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ORIGINAL PAPER

Phylogeography and demographic history of *Atherina presbyter* (Pisces: Atherinidae) in the North-eastern Atlantic based on mitochondrial DNA

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Abstract A fragment of the mitochondrial control region was used to assess phylogeographic patterns and historical demography of the sand-smelt *Atherina presbyter* in the North-eastern Atlantic, covering its geographical range. A striking result is the highly marked differentiation between the Canary Islands population and western European ones. A genetic structure among European populations of *A. presbyter* was revealed, with a pattern of isolation-by-distance or a gradient effect at a scale of hundreds kilometres, an uncommon pattern likely related to the biological and life-history traits of the sand-smelt. The northern European populations present a much lower genetic diversity when compared to southern populations, which is consistent with a recent colonization from southern populations. The results showed signs of Pleistocene signatures, with the population age estimates for the European populations being clearly older than the Last Glacial Maximum (18,000 years bp). Nevertheless, paleotemperature reconstructions show that the sand-smelt could not have inhabited the western European shores during the last glacial phase.

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Introduction

Over the past 20 years, several studies of marine fish population genetics have contributed to a better understanding of population structure in the North-eastern Atlantic (e.g. Almada et al. 2008; Blanquer et al. 1992; Debes et al. 2008; Domingues et al. 2007a, 2008; Magoulas et al. 1996; Mork et al. 1985; Nesbo et al. 2000; Stefanni et al. 2007). Much emphasis has been placed on the Atlantic-Mediterranean transition (see Patarnello et al. 2007 for a review), but the effectiveness of this barrier varies greatly when different species are analyzed (e.g. Bremer et al. 2005; Charrier et al. 2006).

Pleistocene glaciations caused drastic changes in sea surface temperature in the western European Atlantic coast (Calvo et al. 2001; Climap 1984). During the last glacial maximum (LGM, *ca.* 18,000 years bp) (Climap 1984) the polar front was located at the western Portuguese coast (Alveirinho-Dias et al. 1997). Therefore, along the Atlantic shores of western Europe, the recovery and northwards expansion of populations of warm-water fish must have

occurred during the current interglacial period (e.g. Debes et al. 2008). In agreement with this climatic history, the majority of the studies found evidence of Pleistocene bottlenecks that were likely caused by periods of strong ocean cooling (e.g. Consuegra et al. 2002; Domingues et al. 2006, 2007b, 2008; Mäkinen and Merilä 2008). However, populations of different species vary in their phylogeographic patterns and we are still far from a comprehensive picture of the evolution of the western European marine icthyofauna during the Pleistocene. Several studies of fish with high dispersal capabilities either due to a long larval period and/or high adult mobility reported the absence of population differentiation along the west European shore (e.g. Almada et al. 2008; Daemen et al. 2001; Debes et al. 2008; Hickerson and Cunningham 2006). In contrast, several other species, namely of estuarine fish, display differences among populations in the same geographical area. Besides the dispersal capability, the differences observed can also be ascribed to factors such as the environmental preferences of a species (which determine what areas can have acted as glacial refugia) (e.g. Consuegra et al. 2002; Domingues et al. 2008) and the numbers of individuals involved in the process of recolonization of formerly glaciated areas (resulting in the different levels of diversity observed) (e.g. Gysels et al. 2004).

The present study focuses on the broad range phylogeography of the sand-smelt *Atherina presbyter* (Cuvier 1829), a species with well known ecological features, reduced dispersal capability and strictly tied to coastal environments. The sand-smelt is one of the two species representing the family Atherinidae in west Europe. It is an inshore marine fish, occasionally entering coastal lagoons and estuaries. Its distribution ranges from the British Isles and southern North Sea to the Canary Islands, Mauritania and Cape Verde (Quignard and Pras 1986), and it has also been reported from the Azores archipelago (Santos et al. 1997). Although very sporadic, records of this species have also been reported from the western Mediterranean (Kiener and Spillmann 1969; Quignard and Pras 1986). The spawning of *A. presbyter* occurs in very shallow waters and the demersal eggs are attached to vegetation. At a temperature of 15°C the larvae hatch with 6.7–7.5 mm total length after 15–16 days (Bamber et al. 1985). Hatching larvae are well developed and ready to start exogenous feeding. This means that the larval stage is likely very short, limiting passive dispersal. Although the adults of sand-smelt are active swimmers in the water column, their migratory movements are probably difficult along exposed shores.

Francisco et al. (2006a) showed that along the Portuguese west coast fish collected in sites separated by 250 Km did not present signs of population differentiation. Meanwhile, a large number of sequences (128) of *A. presbyter* were obtained covering a much broader area in west Europe and the Canaries as well as some samples from the Azores (Astolfi et al. 2005; Francisco et al. 2008). These data were collected by the authors in the framework of a study on the phylogeny of the North-eastern Atlantic and Mediterranean species of the genus *Atherina*. Thus, they were not analyzed to answer phylogeographic and historical demographic questions. In this study we took advantage of the available data re-analyzing them to explore the phylogeography of the sand-smelt at two distinct scales: one with a magnitude of hundreds of kilometres along the Portuguese coast, and another with a magnitude of thousands of kilometres comparing these locations with populations located at southern and northern extremes of the species range. Mitochondrial DNA control region sequences were used to address the following questions: (1) What is the degree of genetic differentiation of *A. presbyter* along its distributional range?; (2) Is there evidence of post-glacial expansion for this species?; (3) Where was the location of the glacial refugium (refugia) where the sand-smelt survived during the Pleistocene glaciations?

Materials and methods

A total of 144 fish were sampled in the North-eastern Atlantic, distributed by six localities: one in the Wadden Sea (Germany), four in Portugal (Aveiro, Fonte-da-Telha, Arade and Castro Marim) and one in Tenerife in the Canary Islands (Spain) (Fig. 1, Table S1 in supplementary material). Some additional samples were added in order to cover a wider geographical range: Swansea (51°36′N, 3°56′W) (N = 3), Arcachon (44°65′N, 1°17′W) (N = 2), Cabo-do-Mundo (41°11′N, 8°42′W) (N = 8), Lagoa-de-Albufeira (38°30′N, 9°10′W) (N = 7), Amoreira (37°22′N, 8°47′W) (N = 8) and Santa Maria in the Azores (36°58′N, 25°10′W) (N = 6) (Fig. 1).

Details on DNA extraction, PCR and sequencing can be found in Francisco et al. (2008). All sequences were aligned using Geneious 3.04 (Drummond et al. 2006).

A maximum parsimony (MP) analysis was performed with heuristic search using PAUP* 4.0b (Swofford 2000). A sequence of *Atherina boyeri* (accession number EF611669) was used as outgroup and two sequences of the sister species *Atherina hepsetus* were included in the ingroup (accession numbers DQ102843 and AY749100). Robustness of the inferred MP tree was tested by using 100 bootstrap replicates (Felsenstein 1985).

Genealogical relationships among mtDNA sequences were examined with the software TCS 1.21 (Clement et al. 2000) to construct a haplotype network with the parsimony method of Templeton et al. (1992). The network was based on a reduced dataset containing only the transversions.

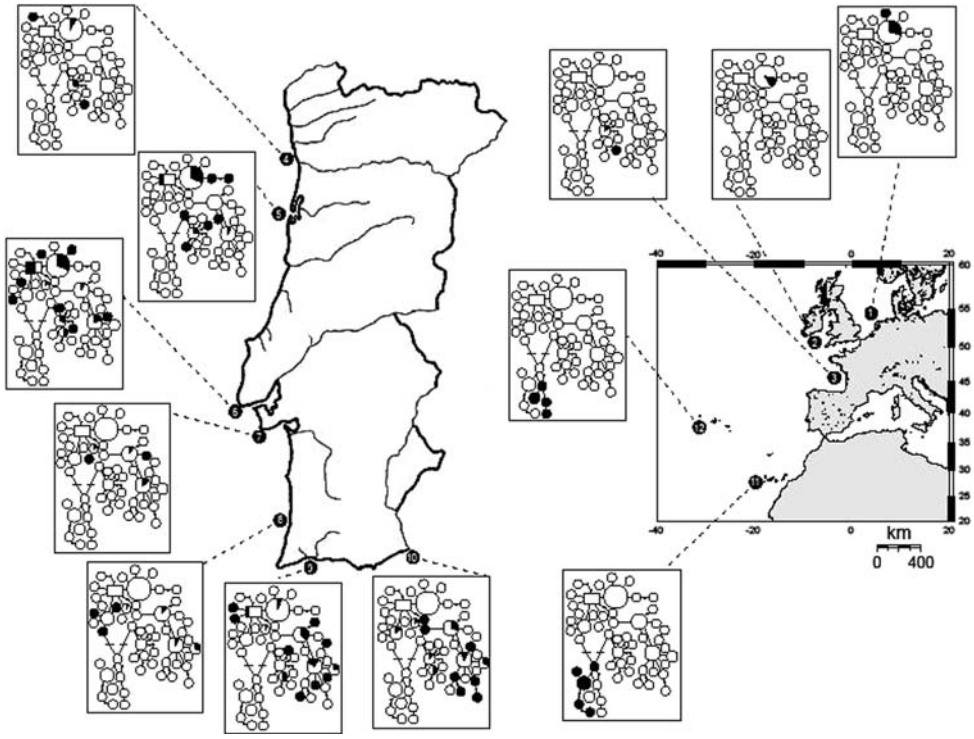

Fig. 1 Location of the 12 sampling sites included in this study: (*1*) Wadden Sea, (*2*) Swansea, (*3*) Arcachon, (*4*) Cabo do Mundo, (*5*) Aveiro, (*6*) Fonte-da-Telha, (*7*) Lagoa de Albufeira, (*8*) Amoreira, (*9*) Arade, (*10*) Castro Marim, (*11*) Tenerife and (*12*) Santa Maria. Statistical parsimony network constructed with the reduced dataset (55 haplotypes, only transversions) for the mtDNA CR of *Atherina presbyter*. The haplotype with the highest outgroup probability is displayed as a square, other haplotypes as *circles*. The size of the *squares* or *circles* is proportional to the haplotype frequency. For each sampling location, *black symbols* represent the presence of the haplotype and *white symbols* its absence

Modeltest 3.7 (Posada and Crandal 1998) was used to identify the appropriated nucleotide substitution model. The selected model was HKY (Hasegawa et al. 1985), with the following parameters: proportion of invariable sites (i) = 0.6639, gamma distribution shape parameter (α) = 0.6928 and transition/transversion rate (ti/tv) = 4.1533.

Bayesian analysis was performed using MCMC as implemented in Mr.Bayes 3.1 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) with four independent runs of four chains of 10,000,000 generations each. Topologies were sampled every 1,000 generations, and a majority-rule consensus tree was estimated after discarding the first 4,000 sampled generations. Finally, phylogenies were edited and displayed using MrEnt v.2. (Zuccon and Zuccon 2008).

Arlequin 3.1 (Excoffier et al. 2005) was used to estimate levels of genetic diversity: percentage of private and shared haplotypes, number of polymorphic sites (S), haplotype diversity (H, Nei 1987) and nucleotide diversity (π, Nei 1987). As the HKY model is not implemented in Arlequin, the genetic pairwise distances between haplotypes were estimated with the Tamura–Nei (TrN) model (Tamura and Nei 1993), using the same parameters for ti/tv rate and α. The Tamura–Nei method was also used to compute the average number of pairwise differences among groups of samples of different localities, after correcting for within group differences as implemented in Arlequin. Population structure was estimated by analysis of molecular variance (AMOVA; Excoffier et al. 1992) and pairwise Φ_{st}. Gene flow (Φ_{ST} and N_m) was also estimated. The Mantel test (Mantel 1967; Smouse et al. 1986) was used to compute correlation between genetic and geographic distances. The significance of the correlation was assessed by performing 10,000 permutations.

The spatial analysis of molecular variance (SAMOVA 1.0) (Dupanloup et al. 2002) was used to identify groups of sampling locations, which are geographically and genetically homogeneous and maximally differentiated from each other. This approach relies on a technique of analysis of molecular variance (AMOVA) (Excoffier et al. 1992). However, in contrast to conventional AMOVA, SAMOVA does not require that the groups are defined a priori, allowing instead the groups to emerge from the data. The most likely number of groups was identified by running SAMOVA with two to four groups and choosing the partition scheme with the highest Φ_{CT} value.

The software package Statistica (version 5.1, StatSoft Inc. 1996) was used to test for correlations between latitude and the genetic diversity indices estimated. The same software was used to perform cluster analysis in order to explore the relationships among localities. The unweighted pair-group average method (Sneath and Sokal 1973) was used and the groups of samples of each locality were defined as the operational taxonomic units (OTUs). As a measure of similarity among OTUs the corrected average pairwise differences among localities based on the Tamura–Nei method were used.

Autocorrelation analysis was applied by using the software AIDA (Bertorelle and Barbujani 1995) with different numbers of distance classes in order to better explore the observed patterns of genetic diversity and to infer hypothesis on the evolutionary processes which shaped them.

Fu's F_s-test (Fu 1997) was also performed with ARLEQUIN 3.1 to test for population expansion. The same software was used to compute the mismatch distributions and test for evidence of pure demographic expansion (Rogers 1995; Rogers and Harpensding 1992) and spatial expansion (Excoffier 2004; Ray et al. 2003). The Kolmogorov–Smirnov (K–S) two-sample test implement in STATISTICA was used to test for significant differences between the two models.

Results

The 178 sequences obtained define 109 haplotypes with an overall haplotype diversity of 0.976 (SE 0.007) and nucleotide diversity of 0.033 (SE 0.017) (Table S1). A total of 80 polymorphic sites and 96 mutations (60 transitions, 31 transversions and 5 indels) were found out of a 371 bp fragment of the mtDNA control region.

The phylogenetic analyses (MP and Bayesian trees) confirmed the monophyly of *A. presbyter* and revealed two strongly supported clades within this species (Fig. 2). The first clade clustered together all the fish from the Portuguese coast and northern European locations (bootstrap value bv = 79, posterior probability pp = 0.97). Within this clade no clear geographic pattern was observed. Some branches are well supported by both methods which document that this clade underwent substantial diversification. However, the haplotypes present in each branch are often shared by different west European locations. The second clade contained all the Macaronesian sequences (bv = 97, pp = 1.00). Within this last clade, the Azorean sequences formed a branch which was well supported by the MP tree (bv = 99), but was recovered in the Bayesian analysis with moderate support (pp = 0.69).

The statistical parsimony network (SPN) constructed with the complete unreduced dataset for the CR sequences of *A. presbyter* is largely congruent with the results of phylogenetic analyses. Under the confidence limit of 95% (Templeton et al. 1992), three unconnected networks were observed: one including all west European samples (from Portugal to Germany, and the British Isles), and two other grouping the fish from the Canaries and the Azores, respectively (data not shown). Even with the reduced dataset (55 haplotypes, only transversions) the confidence limit of 95% did not link all the haplotypes. Thus, in order to obtain a single network the maximum number of connections was set at 15 substitutions. The network thus obtained revealed the same two geographically distinct groups of haplotypes (Fig. 1), with some indication of separation between the Canaries and the Azores. The northern European samples (Arcachon, Swansea and Wadden Sea, N = 24) corresponded only to four haplotypes in Fig. 1 (six haplotypes, when both transitions and transversions are considered). Of these, three were shared with Portugal, and three were private but were only a single step away from three distinct haplotypes found in the Portuguese coast. Out of the 24 northern European fish, 17 (15 from Wadden Sea and 2 from Swansea) shared the same haplotype, which is also observed in fish from three Portuguese locations (one from Cabo-do-Mundo, six from Aveiro and one from Fonte-da-Telha). Haplotypes from Portuguese locations did not show clear geographic patterns in the SPN. The sample from Azores has 100% private haplotypes which were closer to the Tenerife population than to the European ones. The haplotype inferred to be the ancestral one was haplotype 12 (outgroup weight 0.090) shared by 13 individuals (12 from western Portugal and one from southern Portugal). Interestingly, the samples of the Mediterranean *A. hepsetus* were separated by a number of steps from the ancestral haplotype which is not markedly different from that separating European and Macaronesian fish (data not shown).

Measurements of genetic diversity for each collection site of *A. presbyter* are shown in Table 1. A prominent feature emerging from the inspection of this table is the low-diversity of the Wadden Sea samples stressed above. When compared to the other locations, the Wadden Sea population presented the lowest percentage of private haplotypes (50%) and the lowest haplotype (0.380) and nucleotide (0.001) diversity indices. A significantly negative correlation was found between latitude and haplotype diversity ($r = -0.832$, $P < 0.05$); nevertheless, this is not a simple relation, as there is also a decrease in the haplotype diversity for Tenerife. No correlation was found between latitude and the nucleotide diversity ($r = -0.447$, $P > 0.05$) or the number of polymorphic sites ($r = -0.438$, $P > 0.05$).

The Mantel test (Tenerife excluded) revealed a significant correlation between genetic and geographic distances ($r = 0.825$, $P = 0.009$). The spatial autocorrelation analyses (SPA) performed (Tenerife excluded) with different distance classes yielded II values ranging from significantly positive to significantly negative (Bonferroni's $P < 0.05$) as the distance increased (data not shown).

In Table 2 the pairwise Φ_{ST} based on the TrN distance and number of migrants per generation for all six pairs of collection sites are presented. It is interesting to note that geographically close sampling locations (Aveiro/Fonte-da-Telha and Arade/Castro Marim) did not reveal significant Φ_{ST} values. When analysing locations separated by greater distances the results were significant, confirming the trend detected by the Mantel tests. The greatest differences for

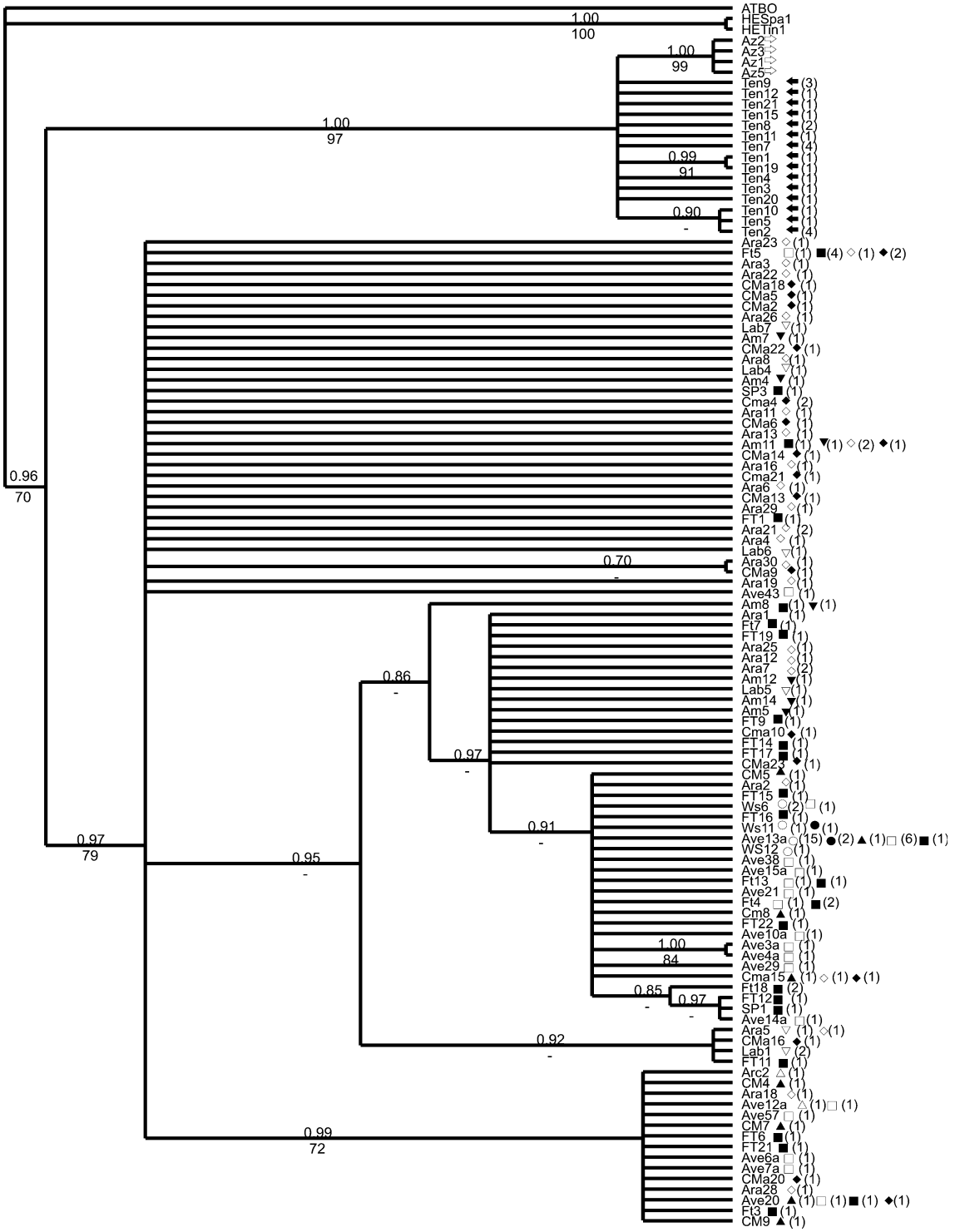

Fig. 2 Phylogenetic relationships of *Atherina presbyter* for the mitochondrial control region. The haplotypes labels are as indicated in Table S1. Bayesian tree with posterior probabilities (above the nodes) and Maximum Parsimony's bootstrap values (bellow the nodes). *Atherina boyeri* (ATBO) was used as outgroup. Symbols in front of each haplotype indicate its presence in the sampling locations, with number of specimens between brackets: *white circle* (Wadden Sea), *black circle* (Swansea), *white triangle up* (Arcachon), *black triangle up* (Cabo do Mundo), *white square* (Aveiro), *black square* (Fonte-da-Telha), *white triangle down* (Lagoa de Albufeira), *black triangle down* (Amoreira), *white lozenge* (Arade), *black lozenge* (Castro Marim), *white arrow* (Santa Maria) and *black arrow* (Tenerife)

Table 1 Diversity measures for the populations of *Atherina presbyter*: number of individuals (N), number of haplotypes (N_h), percentage of private haplotypes ($\%P_h$), percentage of shared haplotypes ($\%S_h$), number of polymorphic sites (S), haplotype diversity (h) and nucleotide diversity (π)

Sampling site	Coordinates	N	N_h	P_h (%)	S_h (%)	S	h	π
Europe	–	120	75	100.00	0.00	62	0.961	0.020
Wadden Sea	53°33′N, 8°35′E	19	4	50.00	50.00	4	0.380	0.001
Western Portugal	–	53	36	86.11	13.89	38	0.968	0.020
Aveiro	40°38′N, 8°45′W	24	19	68.42	31.58	32	0.946	0.020
Fonte-da-Telha	38°32′N, 9°13′′W	29	24	70.83	29.17	29	0.980	0.020
Southern Portugal	–	48	40	91.67	8.33	46	0.991	0.017
Arade	37°08′N, 8°32′W	28	25	84.00	16.00	40	0.992	0.019
Castro Marim	37°12′N, 7°25′W	20	18	77.78	22.22	28	0.989	0.016
Tenerife	28°03′N, 16°34′W	24	15	100.00	0.00	16	0.942	0.009

Table 2 Geneflow among populations of *Atherina presbyter* represented by N_m (above diagonal) and Φ_{ST} (below diagonal), and based on the Tamura–Nei (TrN) model

	WS	Ave	FT	Ara	Cma	Ten
WS		2.638	1.080	0.490	0.317	0.037
Ave	0.159*		11.556	2.109	1.566	0.114
FT	0.316*	0.041		8.892	5.003	0.126
Ara	0.505*	0.192*	0.053*		inf	0.125
Cma	0.612*	0.242*	0.091*	−0.016		0.100
Ten	0.930*	0.814*	0.798*	0.800*	0.834*	

Significant values of probability P are shown with an *. *WS* Wadden Sea, *Ave* Aveiro, *FT* Fonte-da-Telha, *Ara* Arade, *CMa* Castro Marim, *Ten* Tenerife

Table 3 Corrected average pairwise difference for the collecting sites of *Atherina presbyter*

	WS	Ave	FT	Ara	Cma	Ten
WS						
Ave	0.845*					
FT	2.232*	0.313				
Ara	4.543*	1.692*	0.402*			
Cma	5.046*	2.118*	0.680*	−0.097		
Ten	26.351*	22.941*	21.655*	21.128*	21.996*	

Significant values of probability P are shown with an *. *WS* Wadden Sea, *Ave* Aveiro, *FT* Fonte-da-Telha, *Ara* Arade, *CMa* Castro Marim, *Ten* Tenerife

this divergence measurement were found between the European populations and the Canaries (maximum pairwise Φ_{ST} between Tenerife and the Wadden Sea = 0.930). Gene flow was found to be low between Tenerife and the other populations studied, with the N_m bellow the threshold value of one migrant per generation (average = 0.101). The N_m was greater than one for all pairs of Portuguese locations, and in the comparisons involving the Wadden Sea and western Portugal. It was very high between Aveiro and Fonte-da-Telha and between Arade and Castro Marim, where it was computed as infinite. The corrected average pairwise differences (Table 3) were relatively low among the continental populations (average = 1.777), with a minimum value of −0.097 between Arade and Castro Marim, and a maximum value of 5.046 between Wadden Sea and Castro Marim. When involving the Tenerife sequences, the corrected average pairwise differences were much higher (average = 22.814), with a maximum value between this and the Wadden Sea population (26.351).

The SAMOVA procedure based on the pairwise differences yielded a maximized Φ_{CT} (0.729) for the two groups: Tenerife versus the other five continental populations, although this was not a significant result ($P = 0.176$). A significant value was observed for the four gene pools: Wadden Sea, Aveiro + Fonte-da-Telha, Arade + Castro Marim and Tenerife ($\Phi_{CT} = 0.625$, $P = 0.022$). The analysis of molecular variance computed with the Tamura–Nei (TrN) model showed a significant genetic structure along the studied area ($\Phi_{ST} = 0.609$, $P < 0.001$); when the same analysis is performed without the Canaries population the population structure is still recovered (($\Phi_{ST} = 0.201$, $P < 0.001$). The AMOVA result for the four groups recovered by SAMOVA, was also significant ($\Phi_{CT} = 0.634$, $P = 0.023$). The formation of these four populations along the studied area is supported by cluster analysis (Fig. 3). Given the concordance between the pairwise Φ_{st}, SAMOVA/AMOVA and cluster analysis, we decided to pool together Aveiro with Fonte-da-Telha (western Portugal) and Arade with Castro Marim (southern Portugal). Thus, for further analyses on population comparisons and historical demography the following four populations were considered: Wadden Sea, western Portugal, southern Portugal and Tenerife. We also decided to estimate historical demographic parameters for the west European coast as a whole, since this was found to also be a well supported group (phylogenetic trees, SPN, cluster analysis and SAMOVA).

Fu's neutrality test yielded significant negative results for every population analyzed, suggesting demographic expansion (Table 4). The mismatch distribution analyses showed

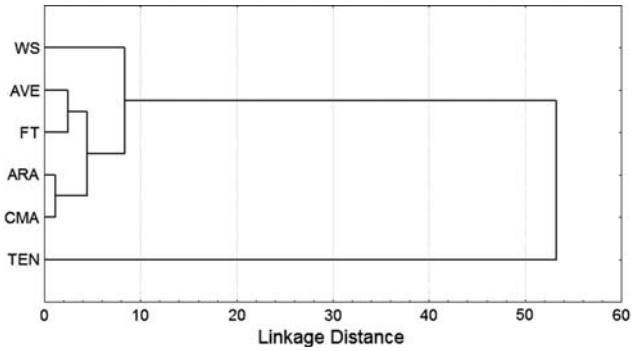

Fig. 3 Cluster analysis for the populations of *Atherina presbyter* based on the mitochondrial control region. Grouping based on the unweighted pair-group average method. *WS* Wadden Sea, *Ave* Aveiro, *FT* Fonte-da-Telha, *Ara* Arade, *CMa* Castro Marim, *Ten* Tenerife

different results for the five populations studied (Table 4). On one hand, for Wadden Sea, western Portugal, Tenerife and the European coast as a whole the mismatch distributions did not differ significantly from both models of sudden demographic and spatial expansion. On the other hand, the mismatch distribution of southern Portugal significantly differed from the purely demographic model, but not from the spatial model. The K–S test showed no significant differences between the distributions generated by the two models, except for southern Portugal ($P < 0.001$). Nevertheless, the data were better fitted to the sudden expansion model for Wadden Sea, western Portugal and the European coast, and to the spatial expansion model for southern Portugal and Tenerife.

In order to compute estimates of effective population size and their changes with time, we used a 10% divergence per million years rate (Bowen et al. 2006). The Wadden sea population seems to be quite young. The expansion was estimated to have taken place at 14–25 thousands years ago (kya) (demographic and spatial model, respectively). The graphic clearly showed a mark of a very recent colonization phenomenon with a peak around the 0 mutational steps (Fig. 4). For the remaining populations, the estimates point to much greater ages, with a maximum of 138–282k ya for western Portugal. The mismatch graph from the Portuguese populations and the European coast revealed one peak around the nine differences, and a second one around 1, 2 and 3 (western Portugal, European coast and southern Portugal, respectively), indicating a second bottleneck episode in their history. The Tenerife population mismatch graph showed a single peak around the three differences. Globally, the confidence intervals of parameters estimated by both sudden expansion and spatial models overlapped and pointed to the fact that these *A. presbyter* populations are very ancient, having diverged long before the LGM.

Table 4 Demographic parameters of *Atherina presbyter* based on mtDNA control region

Population	Wadden Sea	Western Portugal	Southern Portugal	Europe	Tenerife
F_s	−1.550*	−21.665*	−25.092*	−24.764*	−7.267*
Arlequin—mismatch distributions					
Sudden expansion model					
τ (95% CI)	0.932 (0–3.093)	10.477 (4.014–15.465)	2.422 (1.674–3.188)	9.945 (4.600–14.244)	3.361 (1.645–4.525)
t (95% CI)	25k y (0–83)	282k y (108–416)	na	268k y (124–384)	90k y (44–122)
θ_0	0.028	0.000	0.000	0.000	0.007
N_0	755	0	na	0	189
θ_1	0.750	15.992	99,999	18.672	498.750
N_1	20,216	431,051	na	503,288	13,443,396
SSD	0.000	0.005	0.116*	0.005	0.015
Hri	0.161	0.008	0.008	0.008	0.053
Spatial expansion model					
τ (95% CI)	0.530 (0–2.788)	5.117 (2.201–24.309)	2.421 (0.948–12.954)	6.344 (3.798–13.113)	3.379 (1.267–4.689)
t (95% CI)	14k y (0–75)	138k y (59–655)	65k y (26–349)	171k y (102–353)	91k y (34–126)
θ	0.191	6.077	5.938	3.682	0.001
N	5,148	163,801	81	99,245	27
M	2.722	5.557	99,999	8.844	40.581
Nm	1.361	2.779	49,999	4.422	20.291
SSD	0.000	0.013	0.009	0.013	0.014
Hri	0.161	0.008	0.008	0.008	0.053

F_s (Fu's neutrality test)

Estimates of population parameters with Arlequin for sudden expansion and spatial models: τ (tau), t (time since the expansion), θ_0 and θ_1, N_0 and N_1 (female effective population size before and after expansion), M (migration rate), N_m (number of migrants), SSD (sum of square deviations), and Hri (Harpending's Raggedness index). Significant values of probability P are shown with an *. *na* non applicable

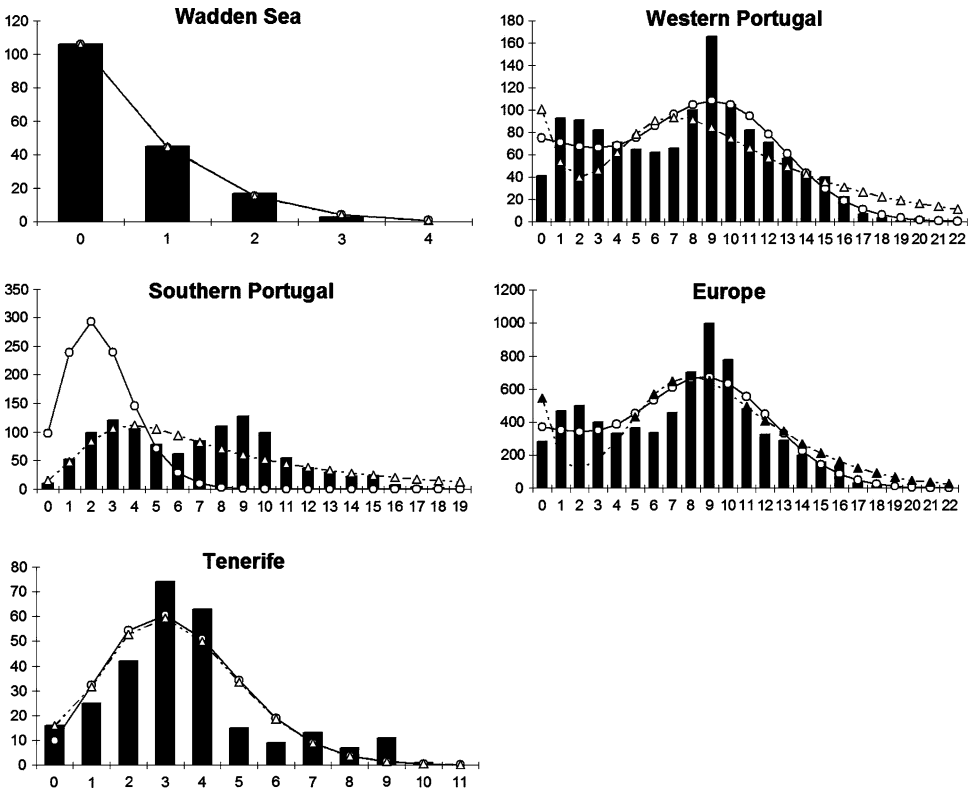

Fig. 4 Mismatch distributions for the populations of *Atherina presbyter* based on the mitochondrial control region. The bars represent the observed frequency of the pairwise differences among haplotypes; the lines indicate the expected distributions based on the models of sudden population expansion (*open circles*, *solid line*) and spatial expansion (*open triangles*, *dashed line*)

Discussion

The degree of genetic differentiation of a species is determined by its demographic history and the amount of contemporary gene flow (Templeton et al. 1995). The present study constitutes the first work analysing the phylogeography and historical demography of *Atherina presbyter* covering its geographical range.

A striking result, supported by every analyses performed, is the highly marked differentiation between the Canary Is. population and the western European coastal ones. The distance of the clade comprising fish from the Azores and the Canaries to the European clade is of a magnitude comparable to that separating *A. presbyter* from *A. hepsetus* (Francisco et al. 2008). These two sand-smelts are sister-species and may represent the result of a vicariant process that may have isolated an ancestral sand-smelt stock in refugia in warm-water pockets in the Atlantic and the Mediterranean, respectively, during glacial events. Indeed, the phylogenetic analysis presented in Fig. 2 is consistent with the formation of three distinct clades from a common ancestor: *A. hepsetus* in the Mediterranean, a stock present in Macaronesia and one which colonized west Europe, a finding also supported by Francisco et al. (2008). Analyses of meristic characters of the fish from the Canaries confirmed their affinity to *A. presbyter* and distinctiveness from the west African *Atherina lopeziana* (A. Brito, unpublished). Nevertheless, as there are no genetic data on this last species, the identity of the Canary's fish can be questioned, as well as that of the Azorean ones. The taxonomic status of these individuals must be carefully revised, as there is still much work needed around the African coast and Macaronesian islands. Previous phylogeographic studies of other small coastal species using mtDNA and nuclear data showed similar results (e.g. blenniids in Almada et al. 2001; *Parablennius parvicornis* in Almada et al. 2005; *Trypterygion delaisi* in Domingues et al. 2007b; Santos et al. 1995 and references therein). In these studies fish from the Canaries, Madeira and Azores typically displayed close connections and were markedly distinct from those from the Mediterranean and Atlantic shores of Europe, the Azores tending to be colonized from Madeira. This pattern means that for many warm-water species Madeira, and eventually part of the Canaries, may have acted as glacial refugia or stepping stones for fish coming from west Africa. From these archipelagos, the Azores were likely colonized via eddies which are common in the area (Santos et al. 1995). This would explain the paradoxical situation already noted by several marine biogeographers, namely Briggs (1974). This author noted that although the prevailing ocean currents at the Azores come from the west and are branches of the Gulf Stream, the affinities of the Azorean ichthiofauna are with the east and not with America. In this context, a phylogeographic investigation of the relationships of the sand-smelt from Madeira is highly desirable.

An interesting finding emerging from this work is the significant correlation between genetic and geographical distances among the European populations. This result suggests that the genetic structure of *A. presbyter* along the western European shores is compatible with an isolation-by-distance (IBD) pattern, or a cline resulting from the south-north colonization. The SPA supports the gradient hypothesis, typically generated by a population expansion followed by continuous founder effects (Sokal 1979). The significantly negative correlation between latitude and the haplotype diversity also agrees with this last hypothesis. As for other North-eastern Atlantic taxa (e.g. Gysels et al. 2004; Helberg et al. 2001; Quesada et al. 1995), the mtDNA data revealed significantly negative correlation between latitude and the haplotype diversity.

The Wadden Sea population of *A. presbyter* presents a much lower genetic diversity when compared to southern populations. According to Grant and Bowen (1998), these low-values of haplotype and nucleotide diversity are indicative of a prolonged or severe demographic bottleneck in recent times. This pattern is consistent with a recent colonization of northern European coasts from southern areas (Hewitt 1999). Indeed, most northern European fish share haplotypes with southwest Europe, with the few private haplotypes being derived by a single mutational step from other Portuguese haplotypes. As we can see from the mismatch analysis, the Wadden Sea population presents a rather small range in the number of differences suggesting a very recent history (probably less than 10k y old). Compared to the other populations the female effective population size and age of population are much smaller for the Wadden Sea. Taken together, the results are supportive of a very recent history for this northern European population. In fact, and according to Climap (1984), the Wadden Sea was covered by ice at the LGM, which means that the sand-smelt was certainly extirpated from the area. It is known however, that, after the LGM and before the last 10k y, additional episodes of severe cooling took place, of which the Younger Dryas (13k y before present) was but one example. This means that, regardless of eventual recolonizations in intervening warming episodes, a succession of local extinctions may have taken place in northern Europe, so that the extant populations may be present in the North Sea since much recent times. Debes et al. (2008) refer that the European sprat, a cold-water tolerant species, could only have recolonized the North Sea 7,500 years before present. It is probable that *A. presbyter*, whose distributional range has its northern limit in the southern North Sea, was only able to recolonize the area much later, and we can even consider the possibility of the present-day Wadden Sea population being more recent than the 17th century, when the Little Ice Age (LIA) ended (Crowley 2000).

The high values of haplotype diversity and low values of nucleotide diversity revealed by Tenerife's and Portuguese populations of sand-smelt are an expected signature of rapid population growth from an ancestral population with small *N* (Grant and Bowen 1998). This conclusion is supported by Fu's neutrality test. Nevertheless, mismatch analyses do not point to recently funded populations for these locations: age of population (138k ya for western Portugal, 65k ya for southern Portugal and 91k ya for Tenerife), female effective population size (431,051 for western Portugal, and 13,443,396 for Tenerife), ancient peaks around the nine differences for the mismatch graph of the Portuguese populations, and depth of these distributions which include fish separated by up to 21 steps in the case of western Portugal. Because of the uncertainty of the divergence rate and the use of a single marker, estimates of population size and age must be viewed as merely indicative. However, even if we adopt a much higher divergence rate (e.g. 18.6% in Domingues et al. 2005) the conclusion that the history of these populations is much older than the last glaciation still holds.

The present study raises two general issues concerning the phylogeography of temperate marine inshore fish. One involves the signatures of Pleistocene glaciations, and the other refers to the spatial scales of analysis. We will address them in turn.

At the LGM, the sea surface temperature along the western Portuguese shore was likely lower than those currently prevailing in the distributional range of *A. presbyter*. In deed, Alveirinho-Dias et al. (1997) showed that the polar front was located at the latitude of western Portugal (between 37°N and 40°N) at the LGM, which means that the northern limit of the sand-smelt must have been located further south during that period. Thus, like the majority of warm-water fishes, *A. presbyter* was likely extinct along most of the coast of west Europe during the last glaciation. Interestingly, in agreement with our findings for the sand-smelt, several warm-temperate fish species present estimated age of populations clearly older than the LGM, even though they could not have inhabited the north-eastern Atlantic during the last glacial phase (e.g. bluefin tuna *Thunnus thynnus* and swordfish *Xiphias gladius* in Bremer et al. 2005; white seabream *Diplodus sargus* in Domingues et al. 2007b; common goby *Pomatoschistus microps* in Gysels et al. 2004). Why should the estimated population ages for temperate fish be much older than the LGM, if their west European populations must have gone extinct at this period? Assuming that the paleotemperature reconstructions are correct and local extinctions did indeed take place, two non-mutually exclusive hypotheses can be advanced. First, the computer programs based on the mismatch distributions and coalescence may be integrating the combined effects of consecutive expansions and contractions during the recurrent Pleistocene glacial cycles. For

instance, some programs assume explicitly that populations are stable or are experiencing a prolonged process of shrinkage or growth (Kuhner 2006, 2009). A detailed and exhaustive investigation of the implications of repetitive demographic oscillations based on simulations will, in the future, help to clarify the meaning of the estimates of the currently available historical demographic tools. A second hypothesis is that the southwards and northwards migrations of this species, as the water temperature dropped and rose, might have been so fast that they left no clear signature discernable by comparing present-day samples collected at different sites. Indeed, the estimates derived from the samples of each location may not reflect what happened in the past in that area, but rather the signatures of the demographic events that took place in the locations where the populations survived during the glacial retreats. In the case of *A. presbyter*, it is plausible that the species might have simply moved south of Iberia, having recolonized the area in the current interglacial (Francisco et al. 2006a, 2008). It is interesting to note that for the southern Portugal population the data conform to the spatial expansion model and the demographic one is rejected. For the other locations, the spatial expansion model also fitted the data although the sudden expansion model could not be rejected. The Iberian Peninsula constituted an important glacial refugium for the Atlantic salmon *Salmo salar* (Consuegra et al. 2002). These authors, using ancient salmon DNA, provided evidence suggesting that the present-day genetic make-up of the salmon populations of northern Spain differs from their constitution during the last glaciation. At the same time, the present peak of genetic diversity in the salmon populations is located at the latitude of the British Isles, an area where the glacial populations were, at best, residual, but which is now at the centre of the species distributional range. For the sand-smelt the highest genetic diversity levels were found in Portugal, which is at or near the centre of the species current distribution. The genetic diversity decreases sharply towards the northern limit of the species (Wadden Sea). It also decreases to the Canaries, near the southern limit of its range. However, in this last case, a note of caution is necessary because we are dealing with an insular population, whose effective size and diversity may have been affected by isolation and the limited habitat available. Data from the African coast will help to clarify this issue. In summary, we suggest that for *A. presbyter*, the hypothesis of a spatial expansion after the last glaciation, probably combined with more or less accentuated demographic oscillations, seems to be a plausible scenario.

Our work reveals the existence of a gradient effect and/or isolation-by-distance at a scale of few hundreds of kilometres (over 300 km, e.g. between Fonte-da-Telha and Arade). For the same species, Francisco et al. (2006a) did not find genetic differentiation for smaller geographic scales (less than 250 km). Several other studies using the same marker and similar sampling effort could not find genetic differentiation, even for much wider spatial scales (several hundreds to thousands of kilometres) (e.g. Almada et al. 2008; Debes et al. 2008; Francisco et al. 2006b). Why does the sand-smelt present such an uncommon pattern? The extent of gene flow may be affected by distinct biological characteristics, such as larval and adult ecology (Riginos and Victor 2001). *A. presbyter* shows several characteristics that may be relevant to this issue. It spawns demersal eggs attached to vegetation in very shallow waters, from which advanced larvae ready to start exogenous feeding hatch. These larvae probably exhibit active behaviours that promote their retention in inshore waters. They congregate in large numbers in the intertidal areas and waters which are very close to shore during all summer in Portugal (Henriques and Almada 1998). Through out the entire breeding season the frequency of adult visits to the littoral is very high, and they seem to stay in the area, spawning repeatedly (V.C. Almada, unpublished). Near shore retention of larvae and juveniles and some site fidelity of the adults during breeding season may favour the development of genetic differentiation among fish from different locations. On the contrary, species in which individuals living in a vast area congregate in restricted places for mass spawning (e.g. Daemen et al. 2001) and/or those who passively disperse eggs and larvae in the plankton, will fail to display detectable population structure at much broader scales (e.g. Debes et al. 2008).

In future studies, the combined use of data from mitochondrial and nuclear markers and other biological data may help in clarifying the role of the several factors that affect present-day demographic structure of *A. presbyter*. In open systems like the ocean, for species that tend to display poorly defined population boundaries, and especially if organisms rapidly track climatic oscillations, the ways in which phylogeographic and historical demographic tools must be applied is an area that deserves to be explored in detail in the future.

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