Mitochondrial phylogeography of the European sprat (*Sprattus sprattus* L., Clupeidae) reveals isolated climatically vulnerable populations in the Mediterranean Sea and range expansion in the northeast Atlantic

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Abstract

We examined the genetic structure of the European sprat (*Sprattus sprattus*) by means of a 530-bp sequence of the mitochondrial control region from 210 fish originating from seven sampling localities of its distributional range. Phylogeographical analysis of 128 haplotypes showed a phylogenetic separation into two major clades with the Strait of Sicily acting as a barrier to gene flow between them. While no population differentiation was observed based on analysis of molecular variance and net nucleotide differences between samples of the Baltic Sea, the North Sea and the Bay of Biscay nor between the Black Sea and the Bosporus, a strong population differentiation between these samples and two samples from the Mediterranean Sea was found. Further, the biggest genetic distance was observed within the Mediterranean Sea between the populations of the Gulf of Lyon and the Adriatic Sea, indicating genetic isolation of these regions. Low genetic diversities and star-like haplotype networks of both Mediterranean Sea populations point towards recent demographic expansion scenarios after low population size, which is further supported by negative F_S values and unimodal mismatch distributions with a low mean. Along the northeast Atlantic coast, a northwards range expansion of a large and stable population can be assumed. The history of a diverse but differentiated Black Sea population remains unknown due to uncertainties in the palaeo-oceanography of this sea. Our genetic data did not confirm the presently used classification into subspecies but are only preliminary in the absence of nuclear genetic analyses.

Keywords: Clupeidae, fish, ice ages, isolation, mtDNA, phylogeography, range expansion

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Introduction

Starting in the Pliocene [~2.5 million years before present (BP)] a global cooling began with about 40 glacial and interglacial phases. As a consequence, not only water temperature but also sea level underwent considerable changes over the ongoing Quaternary. During the last glacial maximum (LGM, ~20 000 BP) sea level was about 120 m lower than at present (Fairbanks 1989; Lambeck & Chappell 2001) causing the isolation of marginal seas with shallow sills. In Europe, the Baltic Sea was isolated until it was connected with the North Sea around 10 000 BP (Andrén *et al.* 2000). The channel of Dover, which now connects the Atlantic with the southern North Sea, lay dry during times of sea-level regression (Streif 2004). The Black Sea is assumed to have repeatedly been isolated from the Mediterranean Sea during the last glacial cycle, since temporal changes in salinity are recorded after a first intrusion of saline water 300 000 BP (Degens & Ross 1972; Schrader 1979). A final reconnection with the Mediterranean Sea occurred around 10 500 BP via the Marmara Sea and the Bosporus (Aksu *et al.* 2002). During the LGM, the polar front was located off Portugal, resulting in considerably colder waters and a lower primary production than today in the Bay of Biscay (Loncaric *et al.* 1998).

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As a consequence, glacial changes had a major impact on the distribution of living organisms, shaping also their genetic structure (Hewitt 2000). Recently observed differences in the phylogeographical patterns of marine organisms in European coastal waters may therefore be regarded as a corollary of their species-specific dispersal abilities and capacities for physiological adaptation. In the relatively young ecosystem of the Baltic Sea, for instance, genetic diversity is on average lower than in the North Sea with clinal patterns of differentiation towards the Atlantic (Johanneson & André 2006). In contrast, genetic divergence between the North Sea and the Atlantic is rare, although for some species, survival within the glacial North Sea or within the Hurd Deep, a seafloor depression of the nascent English Channel, has been proposed on the basis of private haplotypes and/or increased genetic diversity (e.g. *Pomatoschistus minutus*, Gysels *et al*. 2004a; *Pomatoschistus microps,* Gysels *et al*. 2004b; *Palmaria palmata,* Provan *et al*. 2005; *Raja clavata*, Chevolot *et al*. 2006). The Iberian Atlantic coast is believed to be another glacial refugium and the origin of range expansions to the north after deglaciation (e.g. *Salmo salar*, Consuegra *et al*. 2002; *P. microps,* Gysels *et al*. 2004b; *R. clavata*, Chevolot *et al*. 2006).

The gateway between the Atlantic Ocean and the Mediterranean Sea (the Strait of Gibraltar, or rather the Almerian–Oran front) has been found to be a barrier to gene flow for some species but not for others without any relationship to respective biological traits (reviewed in Patarnello *et al*. 2007). Within the Mediterranean Sea, the most pronounced genetic structuring in fish species throughout European marine waters is observed. Very divergent populations have been identified for some species in the Adriatic Sea (e.g. *Plathichthys flesus*, Borsa *et al*. 1997; *P. minutus*, Gysels *et al*. 2004a; *Solea solea*, Rolland *et al*. 2007), and genetic differentiation between the western and the eastern Mediterranean basins is widespread (e.g. *S. solea*, Kotoulas *et al*. 1995; *Trisopterus minutus*, Mattiangeli *et al*. 2003; *Psetta maxima*, Suzuki *et al*. 2004).

The European sprat (*Sprattus sprattus*), a small schooling pelagic clupeid fish species, inhabits European coastal waters and marginal seas. Sprat is commercially exploited and of high economic interest with a mean of 671 000 tonnes per year during the last reported decade (1995–2005; FAO 2008). The species' distribution extends from central Norway (Naevdal 1968) to the Kvark in the Baltic (Aro 1989), over the whole North Sea and around the British Isles including the Orkneys (Rogers *et al*. 2001) down to the Bay of Biscay. A somewhat discontinuous distribution can be found around the Iberian Peninsula, where spawning seems to be associated with the main river mouths. In the Mediterranean Sea, it is found north of 39° latitude, while reproduction has only been confirmed for the Gulf of Lyon and the northern Adriatic Sea (Demir 1963). The whole Black Sea is inhabited by sprat (Daskalov 1999) and its occurrence is also known

for the Marmara Sea (Deval *et al*. 2002). Small clupeid fishes respond dramatically and quickly to changes in physical oceanographic conditions (Hunter & Alheit 1994), and thus, the genetic structure of sprat most probably bears the imprint of glacial times. Within the Mediterranean Sea, sprat is assumed to be a remnant of a boreal fish fauna (Tortonese 1964), a biogeographical category which probably originated in the Würm glacial period (Bianchi & Morri 2000). Depending on the author, it is either considered as a cold-water (Daskalov 1999) or as a warm-water species (Nissling 2004) due to its wide temperature tolerance of 5–21 °C (Berg 1949). In the cold Baltic Sea, sprat favours a temperature of at least 4 °C (a temperature which caused seriously elevated egg mortalities in laboratory studies: Thomson *et al*. 1981; Milligan 1986), and it never lives in water colder than 3.5 °C (Rechlin 1967). An upper temperature limit is not yet clearly defined, but in the above-mentioned studies, elevated egg mortalities were found at temperatures of around 18 °C and beyond. Temperature preferences for sprat from the Black Sea are reported between 8 °C and 18 °C (Cautis 1958). Sprat undertakes large migrations that are assumed to be environmentally driven or age-dependent (Rechlin 1986). It is largely euryhaline (ICES 2008) and can also be found in estuaries (e.g. Costa & Cabral 1999). Reproduction occurs over an extended period where each individual spawns 30 000–40 000 pelagic eggs over about 3 months in eight to 11 batches (Makarchouk 2002). Spawning places vary over the years (ICES 1990) and spawning occurs year-round with a tendency towards summer in the north and towards winter in the south. Sprat is regarded as vulnerable to a temporal mismatch of hatching larvae and food supply (Nissling 2004). Larvae mostly feed on nauplia (Blaxter & Hunter 1982), while adults feed exclusively on zooplankton (Cardinale *et al*. 2002). Sprat is small, reaching 12–16 cm in length, and short-lived with a general upper age limit of 5 years (Bailey 1980). Maturity is reached in the first or, more often, the second year (ICES 2008). Abundances vary extremely, e.g. a 10-fold increase was reported within the 1970s for the North Sea (Munk 1991).

Different sprat subspecies have been proposed over its distribution range. Three groups are generally accepted. They can be morphometrically differentiated by the mean number of postpelvic scutes (x) after Whitehead (1985) with *S. s. balticus* Schneider, 1908 ($x < 11.5$) in the Baltic Sea, *S.* s. *sprattus* Linnaeus, 1758 ($x > 11.5$) in the northeast Atlantic and the North Sea and *S. s. phalericus* Risso, 1827 ($x = 11.3$) in the Mediterranean and the Black Sea. Additional taxonomic subdivisions in the Mediterranean and the Black Sea have been proposed (see Demir 1963 and references therein).

The present study focuses on the broad range phylogeography of *S. sprattus* by means of sequence data of the 5'-end of the mitochondrial control region. Inferred historical events for sprat and their comparison with known patterns in other species might enhance our knowledge of the
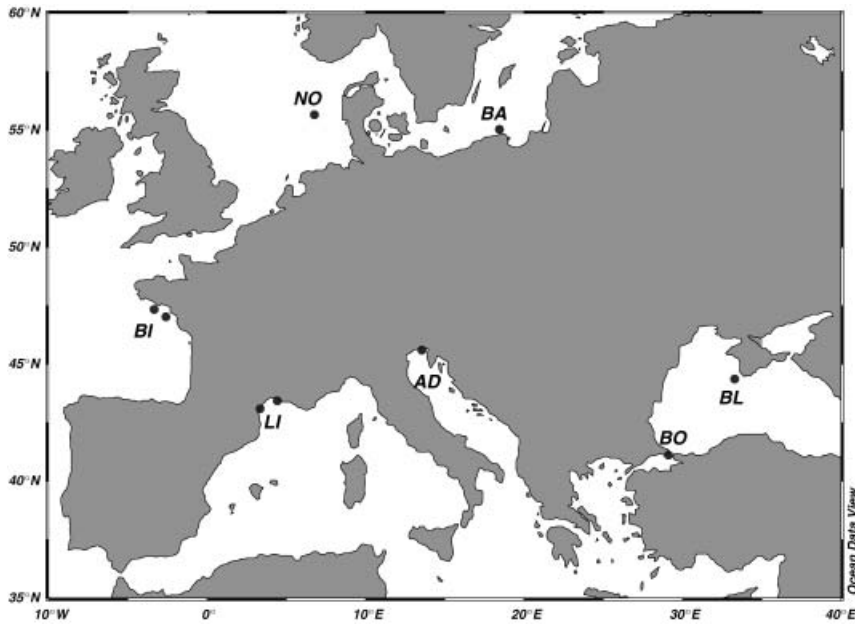
Fig. 1 Geographical positions of sampling localities of *Sprattus sprattus*.

Table 1 Sampling information for *Sprattus sprattus*

Locality	Abbreviation (ID)	Sample size	Date of collection	Geographical coordinates	
				Latitude	Longitude
Baltic Sea	BA	30	May 2006	18.747°E	55.083°N
North Sea	NO	30	July 2005	6.777°E	55.667°N
Bay of Biscay	BI	30	March 2006	3.267°W	47.309°N
Gulf of Lyon	LI	30	July 2006	3.818°E	43.267°N
Adriatic Sea	AD	30	December 2005	13.563°E	45.603°N
Bosporus	BO	30	June 2006	29.119°E	41.201°N
Black Sea	BL	30	June 2006	33.500°E	44.617°N
Overall	All	210	—	—	—

phylogeography of marine species within glacially influenced European coastal waters. In particular, we address the following aspects:

1 Genetic differentiation — as sprat has an extremely high potential dispersal ability, high abundance and can tolerate salinities of a great range, we expect this species to show no or little genetic differentiation within northern areas that were recolonized after the last deglaciation.
2 Does the phylogeographical analysis reveal isolated populations in the Mediterranean in accordance with the category of glacial remnants, and does the sprat show patterns congruent with those known in other marine species?
3 Do the existing subspecies designations reflect evolutionary entities as revealed by genetic data from throughout the species' distribution range?

Materials and methods

Sampling

Samples of 210 European sprat were collected by research vessels or local fishermen between 2005 and 2006. Seven sampling sites roughly covered the species' whole distribution range from the Baltic to the Black Sea (Fig. 1, Table 1). A sampling site in the southern Black Sea was situated just at the entrance of the Bosporus and is further referred to as Bosporus. Samples from the Bay of Biscay and the Gulf of Lyon originated from two different positions and were pooled for analysis as geographical distances for pooled positions are negligible compared to distances between major localities (compare Fig. 1). In these cases the respective mean geographical coordinates were used for following statistics. A pairwise comparison of Φ_{ST} values (calculated as below)

between these subsamples resulted in negative and nonsignificant values [P = 0.97 (BI) and 0.86 (LI)]. Samples from around the Iberian Peninsula could not be obtained due to the recently rare appearance of the species (F. Soborido-Rey, H. Cabral, personal communication). Pieces of muscle tissue were taken from each fish and separately stored in 99% ethanol.

DNA analysis

Total genomic DNA was extracted using a Chelex-Proteinase K protocol. Each sample (1–5 mg) was incubated in 12 µL of Proteinase K (20 mg/mL) (Roche) and 300 µL of 5% Chelex 100 solution (Biorad) at 55 °C and shaking at a 1000 r.p.m. for 3 h with a subsequent denaturation step at 98 °C and 1200 r.p.m. for 25 min in a thermomixer. After centrifugation for 10 min at 13 000 r.p.m. 1 µL of supernatant (100–500 ng DNA) was used in subsequent polymerase chain reactions (PCR). PCR of the 5′-end of the mitochondrial control region was first performed using primers B and Q (Lee *et al*. 1995). A PCR product of about 670-bp could be amplified but generated subsequent sequencing problems due to by-products. Still, both strands of several individuals were successfully sequenced on an ABI 3730 XL sequencer. Mitochondrial identity of the fragment was confirmed by a BLAST search (Altschul *et al*. 1990) on the NCBI webpage (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi), which yielded the highest similarity with a control region sequence of the Atlantic herring (*Clupea harengus*). Based on sequences of two individuals from geographically distant localities (Baltic Sea and Black Sea), new primers were designed, tRP-17: 5′-AGCTCCCAAAGCTAGGATTCTG-3′ and CR552: 5-GATATAGTGCACCAAGGGAGGGAA-3′. Each reaction (50 µL) was performed using QIAGEN PCR Buffer (1×) with additional MgCl$_2$ (final concentration of 1.75 mM), 0.2 mM of each dNTP, 0.1 µM of each primer, 1.75 U *Taq* Polymerase (QIAGEN) and 1 µL supernatant of Chelex extraction solution. PCR conditions were as follows: initial denaturation at 94 °C for 3 min, 31 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, elongation at 72 °C for 1 min, and a final elongation at 72 °C for 3 min. With the latter primer pair, the samples yielded products of about 620 bp appearing as single bands on a 1.5% agarose gel after electrophoresis and staining with ethidium bromide. All products were purified with QIAquick (QIAGEN) and subsequently sequenced in the reverse direction with primer CR552. All chromatograms were checked by eye and ambiguous sites were confirmed by sequencing in the forward direction with primer tRP-17 after repeating the PCR for the respective individuals.

Statistical analysis

All sequences were aligned manually with the program BIOEDIT version 7.0 (Hall 1999). A short sequence of the tRNA^{Pro} gene was deleted from the 5′-end of the alignment to prevent any bias of sequence parameter estimates of the control region. A model of base substitution between haplotypes was estimated using PAUP 4.0 beta (Swofford 2003) and MODELTEST 3.7 (Posada & Crandall 1998) with the AIC (Akaike 1974) and the AICc (Hurvich & Tsai 1989) as information criteria. Sample size must be given when using the AICc, but as this is an unspecified parameter in sequence analysis (Posada & Buckley 2004), we performed calculations with a small (number of haplotypes) and a big sample size (number of haplotypes × number of nucleotides in the alignment) to evaluate if different models are selected. The chosen model under both criteria was GTR + I + G (Tavaré 1989), which is not implemented in most software programs used in subsequent analyses. In these cases, the next simpler model available (TrN) (Tamura & Nei 1993) with shape-parameter (α) of the gamma distribution of site substitution heterogeneity was used to correct genetic distances whenever possible. To describe genetic diversity of all samples and for samples of each locality, haplotype diversity (h) and nucleotide diversity (π) (Nei 1987) were estimated with ARLEQUIN version 3.1 (Excoffier 2005). Genetic distances between samples were calculated as net nucleotide differences (Da) (Nei & Lin 1979) with ARLEQUIN. Genetic differentiation was calculated by means of pairwise Φ_{ST} values (Excoffier *et al*. 1992). The values and corresponding probabilities were obtained with 10 000 permutations with ARLEQUIN and the alpha value was Bonferroni-corrected for multiple tests (Rice 1989). A pattern of isolation by distance (Wright 1943; Kimura & Weiss 1964) was tested for by a Mantel test with 10 000 permutations with ARLEQUIN. Pairwise matrices used consisted of Φ analogues of linearized pairwise F_{ST} values ([$\Phi_{ST}/(1 - \Phi_{ST})$], Slatkin 1995) and pairwise natural logarithms of geographical distances between localities. Biologically relevant geographical distances between localities were estimated with the program NETPAS DISTANCE 2.0 (Seafuture Inc.) as the most direct natural shipping distances.

Phylogenetic analysis of haplotypes stayed unrooted, since sequences of the congeneric species *Sprattus antipodum* and of species of the most closely related genus *Clupea* (Lavoué *et al*. 2007) could not be reliably aligned. Phylogenetic relationships between haplotypes were visualized with the program SPLITSTREE 4.0 (Huson & Bryant 2006) based on an unrooted tree estimated by the program MRBAYES 3.1.2 (Ronquist & Huelsenbeck 2003) under the chosen model of substitution. The calculation strategy involved two runs with five chains each, sampling every 1000 generation and an automatic stop after reaching a mean standard deviation between split frequencies < 0.01. Parameters and trees were estimated from 14 480 samples after discarding the first 25%. The shape parameter (α) of the gamma-distribution as estimated by the program was used within the above calculations to correct values whenever possible.

As a second tree-reconstructing algorithm, we applied maximum parsimony as implemented in the software package PHYLIP 3.66 (Felsenstein 2006), and the robustness of the resulting topology was tested for with 1000 bootstrap replicates. As trees might be inadequate to visualize an intraspecific phylogeny, a statistical parsimony (Templeton *et al.* 1992) haplotype network was constructed with the program TCS 1.21 (Clement *et al.* 2000) under a 95% probability criterion of connection. As the network contained too many ambiguous connections between haplotypes ('loops') to be visualized properly, they were resolved by hand using criteria described in Pfenninger & Posada (2002). Population structuring was tested for with the SAMOVA approach using SAMOVA 1.0 (Dupanloup *et al.* 2002) by maximizing F_{CT}, the proportion of total genetic variance due to differences between groups of populations, and still obtaining significance of this parameter. The inferred structure was additionally tested with an analysis of molecular variance (AMOVA) (Excoffier *et al.* 1992) based on corrected Φ estimates as SAMOVA 1.0 only allows the use of uncorrected *p*-distances, which might be inadequate for sequences of the control region. Samples from localities that did not show any differentiation were pooled for analysis of their history. The mismatch distributions (Harpending 1994), the frequency distribution of numbers of pairwise differences between haplotypes of a population, and simulated distributions under the model of demographic expansion (Rogers & Harpending 1992) with corresponding parameters [$\tau = 2ut$, θ_0 and θ_1 ($\theta = 2N_{ef}u$)] were estimated for each inferred population after Schneider & Excoffier (1999) with ARLEQUIN. Goodness of fit between observed and simulated distributions was estimated by a least-square approach with 10 000 bootstrap replicates. To test for differences of observed distributions between pooled samples from localities of each inferred population, the Kolmogorov–Smirnov two-sample test of differences between empirical distributions of pairwise samples as implemented in the program STATISTICA 6.1 (Statsoft) was used. We additionally used Fu's F_S test (Fu 1997) as implemented in ARLEQUIN to detect evidence of a recent demographic expansion within each inferred population.

Results

Summary statistics

A 530-bp sequence of the 5′-end of the mitochondrial control region from 210 individuals revealed 128 haplotypes defined by 82 segregating sites (of which 52 were parsimony-informative) with 92 substitutions. All sequences were deposited at GenBank (Accession nos EU769294–EU769503). Three gaps were observed: single indels at position 37 and 44 and a large indel of 72 bp beginning at position 165 which was found exclusively within sequences of five individuals from the Gulf of Lyon. The large indel consisted of a perfect

Table 2 Summary statistics for *Sprattus sprattus* with number of haplotypes, number of segregating sites (S), haplotype diversity (*h*) and nucleotide diversity (π) with standard deviation (SD) of each sampling locality and for all samples. Abbreviation IDs are as in **Table 1**

ID	No. of haplotypes	S	$h \pm SD$	$\pi \pm SD$ (%)
BA	22	36	0.945 ± 0.033	0.94 ± 0.52
NO	26	27	0.989 ± 0.013	0.95 ± 0.53
BI	26	36	0.984 ± 0.016	0.95 ± 0.53
LI	18	16	0.894 ± 0.045	0.44 ± 0.28
AD	14	23	0.784 ± 0.078	0.89 ± 0.50
BO	21	30	0.954 ± 0.027	1.47 ± 0.78
BL	16	30	0.920 ± 0.033	1.31 ± 0.71
All	128	82	0.985 ± 0.003	1.99 ± 1.01

double duplication of positions 165–200 of the alignment. All indels were treated as single events and replaced by nucleotides representing transversions. Within the following alignment, transitions were observed four times more often than transversions as estimated by MRBAYES. A pronounced heterogeneity of substitution rate over sites was indicated by a low estimate of the shape parameter of the gamma distribution ($\alpha = 0.08$). A bias of base composition was observed (AT content = 59%).

Overall values for haplotype (*h*) and nucleotide (π) diversities were 0.98 and 1.99%, respectively. Values for *h* for localities were in the range of 0.728–0.989 (Table 2). Lowest values were observed for localities of the Mediterranean Sea (Adriatic Sea, 0.784; Gulf of Lyon, 0.894) with intermediate values for the Baltic (0.945) and the Black Sea (Bosporus, 0.954; Black Sea, 0.920) and highest values for the North Sea (0.989) and the Bay of Biscay (0.984).

Nucleotide diversities (π) of localities ranged between 0.44% and 1.47% (Table 2). Again, lowest diversities were observed within the Mediterranean with the lowest value for the Gulf of Lyon. Within the Baltic, the North Sea and the Bay of Biscay, π was intermediate and similar ($\pi = 0.94$–0.95%). The Bosporus and the Black Sea showed the highest nucleotide diversities with $\pi = 1.47\%$ and 1.31%.

Population structure and differentiation

Inferred population structure was similar with all methods used. Pairwise Φ_{ST} values and net nucleotide differences (*Da*) between localities showed the same pattern (Table 3) which was confirmed by SAMOVA and AMOVA approaches (see below). Φ_{ST} calculations based on the TrN substitution model with a gamma correction between pairwise sampling localities resulted in values between –0.018 and 0.789 (Table 3). Between the Baltic, North Sea and the Bay of Biscay nonsignificant (Bonferroni-corrected) and low values ranging

Table 3 Pairwise Φ_{ST} values between sampling localities based on 10 000 permutations (below diagonal) with significant values after Bonferroni correction (all $p \leq \alpha_{0.001}$) in bold, pairwise net nucleotide differences (Da) and (in parentheses) number of shared haplotypes between sampling localities (above diagonal). Abbreviation IDs are as in **Table 1**

ID	BA	NO	BI	LI	AD	BO	BL
BA	—	0.14 (2)	0.02 (1)	1.92 (0)	10.12 (1)	8.13 (0)	7.62 (0)
NO	0.027	—	0.08 (5)	2.00 (0)	10.09 (1)	8.78 (0)	8.19 (0)
BI	0.003	0.160	—	1.68 (0)	10.78 (0)	8.60 (0)	8.01 (0)
LI	**0.344**	**0.352**	**0.314**	—	13.26 (0)	10.98 (0)	10.37 (0)
AD	**0.676**	**0.674**	**0.689**	**0.789**	—	4.37 (0)	4.15 (0)
BO	**0.560**	**0.578**	**0.573**	**0.684**	**0.411**	—	–0.13 (6)
BL	**0.561**	**0.577**	**0.572**	**0.690**	**0.415**	–0.018	—

from 0.003 to 0.16 were calculated. Between the Bosporus and the Black Sea a negative value, not significantly different from zero, was calculated. All other comparisons resulted in higher and highly significant values after Bonferroni-correction. The highest distance as measured by Φ_{ST} was found between the two localities of the Mediterranean Sea. Corresponding pairwise distances as measured by Da resulted in a similar pattern as observed for Φ_{ST}-comparisons: low values between the Baltic, North Sea and the Bay of Biscay (0.02–0.14) and a negative value for the comparison between the Bosporus and the Black Sea (Table 3). All other comparisons yielded higher values with the highest distance between the localities in the Mediterranean Sea. Testing for a grouping of populations with the SAMOVA approach based on pairwise differences resulted in the highest significant Φ_{CT} (0.558; $P = 0.003$) for a structuring into four groups by pooling the samples from the Baltic, North Sea and the Bay of Biscay ('Northern Group') as well as the samples from the Bosporus with the Black Sea ('Black Sea Group'). The other two sampling localities from the Adriatic Sea and the Gulf of Lyon remained as single populations. Additional calculation for this structure with AMOVA using the TrN model with a gamma distribution resulted in a corrected Φ_{CT} of 0.587 ($P = 0.004$) and Φ_{ST} of 0.589 ($P = 0.000$) supporting the observed pattern of pairwise comparisons (see above) and thus a structuring into these four groups. For this structure, only 0.2% of the total genetic variance was observed among localities within groups ($\Phi_{SC} = 0.005$, $P = 0.349$).

Phylogenetic relationships between haplotypes and populations

The phylogenetic tree obtained by Bayesian analysis under the GTR + I + G model revealed two major clades with the occurrence of one intermediate haplotype (no. 92) and two divergent haplotypes (no. 90 and no. 119) (Fig. 4). This finding was supported by the statistical parsimony network (Fig. 3) where the two divergent haplotypes could not be joined into the network using the mutational connection

limit of a probability of 95%. A maximum parsimony (MP) approach only supported the separation between the two major clades with a bootstrap value of 639 (not shown). The two major clades are separated by five mutational steps (two transitions and three transversions) three of which are fixed differences between the major clades. Clade 1 consists of all haplotypes that were found within the Baltic, the North Sea, the Bay of Biscay, the Gulf of Lyon and one shared haplotype that was also found in two individuals from the Adriatic Sea. Within clade 1, no distinct phylogenetic portioning of geographical localities was found except for the haplotypes that were found in the Gulf of Lyon. Only private haplotypes were found in this locality (Table 3; Fig. 3) and their majority is located in an exclusive branch within the Bayesian tree. However, posterior probability for the first node of this exclusive branch was low (60%), the MP bootstrap approach resulted in collapsing this branch and the construction of the haplotype network resulted in many ambiguous connections between haplotypes from the Gulf of Lyon and the remaining ones of this major clade (not shown). Clade 2 exclusively consisted of haplotypes that were sampled in the Adriatic Sea, the Bosporus and the Black Sea. Most of the haplotypes that were sampled in the Adriatic Sea were found in an exclusive branch within the Bayesian and the MP majority-rule tree (posterior probability = 100%, bootstrap value = 513) and within the haplotype network (no occurrence of ambiguous connections).

Population history

The hypothesis of isolation by distance for the geographical distribution of haplotypes was not supported by the Mantel test, although the result was almost significant ($r = 0.4$, $P = 0.058$).

Pairwise comparisons between the mismatch distributions from subsequently pooled localities in the Baltic, the North Sea and the Bay of Biscay showed no differences within the Northern Group (Kolmogorov–Smirnov test; $P > 0.1$). The Northern Group has a clearly unimodal mismatch
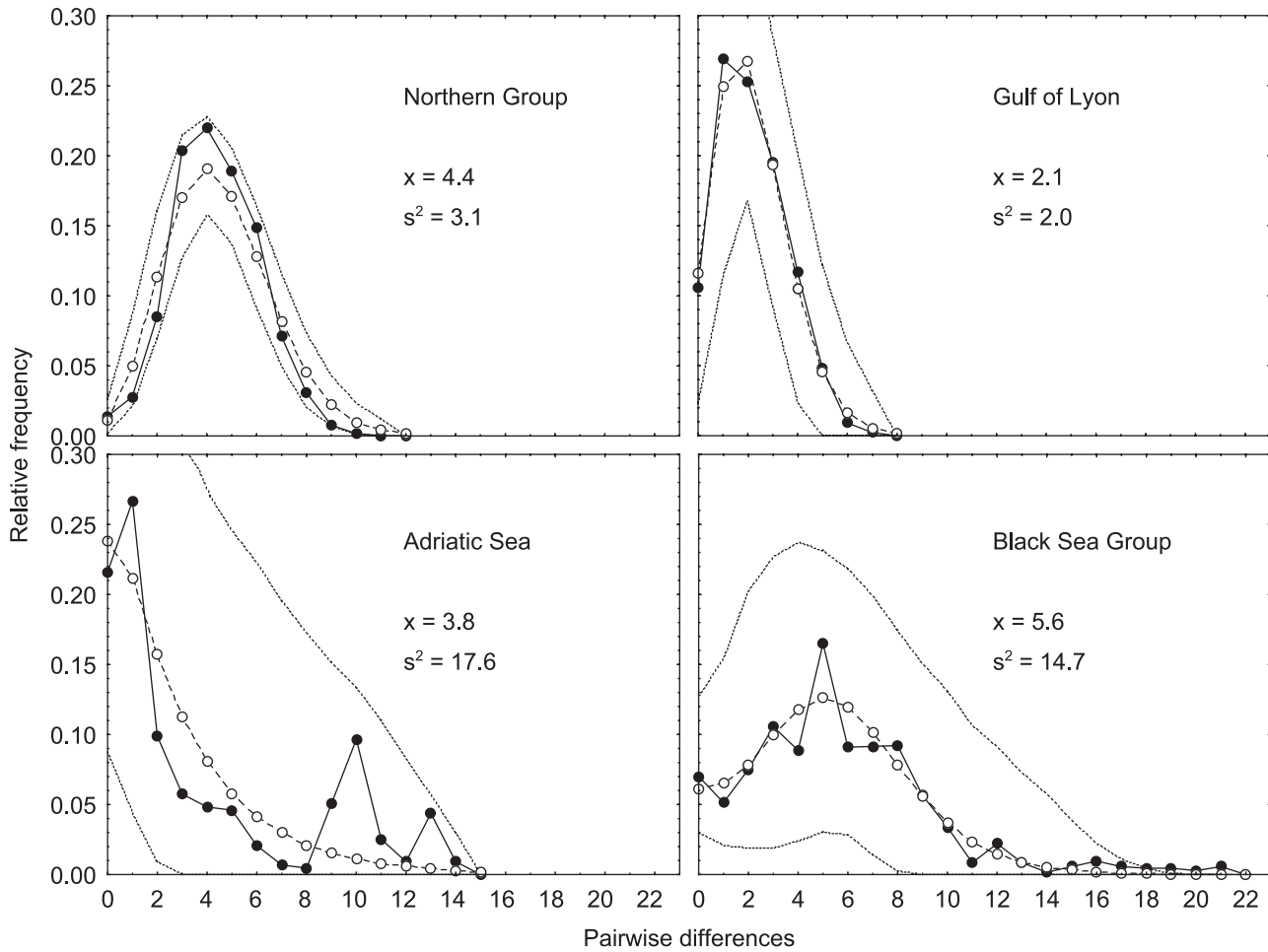
Fig. 2 Observed (filled circles, solid line) mismatch distributions for inferred populations of *Sprattus sprattus* and those simulated under a sudden demographic expansion model (open circles, dashed line). Dotted lines show the confidence intervals ($\alpha = 0.05$) of the observed distribution. Observed mean (x) and variance (s²) of pairwise differences are given for each population.

Table 4 Population statistics and parameters of the demographic expansion model for inferred populations of *Sprattus sprattus*. Abbreviation IDs are as in **Table 1**

	Fu's F_S		Demographic expansion parameters				
ID	F_S	P	τ	θ_0	θ_1	SSD	SSD-P
BA, NO and BI	−25.8	0.000	4.5	0.002	99 999	0.0048	0.031
LI	−15.3	0.000	1.9	0.221	99 999	0.0010	0.848
AD	−4.0	0.041	0.2	2.545	99 999	0.0225	0.615
BO and BL	−15.2	0.000	5.1	1.385	15.8	0.0043	0.676

distribution with a mean of 4.3 (Fig. 2) as typical for populations that experienced a recent demographic expansion, which is also indicated by the lowest value of Fu's F_S ($F_S = -25.8$, $P = 0.000$) (Table 4). The observed distribution differs significantly from a simulated expected distribution under a demographic expansion model [P(SSD) = 0.034] (Table 4), making the estimated corresponding parameters of the model incompatible with the observed distribution. Following

coalescent theory, the shape of the haplotype network contains information about the history of a population. The Northern Group has a pattern called 'star-burst' (Avise 2000), where younger 'satellite haplotypes' radiate with many mutational steps that are explained by sampled haplotypes from central ancestral haplotypes (Fig. 3).

The mismatch distribution from the samples of the Gulf of Lyon is unimodal with a steep leading face and the
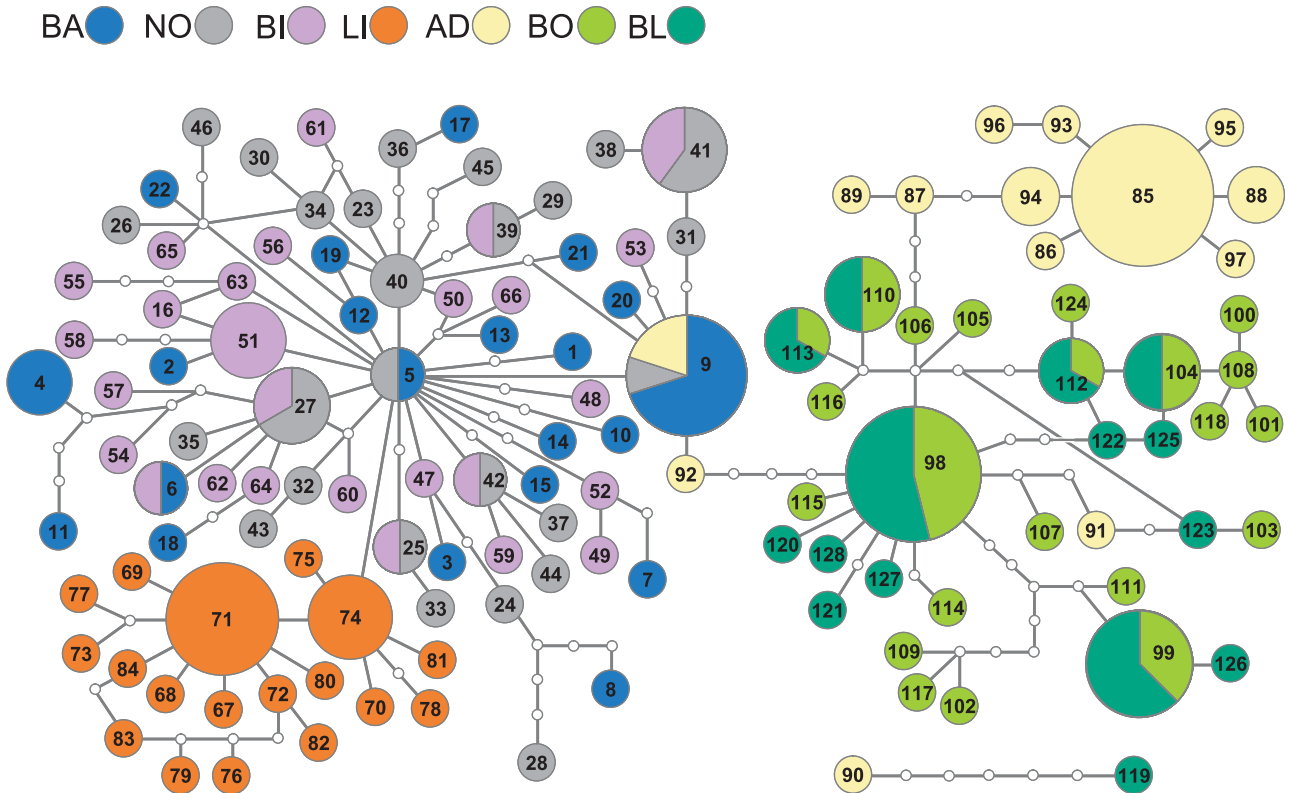
Fig. 3 Haplotype network of *Sprattus sprattus* obtained by statistical parsimony under a 95% probability of connections. Circles represent haplotypes with size proportional to their respective frequencies and numbers refer to haplotypes. Frequency within each sampling locality is indicated by colours as given in the legend. Each connection represents one mutational step; small circles represent unsampled haplotypes.

lowest observed mean of 2.1 (Fig. 2). Haplotypes differ only by few mutations from each other and originate in a star-like manner from two common haplotypes in the network (Fig. 3). A negative F_S was calculated (–15.3, $P = 0.000$) and a recent demographic expansion seems probable [P(SSD) = 0.848].

For the Adriatic Sea, a ragged mismatch distribution with a mean of 3.8 and a high variance was observed (Fig. 2). However, there was no significant deviation from the model of demographic expansion [P(SSD) = 0.615]. Most of the haplotypes from the Adriatic Sea are arranged in a star-like manner in the haplotype network with one central potential ancestral haplotype of high frequency (Fig. 3). One of the haplotypes (no. 91) is only connected to others that were sampled within the Black Sea or Bosporus population, one (no. 92) represents a connecting step to clade 1 and another one (no. 90) could not be joined to the rest of the network.

The comparison of the mismatch distributions of the Black Sea and the Bosporus samples resulted in a highly significant difference (Kolmogorov–Smirnov test; $P < 0.001$), which did not change when omitting the most divergent haplotype (hap 119) from the sample of the Black Sea. The overall mismatch distribution of the Black Sea Group is

relatively flat with a high mean of 5.6 (Fig. 2), but not significantly different from a simulated distribution under a model of recent demographic expansion [P(SSD) = 0.676]. The shape of the corresponding part within the haplotype network shows a star-burst pattern with many missing intermediate haplotypes and one divergent haplotype (no. 119) that could not be joined to the rest of the network (Fig. 3).

Discussion

Overall population structure and divergence between major clades

Four genetically distinct groups of *Sprattus sprattus* (hereafter called groups or populations) defined by a partial sequence of the mitochondrial control region were found. They are referred to as Northern Group (Baltic Sea, North Sea and Bay of Biscay), Gulf of Lyon, Adriatic Sea and Black Sea Group (Bosporus and Black Sea). This structure is supported by pairwise Φ_{ST} and Da comparisons as well as by AMOVA results. The pronouncedly disjunct geographical distribution of phylogenetically separated haplotypes further supports this picture. The lack of isolation by distance, combined
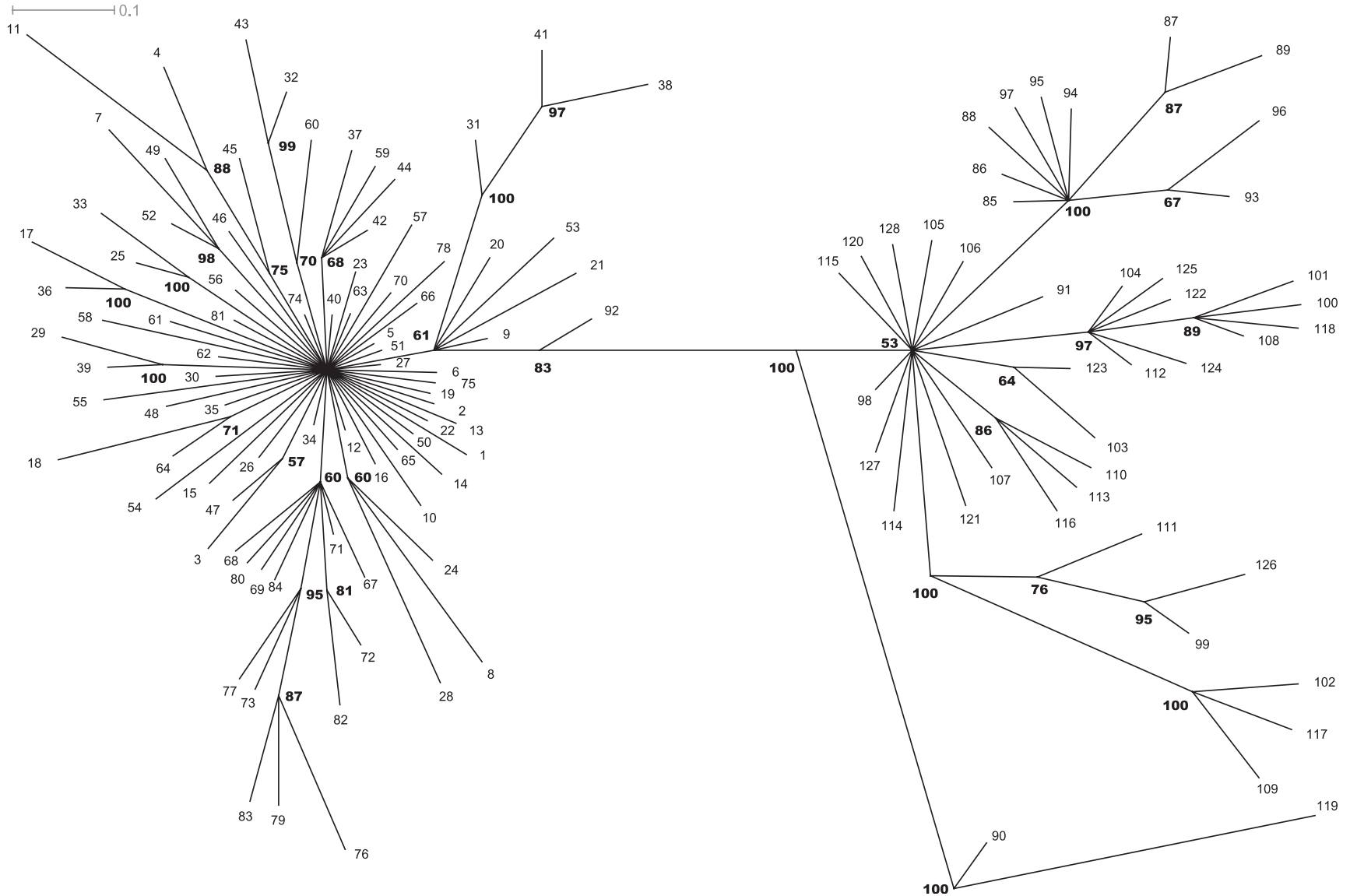
Fig. 4 Unrooted Bayesian haplotype tree of *Sprattus sprattus* obtained under the GTR + *I* + *G* model of substitution with MRBAYES. The scale shows mean expected number of substitutions per site. Bold numbers show posterior probabilities of nodes, other numbers refer to haplotypes as given in Fig. 3. The left clade almost exclusively comprises haplotypes from the Baltic Sea, the North Sea, the Bay of Biscay and the Gulf of Lyon (the latter being mainly represented by the lower branch with a posterior probability of 60). The right clade contains haplotypes from the Black Sea, the Bosporus and the Adriatic Sea. The latter is mainly represented by the upper right branch with a posterior probability of 100.

with the unusual finding of the highest genetic distance between the two Mediterranean populations, implies a complex history of this species. It seems likely that the observed pattern reflects different dispersal abilities through temporally changing physical conditions as assumed for many species within glacially affected regions (Hewitt 2000).

The geological evolution of sea basins coupled with estimates of historic key oceanographic parameters like salinity and temperature together with corresponding tolerances of this species can be a useful guide to the history of *S. sprattus*. We compared conditions of the last glacial period with the emphasis on the LGM to allow for estimates of how strongly fluctuations over glacial periods in general might have influenced the distribution of this species. No confident molecular clock calibration was possible due to absence of unambiguous fossil information. As the estimated rate of control region evolution in fish species varies greatly, we did not assume any of the proposed rates of up to 108% per million years (McMillan & Palumbi 1997).

The phylogeographical pattern is best explained by the incidence of at least two major events. The first was a split between the two major clades, the second one a split within each of them, resulting in the two different Mediterranean populations and the Northern Group and the Black Sea Group, respectively. It should be noted that the split within each major clade might have occurred at different times as divergence (in terms of mean Da or Φ_{ST}) between the Black Sea Group and the Adriatic Sea populations is at least two times higher than between the Northern Group and the Gulf of Lyon population (compare Table 3).

Warming during the last deglaciation within the western Mediterranean is assumed to have increased sea surface temperature by about 9 °C (Cacho *et al.* 2001). This very likely changed the distribution of poikilothermic species that cannot compensate for such differences. The estimated glacial absolute temperatures (winter, ~9 °C; summer, ~13 °C) (Hayes *et al.* 2005) were appropriate for sprat. Its contemporary distribution within the two northern basins of the Mediterranean is at least partly explained by lower temperatures compared to the remaining Mediterranean (Demir 1963). The Italian peninsula acts as a barrier to gene flow between the western and eastern Mediterranean at higher latitudes. This might explain the present isolation of the two major clades as sprat most likely avoids elevated temperatures within the Strait of Sicily and the Strait of Messina that connect the eastern and western basins. A similar general phylogeographical pattern with breaks between the northeastern Atlantic, the western Mediterranean Sea, the Adriatic Sea and the Black Sea was observed for the chaetognath *Sagitta setosa* (Peijnenburg *et al.* 2004) and for flounder *Plathichthys flesus* (Borsa *et al.* 1997). The cause of this pattern in *S. setosa* was thought to be the inability to maintain viable populations in suboptimal geographically intermediate areas. For flounder, sea surface temperature

was assumed to be the main force shaping this structure by limiting dispersal of early planktonic stages. Sinclair & Iles (1989) proposed a general mechanism shaping distinct populations and enhancing intraspecific differences for marine fishes where overall oceanographic conditions are crucial for the persistence of a population. This includes adequate conditions for all life stages from egg to adult, but also retention of planktonic eggs and larvae within the areas that ensure the population's survival through time. The northern basins of the Mediterranean are not only characterized by reduced salinity and temperature (Demir 1963) but also by higher primary production and oceanographic features that favour larval retention (Agostini & Bakun 2002). It can be concluded that distances between adequate habitats in the eastern and western basins would not allow for a recent gene flow between these regions. While the Strait of Sicily between the Gulf of Lyon and the Adriatic Sea is regarded as a potential spawning habitat fulfilling the criteria to support European anchovy (*Engraulis encrasicolus*) populations (Agostini & Bakun 2002), this area with summer water temperatures of 24–25 °C (Locarnini *et al.* 2006) is not suitable for sprat. Reconstruction from foraminiferal assemblages of the LGM of this region resulted in temperatures of 15–17 °C in summer and about 11 °C in winter (Hayes *et al.* 2005). Thus, past conditions might have supported a temporary sprat population and facilitated long-distance dispersal between adequate regions of the western and eastern basins.

The mechanism responsible for the divergence within clade 1 between the Northern Group and the Gulf of Lyon population cannot be definitively concluded as no samples from around the Iberian Peninsula could be obtained. But isolation between the Mediterranean and the Atlantic seems to be very likely as no spawning habitats south of the Tagus River are known to the authors. The occurrence of sprat in Moroccan waters, as described in most text books (e.g. Whitehead 1985) could not be confirmed by literature and data bank searches.

Ramalho (1921) morphometrically examined Portuguese sprat from close to the Tagus River and found a close similarity with fish originating from the Baltic Sea, North Sea and the English Channel with regard to the mean total number of scutes. The mean total number of scutes in the Baltic (33.1–33.8), the North Sea (33.3–33.6) and the English Channel (33.6–33.9) was found to be quite similar compared to those from the Portuguese coast (33.8–33.9). While sprat from the Gulf of Lyon is characterized by a lower mean number of 32.7 scutes, sprat from the Adriatic and Black Sea exhibits an even lower number of 31.3 and 31.1, respectively (Ramalho 1921 and Demir 1963, references therein). These findings correspond well with the phylogeographical structure yielded by the present study, and would preliminarily assign sprat from Portugal rather to the Northern Group than to the Gulf of Lyon population.

Within clade 2, haplotypes from the Adriatic Sea and the Black Sea Group are clearly phylogenetically separated (with the exception of haplotype 90 and 91, see below). For European flounder (*P. flesus*), also regarded as a boreal remnant within the Mediterranean Sea, a single population is assumed for the Aegean Sea and the Black Sea, but the Adriatic Sea was shown to harbour a divergent population based on allozyme and mitochondrial RFLP data (Borsa *et al.* 1997). As the distribution of sprat is discontinuous within the eastern Mediterranean Sea, with reported findings only in the northern Aegean Sea (Deval *et al.* 2002), the most probable cause of the divergent pattern revealed seems to be a barrier to gene flow between the Adriatic and the Black Sea. This barrier might be located, as in the case of the flounder, between the Adriatic and the Aegean Sea. A strong phylogenetic break that corresponds geographically to the Peloponnesian Peninsula between the Adriatic and the Aegean Sea (Nikula & Väinölä 2003) has also been shown for the lagoon cockle *Cerastoderma glaucum*. This might be indicative of a more widespread biogeographical break in this region.

Population history

The Northern Group shows the genetic imprint of a demographic expansion as inferred by a negative F_S test and the unimodal shape of the mismatch distribution, although the estimated parameters of the demographic expansion model did not fit the data as the model was significantly rejected. A star-burst shape of the haplotype network and relatively high diversity indices (overall $h = 0.99$, $\pi = 0.95\%$) indicate a long population history, as this pattern is attributed to stable populations where evolutionary time was long enough to allow for the accumulation of new haplotypes (Avise 2000). Yet, none of the sampling localities seems to have been suitable for this species at the LGM. Colonization of all these regions could not have occurred earlier than 13 000 BP when an abrupt increase in water temperatures in winter from 0 to 10 °C and an enhancement of primary production within the Bay of Biscay was estimated from sediment data (Loncaric *et al.* 1998). The entrance from the Atlantic to the southern North Sea opened around 7500 year BP during transgression (Lambeck 1995). The Baltic was for the first time connected with the North Sea at around 10 000 BP (Sohlenius *et al.* 2001) but only reached an estuarine character around 7600 BP after a long isolated freshwater phase (Lambeck 1999; Andrén *et al.* 2000). The high genetic diversity and star-burst pattern of the haplotype network of this population are most easily explained by the colonization of a large population in the course of a range expansion to the north. A complex mixture of different signals resulting from a spatial and demographic expansion could thus be responsible for the incongruence between the F_S, a unimodal mismatch distribution and the

rejection of the demographic expansion model. Additional adulteration might have resulted from the use of uncorrected distances for the respective statistics in ARLEQUIN. A violation of the infinite sites model by multiple hits for some sites might corrupt the test statistics of the mismatch distribution (Schneider & Excoffier 1999) and the F_S test (Fu 1997). An additional nondetected substructure of the Northern Group might also have enhanced the complexity of its history. For marine fishes and other species, microsatellite-based analyses often uncover population differentiation where mitochondrial sequences do not (e.g. Gonzales & Zardoya 2007). A recent analysis of microsatellites in sprat uncovered significant differences between samples of the Baltic Sea and the North Sea (Limborg *et al.* in preparation). With sequences of the mitochondrial control region in this work, a slightly lower diversity compared to the North Sea and the Bay of Biscay was observed for the Baltic Sea, which is in accordance with a general trend of reduced genetic diversity within the Baltic Sea (Johanneson & André 2006).

The population of the Gulf of Lyon bears the imprint of population growth after low effective population size. A typical star-like pattern of the haplotype network, a negative F_S value, the lowest observed nucleotide diversity ($\pi = 0.44\%$) resulting from closely related haplotypes and a relatively low haplotype diversity ($h = 0.89$) together with a unimodal mismatch distribution suggest this scenario. The estimated relative population size before population expansion ($\theta_0 = 0.221$) is the lowest observed value when ignoring the value of the rejected estimate of the Northern Group (Table 4). The lowest observed mean pairwise differences of the mismatch distribution and the lowest nucleotide diversity indicate that this population is evolutionarily young. Taking into account the occurrence of private haplotypes (Table 3), the Gulf of Lyon must be genetically isolated from all other localities studied. This population is probably an offshoot of the Northern Group as indicated by the phylogenetic relationship of haplotypes (Figs 3 and 4). It can be assumed that some individuals from a common gene pool in the Atlantic invaded the Mediterranean Sea and since then have undergone differentiation by mutation and genetic drift. The estimated relative time of coalescence within the Gulf of Lyon population is $\tau = 1.9$ and lower than the corresponding times for the Black Sea ($\tau = 5.1$) and the Northern ($\tau = 4.5$) populations (although the latter does not seem to fit the model of demographic expansion), suggesting a founder effect of ancestors of the Northern Group. If, however, the signal stems from a bottleneck rather than a founder event, the time of coalescence would not indicate the time of divergence but the time of the bottleneck. For the northwestern Mediterranean Sea, a correlation between recruitment of small pelagic fishes and river runoff, wind mixing and global environmental conditions is known (Lloret *et al.* 2001, 2004) as these are factors responsible for productivity. Combined with temperature changes on a

glacial scale (about 9 °C, see above), an ephemeral existence of a species at its contemporarily southernmost boundary seems possible. For the last 50 000 years, rapid sea surface temperature changes of 4–6 °C in less than a century have reported for this region (Cacho *et al.* 2002). Some extreme and abrupt cooling events (Heinrich events) have also been identified during the last glacial phase that affected the intensity of the basin's thermohaline circulation (Rohling *et al.* 1998). A range shift to the north of this population is impossible as this region is semi-enclosed by land masses. A repeated colonization–extirpation mechanism could thus explain the genetic pattern in relation to that of other localities and also the finding of the biggest genetic distance between the populations of the Gulf of Lyon and the Adriatic Sea.

The genetic pattern of the Adriatic population could be due to different scenarios. A cluster of closely related private haplotypes suggests genetic isolation and a recovery after low effective population size with the accumulation of new mutations that are one or two mutational steps away from a frequent ancestor (Fig. 3). The occurrence of some divergent haplotypes, which is also reflected by peaks of the mismatch distribution at high difference numbers, complicates the interpretation. Two of these haplotypes (no. 9 and no. 92) are more closely related to the Northern Group, one (no. 9) is even shared with the Baltic and the North Sea, and another one (no. 91) is more closely related to the Black Sea Group than to the private Adriatic Sea cluster. One possible interpretation is genetic exchange with both of the other populations or simply the occurrence of sequence homoplasy. Alternatively, incomplete lineage sorting from a common ancient gene pool might have occurred. Usually a relatively low haplotype diversity ($h = 0.75$) and an intermediate nucleotide diversity ($\pi = 0.89\%$) are typical of populations that underwent a bottleneck or founder event (Nei *et al.* 1975). Following this, the divergent haplotypes presently found might be the remnant of a formerly diverse group. This hypothesis may be supported by the fact that one sampled haplotype of the Adriatic Sea (haplotype 90) is more closely related to the most divergent haplotype 119 from the Black Sea than to all other haplotypes. The fact that these two haplotypes are phylogenetically isolated from all others might be an indication of very strong genetic drift (as would be expected in the case of a bottleneck or founder event) in the past. However, a hitherto unknown or extirpated population between the Adriatic and the Black Sea might also account for this pattern. During the LGM, at sea-level low stands, most of the present distribution area of sprat in the shallow northern Adriatic Sea lay dry. Estimates of ancient water temperatures in the southern Adriatic in winter are comparable to today's temperatures but in summer they were 4–6 °C lower (Hayes *et al.* 2005). Present primary production results from the enrichment through input of rivers, mainly of the Po, and temporary wind-driven upwelling (Agostini & Bakun 2002). The ancient Po dis-charged farther south into the Adriatic Sea (Arkhipov *et al.* 1995) and water enrichment might have continued throughout the glacial period, although its magnitude probably differed through time with glacial melt water and changing aridity. Since conditions never seem to have been completely inadequate for sprat, this scenario might favour a bottleneck over a founder event for the Adriatic population.

The comparison of the observed mismatch distributions of the samples from the Bosporus and Black Sea resulted in significant differences between them which might be attributed to a sampling bias or a nondetected substructure of this population. The history of the Black Sea Group is somewhat uncertain because the oceanographic history of this marginal sea is still being debated controversially. Either European sprat inhabited this region during some time of the last glacial in isolation from the Mediterranean Sea or it colonized the Black Sea after the connection between the two seas via the Marmara Sea and the Bosporus was re-established 10 500 BP. One pivotal unknown point for a proper interpretation is whether the Black Sea displayed conditions during phases of isolation from the Mediterranean Sea that would have allowed the survival of sprat. For another small clupeid, the European anchovy (*Engraulis encrasicolus*), a survival during phases of isolation was proposed based on a frequency gradient of haplotypes from two divergent clades from the Black Sea to the Atlantic (Magoulas *et al.* 1996). This 'Black Sea isolation hypothesis' was questioned after reanalysis of the data due to harsh conditions in the Black Sea during glacial times that would not have allowed anchovy to survive (Grant 2005). Air-temperature reconstructions for the Black Sea region of the last LGM are not lower than 5 °C in winter (Mudie *et al.* 2002b): corresponding water temperatures during the last isolation of the Black Sea (22 000–10 500 BP) would have surely allowed sprat to survive, in contrast to anchovy. Regarding salinity during the last isolation, estimates vary considerably. Ryan *et al.* (1997) assume conditions for this time that correspond to a salinity of 0 (Mudie *et al.* 2002a), a value that was also estimated by Llane-Serff & Rohling (1997). A freshwater-lake character of the Black Sea would have rendered the survival of sprat impossible. Mudie *et al.* (2002a) estimated an ancient salinity of around 5, which would have been tolerable for euryhaline sprat. Survival during isolation in the Black Sea as an explanation of divergent phylogenetic lineages in the eastern Mediterranean was also hypothesized for turbot (*Psetta maxima*) (Suzuki *et al.* 2004) that tolerates low salinities and temperatures as it presently occurs in the Baltic Sea under such conditions. If this possibility is excluded, sprat could not have entered the Black Sea before 10 500 BP. The present occurrence of this species in the Aegean Sea has been confirmed (Deval *et al.* 2002) and glacial temperature estimates of 9 °C (winter) and 13–15 °C (summer) for this area (Hayes *et al.* 2005) are

within the ecological range of sprat, rendering a survival in this area before 10 500 BP very likely. The star-burst shape of the haplotype network and the ragged mismatch distribution with high mean and variance would support equilibrium between drift and mutation rather than demographic expansion after low population size and thus a potential survival within the Black Sea. Since for the Northern Group a similar genetic pattern was observed, but a range expansion was inferred, the conclusion for the history of the Black Sea Group remains uncertain.

Taxonomic considerations

The subspecies concept is partly arbitrary since it is based upon 'diagnostic morphological characters' (Mayr 1963), which makes it impossible to consider subspecies real evolutionary units (Mayr 1942, 1963). From an evolutionary or population biological point of view, it is genetic integrity rather than purely taxonomic status that is of interest. A partitioning into presently assumed subspecies could not be confirmed. No genetic evidence for a subspecies in the Baltic Sea or the occurrence of one subspecies in the Mediterranean and the Black Sea could be found. Instead, two major phylogroups with a geographical break between the western and eastern Mediterranean were revealed. The population of the Baltic Sea is assumed to be a subspecies also due to adaptation to low salinity (Aps et al. 1987). Differences in adaptation as well as in morphology are more likely to be mirrored by patterns derived from nuclear markers, which is why our mitochondrial data have to be viewed with caution and only as a preliminary approach to solving this question. Interestingly, however, the diagnostic feature of mean number of postpelvic scutes, as well as the mean number of total scutes, is better reflected by our phylogeographical pattern than in the presently used subspecific taxonomy which may only be, in the case of the Mediterranean subspecies, an a priori biogeographical classification. A tendency towards a slight increase in mean number of scutes from north to south along the European coast, a somewhat lower number in the Gulf of Lyon, opposed by a sharp decrease in the Adriatic Sea and the Black Sea, seems to reflect the major phylogenetic break. Therefore, if a taxonomic subdivision makes sense at all, even if based on mean number of scutes alone, it should be made within the Mediterranean Sea, yielding a western taxon comprising sprat from the Baltic to the Gulf of Lyon and an eastern taxon comprising sprat from the Adriatic to the Black Sea.

Concluding remarks

In conclusion, it can be stated that sprat shows a pronounced range-wide population structure with phylogenetically strongly differentiated haplotypes. This phylogeography is in accordance with similar patterns observed for other species that are at their southern distribution limit within the Mediterranean Sea, such as flounder and the chaetognath *S. setosa*.

An unusual population structure within the Mediterranean Sea with the imprint of population expansion after low population size marks European sprat as trapped by northern land boundaries at the edge of its distribution range. This is in line with the hypothesis of a glacial remnant. With these findings, the Gulf of Lyon and the Adriatic Sea populations could be extremely vulnerable to changes in oceanographic parameters, especially temperature. Given the proposed trend of rising temperature for these regions (CIESM 2002), which has already influenced fish distributions in the northwestern Mediterranean Sea (Sabates et al. 2006), further studies on adaptive traits are necessary to gain insight into the physiological abilities of these populations. Still, under suboptimal environmental conditions, a high fishing pressure might accelerate extirpation of these populations. Sprat landings have already disappeared in some recent years from fishery statistics of the northwestern Mediterranean (Palomera et al. 2007) and from the Adriatic Sea (Grbec et al. 2002). Caution should be exercised when managing these populations in times of inadequate conditions.

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