% molecular clocks respectively. Because the Atlantic–Mediterranean and Indo-Pacific gobies diverged at least 14–15 Ma (McKay & Miller, 1997) and *P. minutus* and *P. lozanoi* diverged between 2.5 and 1.1 Ma (Wallis & Beardmore, 1984; Huyse *et al.*, 2004), a realistic and conservative molecular clock might be between 4% and maximally 6% for the cyt *b* locus and between 6% and 8% for the D-loop. It might be postulated that the fast generation time (1 or 2 years) of gobies and their small body size would compensate for the slower clock generally assumed for fish (Martin & Palumbi, 1993).

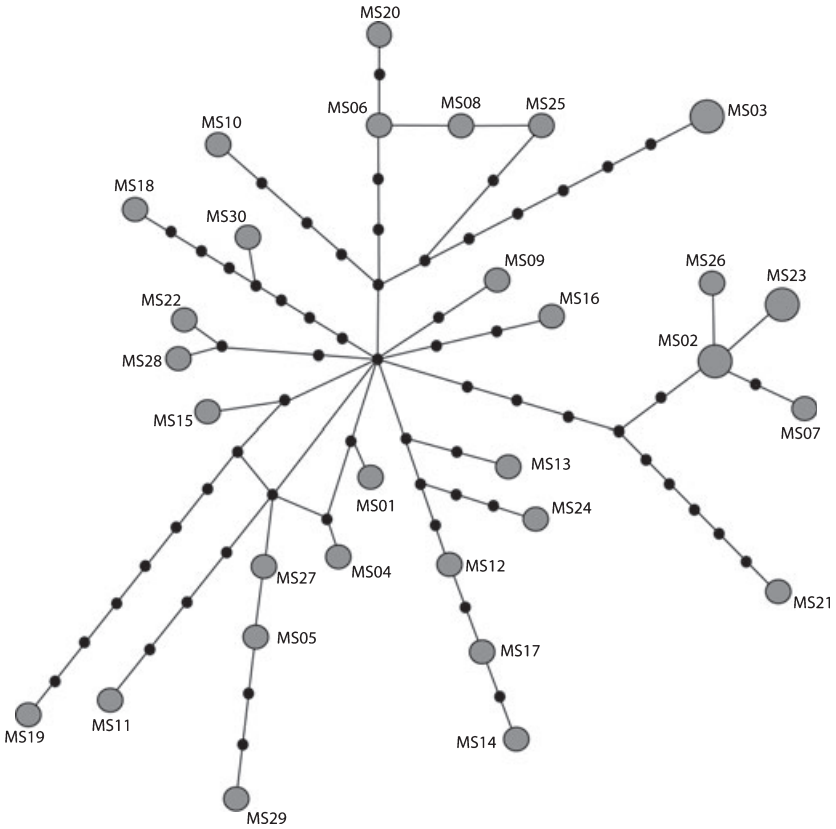

Figure 3 Statistical parsimony network of the cyt *b* haplotypes of the Mediterranean Sea (MS) Clade of *Pomatoschistus minutus*. The size of the circles is proportional to the number of sand gobies sharing that haplotype. The haplotypes are indicated by numbers as given in Table S1.

The tMRCA of all *P. minutus* cyt *b* sequences was estimated as between 0.827 and 0.551 Ma, and those of each clade separately between 0.464 and 0.265 Ma (both respectively with the 4% and 6% clock). This suggests that the divergence between the MS Clade and the AO Clade occurred in the first half of the Middle Pleistocene. The tMRCA of the NA Group and IB Group was estimated at 0.345–0.230 and 0.144–0.096 Ma, respectively (Table 2), also indicating a Middle Pleistocene divergence between the two lineages. Different coalescent models resulted in highly consistent estimates for the tMRCAs. The tMRCAs of the D-loop sequences of all genuine *P. minutus* samples, the MS Clade and the AO Clade were comparable with those estimated from the cyt *b* data (see Table S3 in Appendix S2).

Genetic diversity and population differentiation

For all cyt *b* haplotypes, the nucleotide diversity (π) and the haplotype diversity (h) were 0.01054 and 0.947, respectively. The genetic diversity was higher for the MS Clade ($\pi = 0.01069$ and $h = 0.994$) than for the AO Clade ($\pi = 0.00641$ and $h = 0.931$). The Irish Sea has low π- and h-values, whereas the Bay of Biscay, the southern Baltic, Skagerrak and the southern North seas have high π- and h-values. The Iberian Peninsula and the Northern Baltic have low π- but high h-values (Table 2). There was no significant decrease in nucleotide diversity northwards within the range.

The pairwise F_{ST}-values for the cyt *b* data are given in Table S4 in Appendix S2. The Mantel tests (Mantel, 1967) revealed significant correlations for all data and those of the North Atlantic (respectively, $r^2 = 0.6617$, $P < 0.05$; $r^2 = 0.4220$, $P < 0.05$). The NMDS analysis based on the cyt *b* data (see Fig. S1a,b in Appendix S3) showed different clusters corresponding to the *a priori* groups of *P. minutus* based on its geography (Table 1). The samples WCS, PBS and GOA clustered centrally in the NMDS. These three sampling locations of the Bay of Biscay, Skagerrak and southern Baltic Sea contain haplotypes occupying the entire network of the North Atlantic, in contrast to the other sample locations (BNS, RNS, WIS, BIS, TBS and HBS) (Fig. 4).

The genetic variability and differentiation based on the reconstructed D-loop haplotypes and allozymes were highly consistent with the results of Stefanni & Thorley (2003), Stefanni *et al.* (2003) and Gysels *et al.* (2004c). Additional NMDS analyses for the allozyme data revealed a clear genetic differentiation between Mediterranean Sea and North Atlantic populations, despite the high variability within the latter group (see Fig. S1c in Appendix S3).

Demographic analyses

The mismatch distribution of all sand goby haplotype sequences was clearly bimodal (see Fig. S2a in Appendix S3): one mode corresponded with the number of differences between the MS and AO clades, and the other mode with

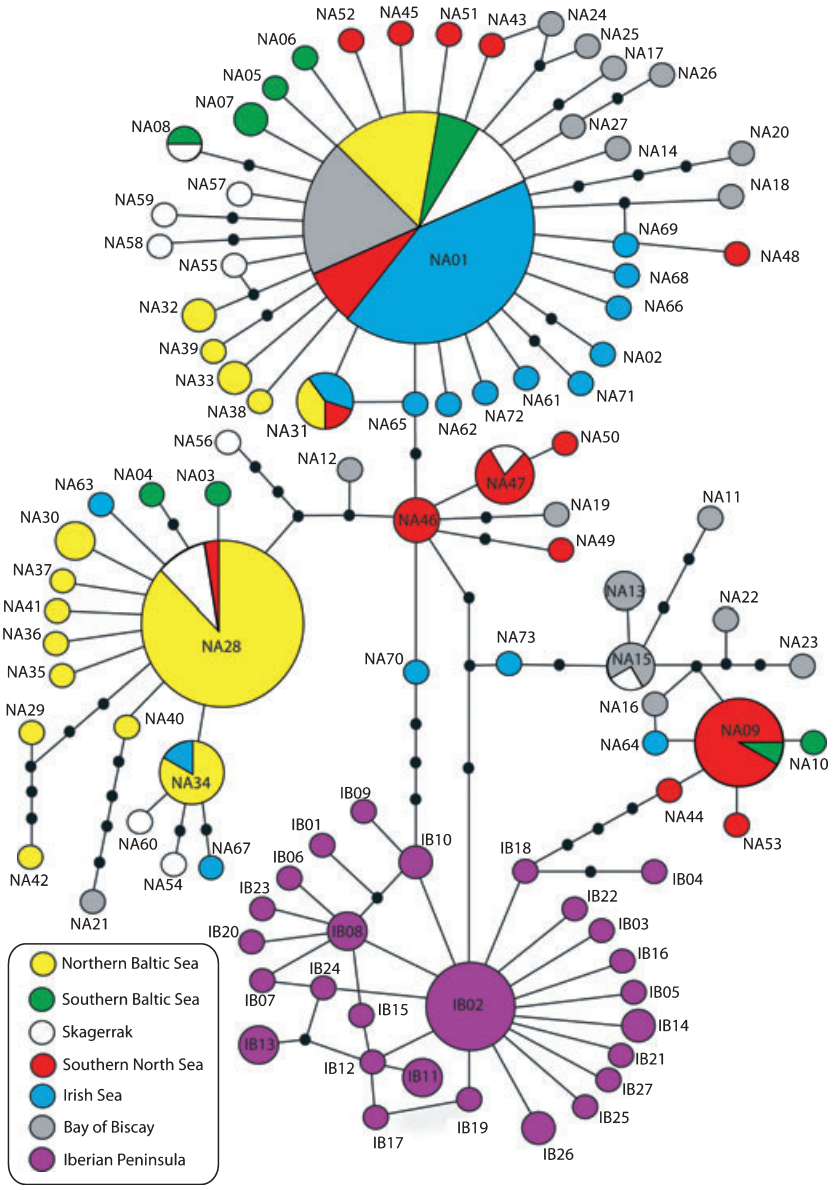

Figure 4 Statistical parsimony network of the cyt *b* haplotypes of the Atlantic Ocean (AO) Clade of *Pomatoschistus minutus*. The size of the circles is proportional to the number of sand gobies sharing that haplotype. The haplotypes are indicated by numbers as given in Table S1.

the number of differences among individuals within both lineages. As expected from the star-like statistical parsimony network (Fig. 3), the mismatch distribution for the MS lineage (see Fig. S2b in Appendix S3) fitted the predicted distribution under a model of sudden expansion. Because of this, and because of the negative neutrality tests ($P < 0.05$) (Table 3), the sudden expansion model could not be rejected for the MS Clade. Its time of expansion was estimated at 0.283–0.187 Ma (Middle Pleistocene) (Table 3). The mismatch analysis of the AO Clade showed a clear bimodal distribution (see Fig. S2c in Appendix S3), and therefore all haplotypes of the IB Group were separated from those of the NA Group. Despite the non-unimodal distribution of the mismatch analysis of the NA Group (see Fig. S2e in Appendix S3), the mismatch distributions of both groups were not significantly different from the predicted distribution under a model of sudden expansion. Moreover, the neutrality tests were significantly negative in both groups (Table 3). The expansions occurred 0.0785–0.0523 Ma (Late Pleistocene) and 0.186–0.0800 Ma (Middle and Late Pleistocene) for the IB Group and the NA Group, respectively. Only for the North Atlantic was a significant difference found between the demographic and the range expansion analyses (Table 3). The various mismatch analyses of the D-loop showed the same global pattern, despite the low number of Mediterranean haplotypes (Table S3 in Appendix S2; Fig. S3 in Appendix S3).

The Bayesian skyline plot (BSP) analysis was used to date shifts in population size of the *P. minutus* clades. The BSP of the MS Clade with the cyt *b* data shows two periods of population growth. The oldest expansion is dated before 110 ka, and a more recent, much smaller, expansion phase occurred between 75 and 30 ka (Fig. 5a). The BSP of the IB Group reveals a growth of population size before 25–17 ka (Fig. 5b). The plot of the NA Group based on the cyt *b* data

Table 2 Diversity indices and times to most recent common ancestor (tMRCAs) for the observed phylogeographic units and the various north-eastern Atlantic marine systems of *Pomatoschistus minutus* based on cyt *b* data.

	N	*R*	*h*	π	tMRCA (4%)	tMRCA (6%)
Total data set	263	130	0.947 (0.009)	0.01054 (0.00062)	0.827 (1.032–0.602)	0.551 (0.688–0.401)
MS-Clade	33	30	0.994 (0.009)	0.01069 (0.00067)	0.465 (0.609–0.306)	0.310 (0.406–0.204)
AO-Clade	230	100	0.931 (0.012)	0.00641 (0.00019)	0.398 (0.516–0.277)	0.265 (0.344–0.185)
IB-Group	45	27	0.925 (0.032)	0.00244 (0.00026)	0.144 (0.212–0.091)	0.096 (0.141–0.061)
NA-Group	185	73	0.897 (0.017)	0.00518 (0.00021)	0.345 (0.503–0.268)	0.230 (0.335–0.178)
Northern Baltic	53	16	0.800 (0.048)	0.00391 (0.00043)	–	–
Southern Baltic	12	9	0.939 (0.058)	0.00563 (0.00107)	–	–
Skagerrak	17	12	0.919 (0.057)	0.00555 (0.00080)	–	–
Southern North Sea	34	15	0.870 (0.044)	0.00504 (0.00033)	–	–
Irish Sea	39	17	0.687 (0.086)	0.00269 (0.00065)	–	–
Bay of Biscay	30	18	0.887 (0.052)	0.00587 (0.00068)	–	–

N, number of individuals per region; *R*, number of cyt *b* haplotypes observed; *h*, haplotype diversity; π, nucleotide diversity; tMRCA in Ma was estimated using molecular clocks of 4% and 6% sequence divergence per million years. The credibility intervals of tMRCA and the standard deviations of haplotype and nucleotide diversity values are given between brackets. MS-Clade, Mediterranean Sea Clade; AO-Clade, Atlantic Ocean Clade; IB-Group, Iberian Peninsula Group; NA-Group, North Atlantic Group.

Table 3 Results of the neutrality tests and mismatch analysis for cyt *b* sequences.

			Demographic mismatch distribution					Spatial mismatch distribution				
	D	F_S	τ	t_e (4%)	t_e (6%)	*P*(rag)	*P*(SDD)	τ	t_e (4%)	t_e (6%)	*P*(rag)	*P*(SDD)
MS-Clade	**−1.732**	**−20.404**	9.623	0.283	0.189	0.923	0.884	9.508	0.280	0.187	0.857	0.765
AO-Clade	**−1.914**	**−24.974**	7.246	0.213	0.142	0.591	0.322	5.846	0.172	0.115	0.943	0.304
IB-Group	**−1.781**	**−26.156**	2.668	0.079	0.052	0.067	0.103	2.669	0.079	0.052	0.059	0.103
NA-Group	**−2.026**	**−25.473**	6.324	0.186	0.124	0.805	0.673	4.074	0.120	0.080	0.933	0.565

D, Tajima's *D*-test and F_s, Fu's F_s-test (bold: *P*-value < 0.05); τ, time since expansion measured in mutational time units; t_e, absolute time (Ma) since expansion estimated with molecular clocks of 4% and 6% sequence divergence per million years; *P*(rag), *P*-value for the raggedness index; *P*(SDD), *P*-value for the sum of the squared deviations under the hypothesis of sudden expansion. MS-Clade, Mediterranean Sea Clade; AO-Clade, Atlantic Ocean Clade; IB-Group, Iberian Peninsula Group; NA-Group, North Atlantic Group.

shows a sharp increase in the effective number of individuals between 70 and 30 ka and between 105 and 40 ka for the 6% and 4% clocks, respectively (Fig. 5c). The results were robust, as different coalescent models in the BEAST analysis resulted in similar estimates. The results of the BSP analyses of the cyt *b* data were comparable with those based on the D-loop data (see Fig. S4 in Appendix S3).

DISCUSSION

Consequences of Pleistocene glaciations for the distribution of *Pomatoschistus minutus*

The phylogenetic analysis clearly shows two monophyletic clades within the species *P. minutus* (Fig. 2). The Mediterranean Sea Clade (MS Clade) is represented only by individuals from the Vaccarès lagoon (Gulf of Lion). The Atlantic Ocean Clade (AO Clade) comprises all populations spanning from the Iberian Peninsula to the Baltic Sea. Miller (1986) previously suggested this division between sand gobies from the Atlantic Ocean, *P. m. minutus*, and those from the Mediterranean and Black seas, *P. m. elongatus* (Canestrini, 1861), based on morphological and ecological differences. Earlier genetic studies based on mtDNA sequences and allozymes failed to prove a strong genetic divergence between the West Mediterranean and the Atlantic sand gobies owing to a limited resolution of the applied genetic markers (Stefanni *et al.*, 2003; Stefanni & Thorley, 2003; Gysels *et al.*, 2004b; Huyse *et al.*, 2004). However, the reanalyses of the D-loop and the allozyme data herein are not in conflict with the new cyt *b* data. Reciprocal monophyly was also shown for the D-loop marker, although with a much lower resolution. This lower resolution in comparison with the cyt *b* marker could be caused by homoplasy at hotspots for substitutions in the D-loop (Tamura & Nei, 1993; Wakeley, 1993). The signal of historical divergence was less pronounced for the allozyme data, which we suggest is because of the higher nuclear population size and the consequent lower genetic drift effects among nuclear markers compared with matrilinear DNA.

The divergence time of the two sand goby clades or evolutionary significant units (ESUs) was dated to the Middle Pleistocene (Table 2). This is consistent with many other

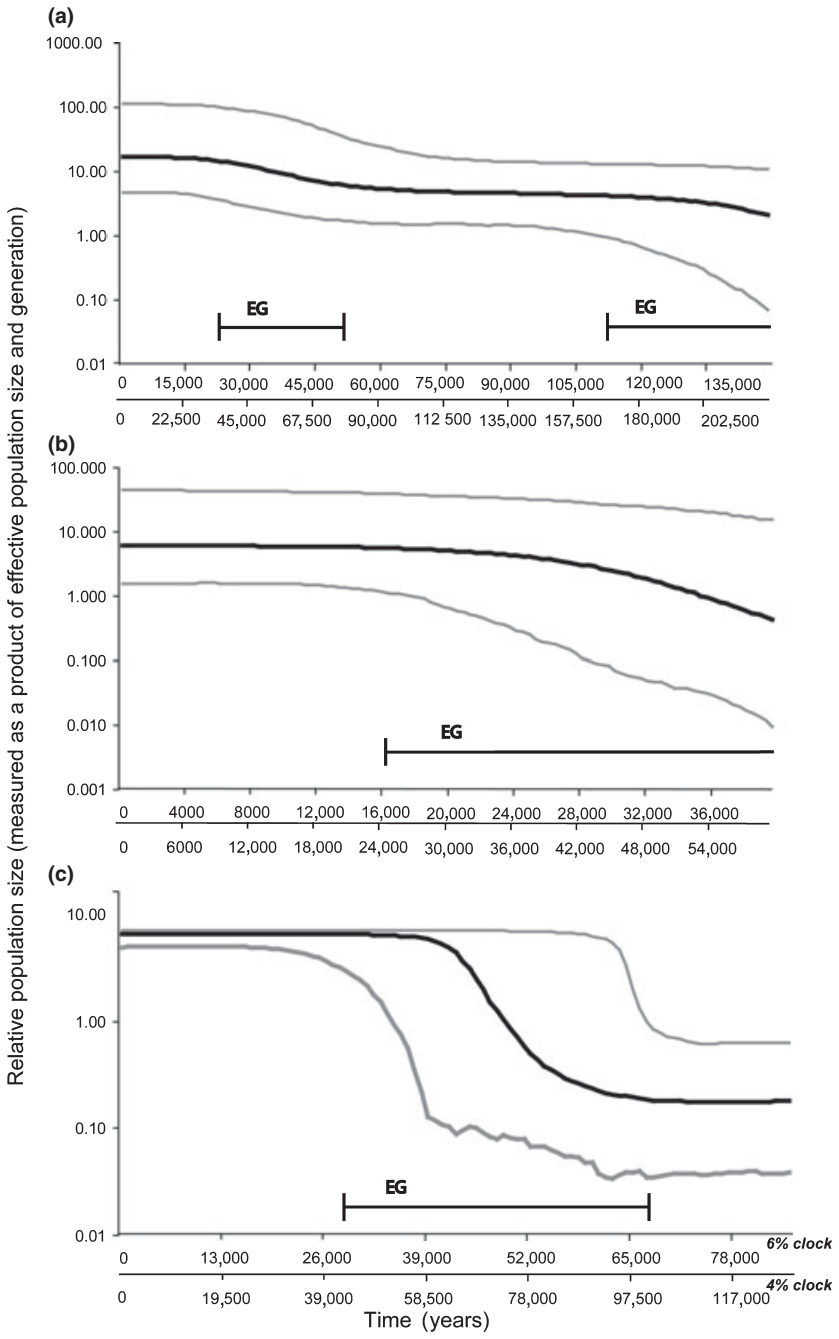

Figure 5 Bayesian skyline plots (BSPs) showing changes in population size through time: (a) Mediterranean Sea (MS) Clade; (b) Iberian Peninsula (IB) Group; (c) North Atlantic (NA) Group. These historical demographic trends of the cyt *b* lineages in *Pomatoschistus minutus* are represented by the 6% and 4% molecular clocks and by indication of the period of expansion growth (EG); the relative population size is measured as a product of effective population size and generation. *x*-axis: time (in years); the upper axis is the time measured with the 6% molecular clock and the lower axis shows the time with the 4% molecular clock; *y*-axis: relative population size (measured as a product of effective population size and generation). The black line represents the median estimate; upper and lower limits (95% HPD) are drawn in grey.

marine organisms for which reciprocal monophyly has been observed between the Mediterranean and Atlantic populations (reviewed in Patarnello *et al.*, 2007). This divergence is probably the result of geographic isolation caused by one of the Middle Pleistocene sea-level drops. Following this differentiation, subsequent gene flow was disrupted between the two clades. Contemporary gene flow is unlikely to occur owing to the discontinuous distribution of the sand goby between the Mediterranean and Atlantic basins (Fig. 1). Hypothesis 1, which concerns the recolonization of the Mediterranean Sea by Atlantic sand gobies, is thus rejected. The MS Clade has the highest diversity, although it has a small distribution range along the north-western Mediterranean Sea coasts and lagoons. Because the highest species diversity of the 'sand goby' group is observed within the Mediterranean Sea (Huyse *et al.*, 2004) and the cyt *b* haplotype diversity of *P. minutus* is the highest in this marine system, the origin of this species is expected to be in the Mediterranean Sea, as suggested by Gysels *et al.* (2004b). Similar to other estuarine systems, the Mediterranean lagoons play an important role in the life cycle of gobies (Bouchereau & Guelorget, 1998). These lagoons, however, suffer from increasing human pressure through overexploitation (gobies are a common by-catch) and reduced water quality (Bouchereau & Guelorget, 1998; Bouchereau, 2001). Moreover, Mediterranean

sand gobies are probably also directly threatened by the contemporary climate change (Philippart *et al.*, 2007, and references therein), as the water temperature approaches critically high values for sand gobies in the Mediterranean system (Pampoulie *et al.*, 1999). A further increase in water temperature could be pernicious as northward migration is excluded.

Two groups were identified within AO Clade: the Iberian (IB) Group and the North Atlantic (NA) Group. The IB Group contained the individuals collected along the coasts of the Iberian Peninsula. The NA Group comprised all populations spanning from the Bay of Biscay to the Baltic Sea (Fig. 4). This divergence is probably the result of population restriction within different refugia during glaciations in the Middle Pleistocene (Table 2). Hypothesis 2, which concerns the absence of population differentiation within the North Atlantic sand gobies, is therefore rejected. All haplotypes of the IB Group are unique for the Iberian Peninsula coast, suggesting a glacial refugium in this region for the sand goby. A growing body of studies on north-eastern Atlantic marine taxa [e.g. seagrasses (Coyer *et al.*, 2003; Olsen *et al.*, 2004), invertebrates (Zane *et al.*, 2000), rays (Chevolot *et al.*, 2006) and teleosts (Consuegra *et al.*, 2002; Cortey & García-Marín, 2002; Gysels *et al.*, 2004a)] suggests an Iberian refugium during the Pleistocene. In contrast to the case for most of the organisms with a presumed Iberian refugium, including the related common goby (*P. microps*) (Gysels *et al.*, 2004a), the Iberian sand gobies appear not to have contributed to the post-glacial distribution expansion in northern Europe.

The northward spatial expansion to the North Sea, Irish Sea and Baltic Sea appears to have involved only the NA Group of the sand goby. The cyt *b* haplotype network of this NA Group showed no regional clustering, but there were population-specific haplotypes scattered throughout the whole network and radiations in the Baltic Sea, Irish Sea, North Sea and Bay of Biscay around a variety of common haplotypes (Fig. 4). This complex pattern suggests different genetic bottlenecks and subsequent recolonization phases, driven by the periodic climate fluctuations during the Middle and Late Pleistocene. It is very difficult and rather speculative to reconstruct these various historical patterns by locating the northern peri-glacial refugia and recolonization routes, based on present-day genetic information alone. However, based on the high present-day genetic diversity and the presence of an assortment of unique haplotypes (Table 2; Fig. 4), the Bay of Biscay and the Hurd Deep in the English Channel appear more likely to have been glacial refugia than south-western Ireland (Table 2, Fig. 4). There is, however, no evidence that the sand goby was able to withstand the much lower temperatures that have been reconstructed for this region of the English Channel for the glacial maxima of the Late Pleistocene. Modelling reconstructions suggest that sea temperatures near the shelf in this region of the English Channel were 1–2°C in winter, rising to 5–6°C in summer during the LGM (Sarnthein, 2001). The difference in the haplotype network between southern and northern Baltic Sea samples (Fig. 4) suggests 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 haplotypes in the northern samples HBS and TBS (haplotypes NA01 and NA28) with unique derived haplotypes suggests that only a few individuals that were able to adapt to the severe abiotic conditions of the northern Baltic (Johannesson & André, 2006) founded the population. A better sampling strategy with a higher spatial resolution in the North Atlantic region and the use of a genetic marker with an adequate variability (e.g. microsatellites) to detect genetic differences are needed to locate the refugial areas more accurately and to understand the complex recolonization routes in the north-eastern Atlantic.

Consequences of the Pleistocene glaciations for the demography of *Pomatoschistus minutus*

The various demographic analyses show that the intra-assemblage genetic structure of *P. minutus* contains signatures of demographic expansion events (Fig. 5; Fig. S2 in Appendix S3; Table 3). For the MS Clade, the main demographic expansion period was consistently dated as having occurred during the Middle Pleistocene using the mismatch analysis. This strongly corroborates the scarce data on the demographic expansion events of other Mediterranean Sea organisms: for the sea urchin *Paracentrotus lividus* (Calderón *et al.*, 2008) and the tuna *Thunnus thynnus thynnus* (Carlsson *et al.*, 2004) the demographic expansion was estimated as occurring between 0.135 and 0.360 Ma. However, the BSP of the cyt *b* sequences also suggests an additional smaller expansion in the Mediterranean region in the past 50,000 years (Fig. 5a).

It is generally assumed that the current demography of Atlantic populations results mainly from the LGM, because this period probably blurred the signals of earlier demographic events. Until now, the demographic expansions of marine species along the north-eastern Atlantic coast have always been dated as pre-LGM events, between 1.7 and 0.11 Ma; for example algae (Provan *et al.*, 2005; Hoarau *et al.*, 2007; Calderón *et al.*, 2008), polychaetes (Jolly *et al.*, 2006), bivalves (Luttikhuizen *et al.*, 2003), urchins (Calderón *et al.*, 2008), crustaceans (Stamatis *et al.*, 2004), rays (Chevolot *et al.*, 2006) and teleosts (Gysels *et al.*, 2004a; Aboim *et al.*, 2005; Bremer *et al.*, 2005; Charrier *et al.*, 2006). For the Iberian sand gobies (IB Group), an expansion period was dated herein as occurring in the Eem interglacial (128–67 ka) or later (Table 3, Fig. 5b), depending on the molecular clock used. For the NA Group, the bimodal mismatch distribution of all North Atlantic haplotypes corresponds to the network analysis, which showed star-like patterns around several ancestral haplotypes (Fig. 4). This suggests lineage sorting followed by a recent expansion period. In the case of a non-unimodal pattern of the mismatch distribution, the BSP is a better method with which to date this expansion period than the mismatch analysis. The BSP ignores the pattern of the mismatch distribution and is based on coalescent events (Drummond *et al.*, 2005). Using BSP, the expansion event for the NA Group was estimated to have

occurred during the Eem interglacial (Fig. 5c). The Eem interglacial followed the Warthe/Saale glaciation (180–128 ka), which is thought to have had more widespread effects on the distribution and demography of organisms than the LGM (Kellaway *et al.*, 1975). This indicates that hypothesis 3 of the present study is not confirmed, and that the LGM did not substantially blur the signals of earlier demographic events. A similar conclusion was also reached in analyses of the demography of marine taxa in the north-eastern Atlantic (Hoarau *et al.*, 2007).

ACKNOWLEDGEMENTS

The authors wish to thank the following persons who kindly provided the samples used in this study: P. Drake (CSIC, Spain), M. Järvi-Laturi (University of Helsinki, Finland), K. Lindström (University of Helsinki, Finland), K. Waligóra-Borek (University of Gdańsk, Poland), L. Kvarnemo (Göteborgs Universitet, Sweden), D. Rodgers (Environment & Heritage Service, UK), I. McCarthy (University of Wales, Bangor, UK), M. Lepage (Ifremer, France), M. Freitas (University of Algarve, Portugal) and A. Crivelli (Station Biologique de la Tour du Valat, France). We are also grateful to T. Huyse and three anonymous referees, whose suggestions greatly improved this manuscript. This work was supported by a PhD fellowship from the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen). This study is part of the WESTBANKS project financed by the Belgian Federal Office for Scientific, Technical and Cultural Affairs (contract number SD/BN/01A).

REFERENCES

Aboim, M.A., Menezes, G.M., Schlitt, T. & Rogers, A.D. (2005) Genetic structure and history of populations of the deep-sea fish *Helicolenus dactylopterus* (Delaroche, 1809) inferred from mtDNA sequence analysis. *Molecular Ecology*, **14,** 1343–1354.

Avise, J.C. (2000) *Phylogeography: the history and formation of species*. Harvard University Press, Cambridge, MA.

Bernatchez, L. & Wilson, C.C. (1998) Comparative phylogeography of Nearctic and Palearctic fishes. *Molecular Ecology*, **7,** 431–452.

Bouchereau, J.L. (2001) Le gobie buhotte *Pomatoschistus minutus* (Pallas, 1770). *Atlas des poissons d'eau douce de France* (ed. by P. Keith and J. Allardi), pp. 336–337. Publications Scientifiques du Muséum National d'Histoire Naturelle, Paris.

Bouchereau, J.L. & Guelorget, O. (1998) Comparison of three Gobiidae (Teleostei) life history strategies over their geographical range. *Oceanologica Acta*, **21,** 503–517.

Bowen, B.W., Bass, A.L., Rocha, L.A., Grant, W.S. & Robertson, D.R. (2001) Phylogeography of the trumpetfishes (*Aulostomus*): ring species complex on a global scale. *Evolution*, **55,** 1029–1039.

Bremer, J.R.A., Viñas, J., Mejuto, J., Ely, B. & Pla, C. (2005) Comparative phylogeography of Atlantic bluefin tuna and swordfish: the combined effects of vicariance, secondary contact, introgression, and population expansion on the regional phylogenies of two highly migratory pelagic fishes. *Molecular Phylogenetics and Evolution*, **36,** 169–187.

Calderón, I., Giribet, G. & Turon, X. (2008) Two markers and one history: phylogeography of the edible common sea urchin *Paracentrotus lividus* in the Lusitanian region. *Marine Biology*, **154,** 137–151.

Carlsson, J., McDowell, J.R., Diaz-Jaimes, P., Carlsson, J.E.L., Boles, S.B., Gold, J.R. & Graves, J.E. (2004) Microsatellite and mitochondrial DNA analyses of Atlantic bluefin tuna (*Thunnus thynnus thynnus*) population structure in the Mediterranean Sea. *Molecular Ecology*, **13,** 3345–3356.

Charrier, G., Chenel, T., Durand, J.D., Girard, M., Quiniou, L. & Laroche, J. (2006) Discrepancies in phylogeographical patterns of two European anglerfishes (*Lophius budegassa* and *Lophius piscatorius*). *Molecular Phylogenetics and Evolution*, **38,** 742–754.

Chevolot, M., Hoarau, G., Rijnsdorp, A.D., Stam, W.T. & Olsen, J.L. (2006) Phylogeography and population structure of thornback rays (*Raja clavata* L., Rajidae). *Molecular Ecology*, **15,** 3693–3705.

Clement, M., Posada, D. & Crandall, K.A. (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology*, **9,** 1657–1659.

Consuegra, S., De Leaniz, C.G., Serdio, A., Morales, M.G., Straus, L.G., Knox, D. & Verspoor, E. (2002) Mitochondrial DNA variation in Pleistocene and modern Atlantic salmon from the Iberian glacial refugium. *Molecular Ecology*, **11,** 2037–2048.

Cortey, M. & García-Marín, J.L. (2002) Evidence for phylogeographically informative sequence variation in the mitochondrial control region of Atlantic brown trout. *Journal of Fish Biology*, **60,** 1058–1063.

Coyer, J.A., Peters, A.F., Stam, W.T. & Olsen, J.L. (2003) Post-ice age recolonization and differentiation of *Fucus serratus* L. (Phaeophyceae; Fucaceae) populations in Northern Europe. *Molecular Ecology*, **12,** 1817–1829.

Domingues, V.S., Bucciarelli, G., Almada, V.C. & Bernardi, G. (2005) Historical colonization and demography of the Mediterranean damselfish, *Chromis chromis*. *Molecular Ecology*, **14,** 4051–4063.

Domingues, V.S., Faria, C., Stefanni, S., Santos, R.S., Brito, A. & Almada, V.C. (2007) Genetic divergence in the Atlantic–Mediterranean Montagu's blenny, *Coryphoblennius galerita* (Linnaeus 1758) revealed by molecular and morphological characters. *Molecular Ecology*, **16,** 3592–3605.

Drummond, A.J. & Rambaut, A. (2006) *BEAST*, 1.4 edn. Available at: http://beast.bio.ed.ac.uk (last accessed 27 October 2008).

Drummond, A.J., Rambaut, A., Shapiro, B. & Pybus, O.G. (2005) Bayesian coalescent inference of past population dynamics from molecular sequences. *Molecular Biology and Evolution*, **22,** 1185–1192.

Ehrenberg, S.Z., Hansson, S. & Elmgren, R. (2005) Sublittoral abundance and food consumption of Baltic gobies. *Journal of Fish Biology*, **67,** 1083–1093.

Excoffier, L. (2004) Patterns of DNA sequence diversity and genetic structure after a range expansion: lessons from the infinite-island model. *Molecular Ecology*, **13,** 853–864.

Excoffier, L., Laval, G. & Schneider, S. (2005) Arlequin (version 3.0): an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online*, **1,** 47–50.

Fu, Y.X. (1997) Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics*, **147,** 915–925.

Guelinckx, J., Maes, J., Geysen, B. & Ollevier, F. (2008) Estuarine recruitment of a marine goby reconstructed with an isotopic clock. *Oecologia*, **157,** 41–52.

Guindon, S. & Gascuel, O. (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology*, **52,** 696–704.

Guindon, S., Lethiec, F., Duroux, P. & Gascuel, O. (2005) PHYML Online – a web server for fast maximum likelihood-based phylogenetic inference. *Nucleic Acids Research*, **33,** W557–W559.

Gysels, E.S. (2003) *Genetic structure and evolutionary patterns of* Pomatoschistus *gobies on the European continental shelf and in the Mediterranean Sea*. PhD Thesis, Katholieke Universiteit Leuven, Leuven, Belgium.

Gysels, E.S., Hellemans, B., Pampoulie, C. & Volckaert, F.A.M. (2004a) Phylogeography of the common goby, *Pomatoschistus microps*, with particular emphasis on the colonization of the Mediterranean and the North Sea. *Molecular Ecology*, **13,** 403–417.

Gysels, E.S., Hellemans, B., Patarnello, T. & Volckaert, F.A.M. (2004b) Current and historic gene flow of the sand goby *Pomatoschistus minutus* on the European Continental Shelf and in the Mediterranean Sea. *Biological Journal of the Linnean Society*, **83,** 561–576.

Gysels, E.S., Leentjes, V. & Volckaert, F.A.M. (2004c) Small-scale clinal variation, genetic diversity and environmental heterogeneity in the marine gobies *Pomatoschistus minutus* and *P. lozanoi* (Gobiidae, Teleostei). *Heredity*, **93,** 208–214.

Hamerlynck, O. (1990) The identification of *Pomatoschistus minutus* (Pallas) and *Pomatoschistus lozanoi* (Debuen) (Pisces, Gobiidae). *Journal of Fish Biology*, **37,** 723–728.

Harpending, H.C. (1994) Signature of ancient population growth in a low-resolution mitochondrial-DNA mismatch distribution. *Human Biology*, **66,** 591–600.

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

Hewitt, G.M. (2004) Genetic consequences of climatic oscillations in the Quaternary. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **359,** 183–195.

Hoarau, G., Coyer, J.A., Veldsink, J.H., Stam, W.T. & Olsen, J.L. (2007) Glacial refugia and recolonization pathways in the brown seaweed *Fucus serratus*. *Molecular Ecology*, **16,** 3606–3616.

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

Johannesson, K. & André, C. (2006) Life on the margin: genetic isolation and diversity loss in a peripheral marine ecosystem, the Baltic Sea. *Molecular Ecology*, **15,** 2013–2029.

Jolly, M.T., Viard, F., Gentil, F., Thiebaut, E. & Jollivet, D. (2006) Comparative phylogeography of two coastal polychaete tubeworms in the Northeast Atlantic supports shared history and vicariant events. *Molecular Ecology*, **15,** 1841–1855.

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

Keith, P., Galewski, T., Cattaneo-Berrebi, G., Hoareau, T. & Berrebi, P. (2005) Ubiquity of *Sicyopterus lagocephalus* (Teleostei: Gobioidei) and phylogeography of the genus *Sicyopterus* in the Indo-Pacific area inferred from mitochondrial cytochrome *b* gene. *Molecular Phylogenetics and Evolution*, **37,** 721–732.

Kellaway, G.A., Redding, J.H., Shephardthorn, E.R. & Destombes, J.P. (1975) The quaternary history of the English Channel. *Philosophical Transactions of the Royal Society of London Series A, Mathematical Physical and Engineering Sciences*, **279,** 189–218.

Larmuseau, M.H.D., Guelinckx, J., Hellemans, B., Van Houdt, J.K.J. & Volckaert, F.A.M. (2008) Fast PCR-RFLP method facilitates identification of *Pomatoschistus* species in the North Atlantic. *Journal of Applied Ichthyology*, **24,** 342–344.

Luttikhuizen, P.C., Drent, J. & Baker, A.J. (2003) Disjunct distribution of highly diverged mitochondrial lineage clade and population subdivision in a marine bivalve with pelagic larval dispersal. *Molecular Ecology*, **12,** 2215–2229.

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

Martin, A.P. & Palumbi, S.R. (1993) Body size, metabolic rate, generation time, and the molecular clock. *Proceedings of the National Academy of Sciences USA*, **90,** 4087–4091.

McKay, S.I. & Miller, P.J. (1997) The affinities of European sand gobies (Teleostei: Gobiidae). *Journal of Natural History*, **31,** 1457–1482.

Miller, P.J. (1986) Gobiidae. *Fishes of the north-eastern Atlantic and Mediterranean* (ed. by P.J.P. Whitehead, M.-L. Bauchot, J.C. Hureau, J. Nielsen and E. Tortononese), pp. 1019–1085. UNESCO, Paris.

Nesbø, C.L., Rueness, E.K., Iversen, S.A., Skagen, D.W. & Jakobsen, K.S. (2000) Phylogeography and population history of Atlantic mackerel (*Scomber scombrus* L.): a genealogical approach reveals genetic structuring among the

eastern Atlantic stocks. *Proceedings of the Royal Society B: Biological Sciences*, **267,** 281–292.

Olsen, J.L., Stam, W.T., Coyer, J.A., Reusch, T.B.H., Billingham, M., Boström, C., Calvert, E., Christie, H., Granger, S., La Lumière, R., Milchakova, N., Oudot-Le Secq, M.P., Procaccini, G., Sanjabi, B., Serrão, E., Veldsink, J., Widdicombe, S. & Wyllie-Echeverria, S. (2004) North Atlantic phylogeography and large-scale population differentiation of the seagrass *Zostera marina* L. *Molecular Ecology*, **13,** 1923–1941.

Palumbi, S.R. (1994) Genetic divergence, reproductive isolation, and marine speciation. *Annual Review of Ecology and Systematics*, **25,** 547–572.

Pampoulie, C., Rosecchi, E., Bouchereau, J.L. & Crivelli, A.J. (1999) Life history traits of *Pomatoschistus minutus* in the Rhone Delta, France. *Journal of Fish Biology*, **55,** 892–896.

Pasquaud, S., Girardin, M. & Elie, P. (2004) Diet of gobies of the genus *Pomatoschistus* (*P. microps* and *P. minutus*), in the Gironde estuary (France). *Cybium*, **28,** 99–106.

Patarnello, T., Volckaert, F.A.M. & Castilho, R. (2007) Pillars of Hercules: is the Atlantic–Mediterranean transition a phylogeographical break? *Molecular Ecology*, **16,** 4426–4444.

Philippart, C.J.M., Anadón, R., Danovaro, R., Dippner, R., Drinkwater, K.F., Hawkins, S.J., Oguz, T. & Reid, P.C. (2007) *Climate change impacts on the European marine and coastal environment – an ecosystem approach*. Position Paper of the Marine Board of the European Science Foundation, Strasbourg.

Posada, D. & Crandall, K.A. (1998) MODELTEST: testing the model of DNA substitution. *Bioinformatics*, **14,** 817–818.

Provan, J., Wattier, R.A. & Maggs, C.A. (2005) Phylogeographic analysis of the red seaweed *Palmaria palmata* reveals a Pleistocene marine glacial refugium in the English Channel. *Molecular Ecology*, **14,** 793–803.

Rambaut, A. & Drummond, A.J. (2007) *Tracer*, 1.3 edn. Available at: http://beast.bio.ed.ac.uk/Tracer (last accessed 27 October 2008).

Rozas, J., Sánchez-DelBarrio, J.C., Messeguer, X. & Rozas, R. (2003) DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics*, **19,** 2496–2497.

Sa-Pinto, A., Branco, M., Harris, D.J. & Alexandrino, P. (2005) Phylogeny and phylogeography of the genus *Patella* based on mitochondrial DNA sequence data. *Journal of Experimental Marine Biology and Ecology*, **325,** 95–110.

Sarnthein, M. (2001) Der Atlantic im Letzen Glazialen Maximum. *Nova Acta Leopoldina*, **88,** 35–43.

Singer, A., Kvarnemo, C., Lindström, K. & Svensson, O. (2006) Genetic mating patterns studied in pools with manipulated nest site availability in two populations of *Pomatoschistus minutus*. *Journal of Evolutionary Biology*, **19,** 1641–1650.

Stamatis, C., Triantafyllidis, A., Moutou, K.A. & Mamuris, Z. (2004) Mitochondrial DNA variation in northeast Atlantic and Mediterranean populations of Norway lobster, *Nephrops norvegicus*. *Molecular Ecology*, **13,** 1377–1390.

Stefanni, S. & Thorley, J.L. (2003) Mitochondrial DNA phylogeography reveals the existence of an Evolutionarily Significant Unit of the sand goby *Pomatoschistus minutus* in the Adriatic (Eastern Mediterranean). *Molecular Phylogenetics and Evolution*, **28,** 601–609.

Stefanni, S., Gysels, E.S., Volckaert, F.A.M. & Miller, P.J. (2003) Allozyme variation and genetic divergence in the sand goby, *Pomatoschistus minutus* (Teleostei: Gobiidae). *Journal of the Marine Biological Association of the United Kingdom*, **83,** 1143–1149.

Swofford, D.L. (2002) *PAUP*. Phylogenetic analysis using parsimony (*and other methods)*. Sinauer Associates, Sunderland, MA.

Taberlet, P., Fumagalli, L., Wust-Saucy, A.G. & Cosson, J.F. (1998) Comparative phylogeography and postglacial colonization routes in Europe. *Molecular Ecology*, **7,** 453–464.

Tajima, F. (1989) The effect of change in population-size on DNA polymorphism. *Genetics*, **123,** 597–601.

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

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, **24,** 1596–1599.

Wakeley, J. (1993) Substitution rate variation among sites in hypervariable region-1 of human mitochondrial-DNA. *Journal of Molecular Evolution*, **37,** 613–623.

Wallis, G.P. & Beardmore, J.A. (1984) An electrophoretic study of the systematic relationships of some closely related goby species (Pisces, Gobiidae). *Biological Journal of the Linnean Society*, **22,** 107–123.

Weinstock, J., Willerslev, E., Sher, A., Tong, W.F., Ho, S.Y.W., Rubenstein, D., Storer, J., Burns, J., Martin, L., Bravi, C., Prieto, A., Froese, D., Scott, E., Lai, X.L. & Cooper, A. (2005) Evolution, systematics, and phylogeography of Pleistocene horses in the New World: a molecular perspective. *PLoS Biology*, **3,** 1373–1379.

Wilson, A.B. (2006) Genetic signature of recent glaciation on populations of a near-shore marine fish species (*Syngnathus leptorhynchus*). *Molecular Ecology*, **15,** 1857–1871.

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

Zhang, D.X. & Hewitt, G.M. (1996) Nuclear integrations: challenges for mitochondrial DNA markers. *Trends in Ecology and Evolution*, **11,** 247–251.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Additional information on the mtDNA amplification and sequencing, and on the analysis of the genetic diversity and population differentiation.
Appendix S2 Supplementary tables.
Appendix S3 Supplementary figures.

Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

BIOSKETCH

Maarten H. D. Larmuseau is a PhD student at the Laboratory of Animal Diversity and Systematics of the Katholieke Universiteit Leuven, Belgium. He is interested in the relationship between phylogeography and local adaptation in marine and freshwater organisms.

Editor: David Bellwood