



Barriers in the pelagic: population structuring of *Calanus helgolandicus* and *C. euxinus* in European waters

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ABSTRACT: Molecular studies of marine plankton have shown that ecological and/or environmental barriers play an important role in separating populations. Calanoid copepods are central in marine ecosystems, and dramatic biogeographical shifts in copepod assemblages associated with recent climate warming have been reported. We examined spatial population structuring in European waters of the Atlantic Ocean and Mediterranean Sea of *Calanus helgolandicus* and its sister species, *C. euxinus*, from the Black Sea based on genetic and morphometric characters. The aims were to identify barriers to dispersal, relate these to hydrographic characteristics and infer historical patterns of distribution and demography. We analysed a 408 bp fragment of the mitochondrial 16S gene (316 individuals), prosome to urosome length relationships (212 individuals) and sea surface temperatures obtained from 19 European sites. Estimates of genetic differentiation between samples and hierarchical analyses of molecular variance indicated strong spatial population structuring between, as well as within, basins. We identified 7 phylogeographic groups: Fjords, Oceanic inflow, NE Atlantic/Tyrrhenian, Adriatic, Mljet Island, Aegean, and Black Sea, which explained 39.7% of the total genetic variation. Based on genetic data, *C. euxinus* is considered to be a differentiated population within the *C. helgolandicus* distribution range because the most important genetic barrier separates western and eastern Mediterranean populations. Morphometric barriers largely reflect sea surface temperature barriers and are not congruent with the main genetic barriers. Contrary to recent findings for *C. finmarchicus*, we conclude that *C. helgolandicus*/*C. euxinus* populations are not connected by high levels of dispersal and have been vulnerable to past climatic changes.

KEY WORDS: *Calanus helgolandicus* · *Calanus euxinus* · 16S rDNA · Mediterranean Sea · North Atlantic Ocean · Black Sea

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INTRODUCTION

Little is known about the origin and maintenance of pelagic (open ocean) biodiversity. Understanding evolutionary processes in the pelagic realm is particularly difficult because barriers to gene flow are often obscure and pelagic taxa typically lack fossil records (Angel

1993, Norris 2000). Molecular markers can provide important insights into fundamental questions, such as those raised by Fleminger & Hulsemann (1987, p. 46): 'Does local patchiness in zooplankton concurring with environmental selection pressures lead to adaptive consequences of evolutionary significance? And more generally, at what geographical scale do we find evi-

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dence of genetic change in conspecific planktonic populations, and what is the significance of such changes relative to the breadth and width of pelagic ecosystems?' Molecular studies of marine plankton have challenged the long standing view of widespread panmictic populations that result in relatively few and slowly evolving species by showing that ecological and/or environmental barriers play an important role in separating populations (e.g. De Vargas et al. 1999, Bucklin et al. 2000a, Goetze 2003, Peijnenburg et al. 2004).

Connectivity (the extent to which populations are connected through exchange of individuals) among populations can be affected by a range of factors, the most obvious of which are physical barriers to dispersal. Biological factors may also play an important role in determining population structuring (e.g. Marshall et al. 2010), but some counterintuitive results have been reported. For instance, Patarnello et al. (2007) investigated the significance of the Atlantic–Mediterranean transition zone across physical barriers such as the Gibraltar Strait and Almeria–Oran Front (AOF) by reviewing 75 genetic studies of marine taxa ranging from plants to marine mammals. They found no obvious relationship between either dispersal ability or life history traits and genetic structuring, and reported patterns of no differentiation (32 studies), weak differentiation (2 studies) and strong differentiation (41 studies) between Atlantic and Mediterranean populations. Similarly, Galarza et al. (2009) who compared gene flow patterns of 7 littoral fish species across 2 oceanographic fronts in the western Mediterranean (the AOF and the Balearic Front) reported diverse patterns for different species and found no correlation between dispersal potential or any life history trait and degree of genetic structuring. Clearly, determining the relative influence of physical and biotic factors on genetic connectivity in marine populations is not a trivial task.

Members of the genus *Calanus* are among the largest copepods and play critical roles in marine ecosystems as grazers of microplankton and as a major food source for larvae, juveniles and adults of commercially important fishes (e.g. Meyer-Harms et al. 1999, Gislason & Astthorsson 2002). Additional interest in *Calanus* populations has come from analysis of long time series such as the Continuous Plankton Recorder survey (CPR) that has shown dramatic biogeographical shifts in calanoid copepod assemblages (Beaugrand et al. 2002, Hays et al. 2005). For example, Beaugrand et al. (2002) showed that in the North Sea, species associated with warm waters such as *C. helgolandicus* have expanded north by 10° of latitude during the last 50 yr.

This study focuses on *Calanus helgolandicus* (Claus 1863) and its sister species in the Black Sea, *C. euxinus* (Hulsemann 1991). The distribution of these species covers a wide range of habitats from open ocean to

coastal environments (Bonnet et al. 2005 and references therein). *C. helgolandicus* occurs from neritic waters off eastern North America across the temperate North Atlantic Ocean to the northern Mediterranean Sea. In the western Mediterranean Sea its abundance is generally low and it appears mainly during winter months, residing at greater depths during the remainder of the year (Andersen et al. 2001). The species dominates the northern shelf and slope regions, but has also been reported on the southern coasts (off Tunisia) probably transported with Atlantic waters through the Strait of Gibraltar. In the eastern Mediterranean, the species is common in the Adriatic and Ionian seas, but it is only present seasonally and in small numbers in the Aegean Sea. The species has also been reported in the Levantine basin, but seasonal reproduction in this basin remains uncertain. In the Atlantic Ocean, *C. helgolandicus* diapauses at great depths (~1000 m) during the winter months (Williams & Conway 1988, Bagoieien et al. 2001), whilst in the Mediterranean Sea, the species probably 'oversummers' at great depths (>1000 m) to avoid high temperatures (Scotto di Carlo & Ianora 1983, Weikert et al. 2001). *C. helgolandicus* and *C. euxinus* are considered cold water or 'boreal' elements in the Mediterranean and Black Sea faunas, which colonised the Mediterranean basin from the Atlantic during glacial periods of the Pleistocene (Angel 1979, Furnestin 1979, Bianchi & Morri 2000). *C. euxinus* is endemic to the Black Sea and was identified as a different species based on differences in prosome to urosome length relationships and integumental pore signatures (Fleminger & Hulsemann 1987). It is worth noting that hydrographic exchange and environmental conditions differ considerably across the distribution of these species and this potentially allows for the adaptive evolution of populations.

The first genetic studies of *Calanus helgolandicus* focussed on molecular identification of co-occurring *Calanus* species in the North Atlantic Ocean (Bucklin et al. 1999, Lindeque et al. 1999). Two more recent phylogeographic analyses of *C. helgolandicus* and *C. euxinus* (Papadopoulos et al. 2005, Unal et al. 2006) reported significant population structuring between Atlantic, Mediterranean and Black Sea populations but could not resolve population structuring within basins because of limited sampling. Based on coalescent analyses of mitochondrial DNA (mtDNA) sequences of cytochrome oxidase I (COI), Papadopoulos et al. (2005) concluded that divergences between NE Atlantic, Mediterranean, and Black Sea populations dated back to the middle Pleistocene. Similar results have been reported for populations of blue mussel *Mytilus galloprovincialis* (Ladoukakis et al. 2002), a planktonic chaetognath, *Sagitta setosa* (Peijnenburg et

al. 2004), common shrimp *Crangon crangon* (Luttikhuisen et al. 2008), and European anchovy *Engraulis encrasicolus* (Magoulas et al. 2006). Papadopoulos et al. (2005) and Unal et al. (2006) both suggested that none of the *Calanus* populations were singled out based on genetic data and therefore called the species status of *C. euxinus* into question. In the present study, we consider *C. euxinus* as the Black Sea population of *C. helgolandicus*, which may or may not have diverged enough to represent a different biological species. To test this, crossing experiments would be necessary, which have not yet been carried out.

The present study has built on the network of sampling stations established by Bonnet et al. (2005) and uses a more intense sampling scheme compared with previous studies, both in terms of geographic and temporal coverage, and numbers of individuals analysed. We collected sequence data for a portion of the mitochondrial 16S gene together with morphometric measurements (prosoma and urosome length) and environmental data obtained from the same sites. The aims of this study are to (1) assess spatial population structur-

ing of *Calanus helgolandicus* and *C. euxinus* based on genetic and morphometric characters, (2) reveal barriers to dispersal and relate these to hydrographic characteristics and (3) infer historical patterns of distribution and demography to provide insights into previous responses of these species to climatic fluctuations in the Pleistocene.

MATERIALS AND METHODS

Sampling. Copepods were collected from 19 sites throughout Europe encompassing the NE Atlantic Ocean, Mediterranean Sea and Black Sea at different times of the year between 1999 and 2006 (Table 1, Fig. 1A). Female *Calanus helgolandicus* (*C. euxinus* in the Black Sea) were collected by net tows and preserved in 95% ethanol.

Molecular analysis. A 408 bp fragment of the 16S mitochondrial gene was amplified for 316 individual copepods (between 4 and 59 copepods per site, Table 1). Each copepod was removed from ethanol and re-

Table 1. *Calanus helgolandicus* and *C. euxinus*. Sampling information including sampling site, location, date, collector's name, June and December mean SST ($^{\circ}\text{C}$), and number of copepods sequenced (n) and measured (N). Estimates of genetic diversity based on 16S mitochondrial DNA sequences are given per sample as nucleotide diversity, π ($\times 10^{-3}$), and haplotype diversity (h)

Site	Latitude	Longitude	Sampling date	Collector	June SST	December SST	n	N	π ($\times 10^{-3}$)	h
Raunefjord	60° 16.0' N	05° 14.0' E	June 2006	J. C. Nejtgaard	11.2	8.1	19	1	1.6	0.56
Gulmarfjord	58° 17.5' N	11° 30.6' E	March 2006	P. Thor	13.1	7.7	18	4	2.5	0.62
Faroe	60° 28.0' N	09° 23.0' W	July 2006	E. Gaard	10.1	8.3	16	2	6.0	0.54
Gijón	43° 34.8' N	05° 36.5' W	May to Sept 2005	J. Cabal	16.1	13.3	22	15	5.5	0.71
Lisbon	38° 40.0' N	09° 26.1' W	May 2005	A. Dos Santos	16.8	14.1	4	10	0.0	0.00
Stonehaven	56° 57.8' N	02° 06.2' W	September 2005	K. Cook	10.2	8.5	13	20	1.1	0.29
Dogger Bank	55° N & 56.4° N	03° E & 05.39° E	August 2005	S. H. Jónasdóttir	12.2	8.6	23	14	2.3	0.46
Helgoland	54° 11.2' N	07° 54.0' W	August 2005	R. Diekman	12.7	7.3	14	8	2.1	0.27
Plymouth	50° 15.0' N	04° 13.0' W	June 2005 & January 2006	D. Bonnet, L. Yebra	14.0	11.6	59	14	2.0	0.42
Roscoff	48° 45.0' N	03° 58.0' W	August 2005	S. Poulet	13.7	11.4	14	20	1.8	0.27
San Sebastián	43° 29.6' N	02° 41.7' W	June 2004	A. Albaina	17.2	13.9	16	16	0.9	0.24
Tyrrhenian	40° 48.5' N	14° 15.0' E	April 2006	M. G. Mazzocchi	19.5	15.0	12	–	3.6	0.56
N Adriatic	44° 57.5' N	12° 42.5' E	March 2006	M. G. Mazzocchi	20.6	14.0	14	15	4.3	0.67
Trieste	45° 42.0' N	13° 43.0' E	January 2006	V. Tirelli	21.5	12.1	14	20	4.0	0.82
S Adriatic	42° 37.0' N	18° 06.0' E	June 2005	D. Lučić	20.1	15.9	11	3	3.0	0.76
Ionian Stns 157 & 73	38° 11.6' N & 38° 09.7' N	20° 56.2' E & 22° 53.5' E	June 1999	A. Ramfos	23.5	18.2	6	2	4.4	0.80
Mljet	42° 46.0' N	17° 22.0' E	June 2005	D. Lučić	21.7	12.9	15	20	5.7	0.79
Aegean Stns 12 & 24	39° 06.3' N & 38° 45.6' N	23° 19.3' E & 23° 11.1' E	May 1999	A. Ramfos	20.2	16.2	9	20	10.6	0.94
Black Sea Stns i & vi	43° 09.8' N & 44° 09.9' N	28° 50.0' E & 29° 30.1' E	June 2006	L. Kamburska	19.0	11.0	17	8	1.4	0.33

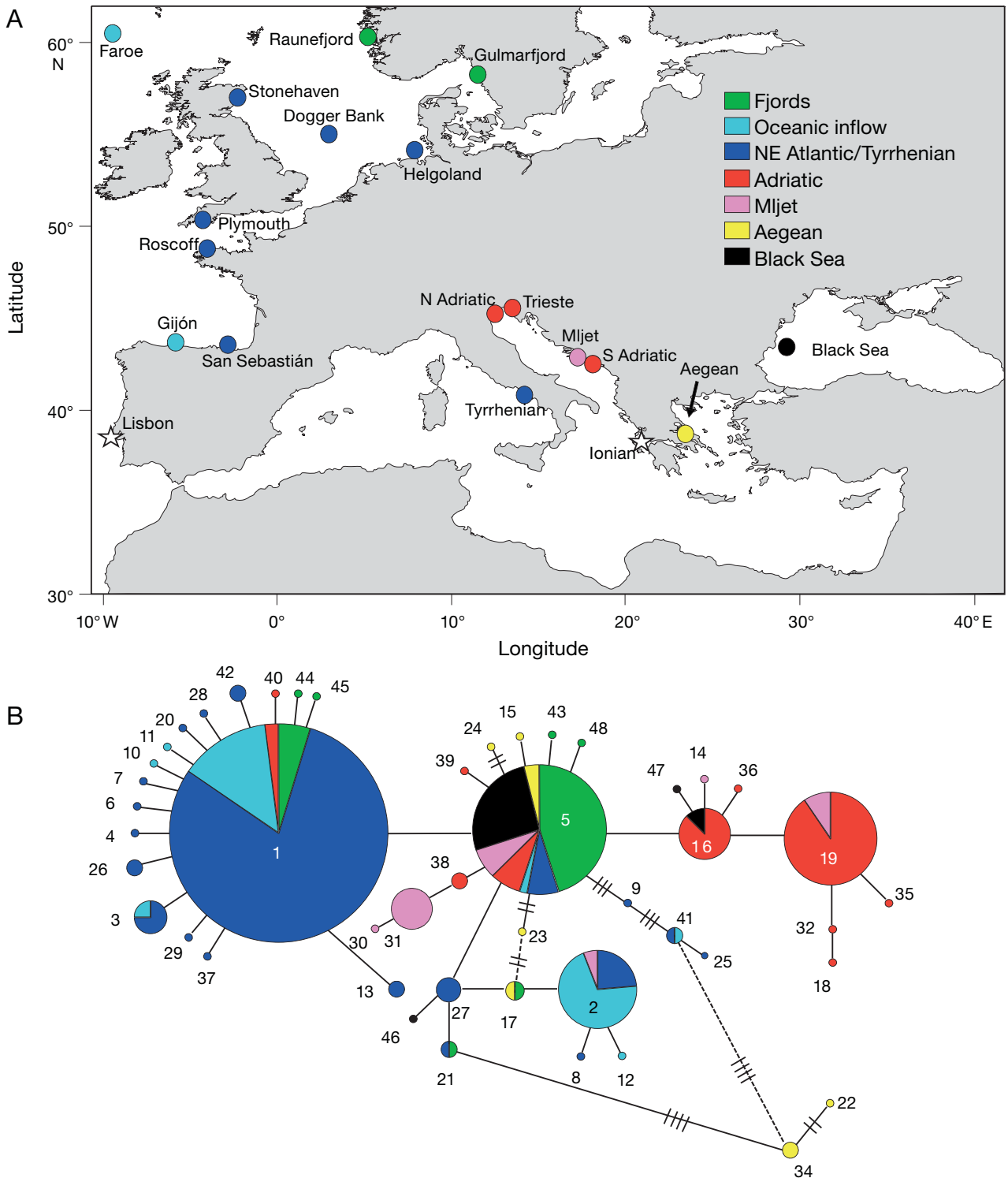


Fig. 1. *Calanus helgolandicus* and *C. euxinus*. (A) Location of sampling stations. Different colours indicate 7 phylogeographic groups: Fjords, Oceanic inflow, NE Atlantic/ Tyrrhenian, Adriatic, Mljet Island, Aegean Sea, and Black Sea. The Lisbon and Ionian samples could not be assigned to a phylogeographic group because of low sample sizes and are shown by star symbols (see text). (B) Minimum spanning network showing evolutionary relationships among 47 mitochondrial 16S haplotypes of *C. helgolandicus* (NE Atlantic and Mediterranean) and *C. euxinus* (Black Sea). Colours correspond to phylogeographic groups shown in (A). Each circle represents a single haplotype and its diameter is proportional to the number of individuals carrying that haplotype, with the smallest circles representing 1 individual. Each line in the network represents a single mutational change; dashes on lines connecting haplotypes represent number of base substitutions >1 between haplotypes

hydrated in 0.5 ml Milli-Q water overnight at room temperature. After rehydration, each individual was placed into a 0.5 ml microcentrifuge tube containing 32 μ l Milli-Q water and 5 μ l 10 \times JumpStart *Taq* DNA polymerase buffer (Sigma Aldrich). Copepods were homogenised with a pellet pestle motor (Anachem) and incubated overnight at 4°C. After incubation, 13.5 μ l master mix [5 μ l of 2mM deoxynucleoside triphosphate (dNTP) (Promega), 4 μ l of each primer (10 μ M): 16SAR (5'-CGC CTG TTT AAC AAA AAC AT-3', Palumbi & Benzie 1991) and 16SB2R (5'-ATT CAA CAT CGA GGT CAC AAA C-3', Lindeque et al. 1999) and 0.5 μ l Jumpstart *Taq* DNA polymerase (Sigma Aldrich)] were added to each tube. Amplification was carried out in a thermocycler (GeneAmp PCR system 9700, Applied Biosystems or PTC-100, MJ Research) following the cycling parameters described in Lindeque et al. (1999). Aliquots (5 μ l) of the amplification reactions were checked by agarose gel electrophoresis. PCR products (2.5 μ l) were cleaned before sequencing by adding 1 μ l ExoSapIT and incubating at 37°C for 15 min and at 80°C for 15 min. Cleaned amplification products were sequenced in both directions with a cycle sequencing kit (BigDye Terminator v. 3.1, Applied Biosystems) on a gene sequencer (ABI 3100, Applied Biosystems).

DNA sequence analysis. All polymorphic sites were manually double-checked and resolved by comparing forward and reverse chromatographs. Sequences were unambiguously aligned with the DnaSP program (Rozas et al. 2003). We estimated genetic variation per sampling site as haplotype diversity, h , (Nei 1987) and nucleotide diversity, π (Tajima 1983, Nei 1987) in Arlequin v. 3.5 (Excoffier et al. 2005). A minimum spanning network of 16S haplotypes was also constructed. Since our data set did not show any signs of sequence saturation, simple pairwise differences between haplotypes were used as a measure of genetic distance. To test for spatial genetic structuring of samples, we used 2 approaches. (1) We calculated pairwise Φ_{ST} values between sampling sites (tested by 10 000 permutations as implemented in Arlequin). (2) We performed hierarchical analyses of molecular variance (AMOVA) in Arlequin to examine the partitioning of the total variance between various groups of samples. Using these approaches, we identified 7 phylogeographic groups. Remaining analyses were carried out on pooled samples per phylogeographic group. Demographic history was examined in terms of mismatch distributions, i.e. the distribution of the observed number of differences among all pairs of haplotypes in Arlequin. Population expansions produce a wave in the mismatch distribution creating a unimodal distribution. L-shaped mismatch distributions with a high probability of identical sequences are expected for populations that have recently undergone a population or genetic bottle-

neck. By contrast, multi-modal distributions are expected for samples drawn from populations with constant population sizes (Rogers & Harpending 1992). To test for deviations of the neutral model (selective neutrality and population equilibrium) we computed Tajima's D (Tajima 1989) and Fu's F_s (Fu 1997) test statistics. The significance of empirical values of D and F_s were tested by comparison of observed values to null distributions generated by 10 000 permutations in Arlequin. Uncorrected genetic distances (p -distances) between all haplotypes were calculated in PAUP v. 4.0b10 (Swofford 1998).

Morphometric analysis. A total of 212 undamaged adult *Calanus* females were selected for morphological analysis from the same samples that were used in genetic analyses (but different individuals). Unfortunately, some samples (e.g. Tyrrhenian Sea) contained no or few adult females (Table 1). Prosome and urosome lengths were measured under a binocular microscope with an eye-piece graticule. Since size is known to vary according to temperature and food conditions, we focussed on prosome and urosome length relationships as suggested by Fleminger & Hulsemann (1987). The significance of the Pearson coefficients of the linear regressions was tested to determine the correlation between prosome and urosome lengths within each group (Student's t -test). The slopes and the origin ordinates of the linear regressions were compared between the groups by means of an analysis of covariance.

Geographic analysis. To visualise where areas of abrupt change (barriers) in the genetic or morphological composition of samples were geographically located and to possibly link these with environmental parameters, we used Monmonnier's maximum difference algorithm as implemented in Barrier v. 2.2 (Manni et al. 2004 and references therein). This algorithm finds the edges associated with the highest rate of change in a given distance measure, which can be genetic, morphological or environmental (we used sea surface temperature, SST). The algorithm is applied to a geometric network that connects all samples using Delaunay triangulation followed by Voronoi tessellation (further details in Manni et al. 2004). The first boundary is traced perpendicular to the edges of the triangulation, starting from the edge for which the distance value is maximum and proceeding across adjacent edges until the barrier has reached the limits of the triangulation or closes on itself. The next barriers are constructed one after another in a hierarchical order. We chose to present 6 genetic barriers because these barriers coincided with significant Φ_{ST} values between samples. Distance matrices (Euclidean distance) of prosome/urosome ratio and SST (composite of June and December averages, Table 1) were computed with PRIMER 6 software (Clarke & Warwick 1994).

RESULTS

Genetic diversity and population structuring

We found 48 different haplotypes in a total data set of 316 mitochondrial 16S sequences collected from samples across the distributional range of *Calanus helgolandicus* and *C. euxinus* (Table 1, Genbank accession numbers FJ861604 to FJ861653). Sixteen of these haplotypes were shared between individuals and 32 represented unique haplotypes, resulting in a highly skewed haplotype distribution (Table A1 in the Appendix). Haplotype diversities ranged from 0.24 (San Sebastián) to 0.94 (Aegean Sea) and nucleotide diversities ranged from 0.0009 (San Sebastián) to 0.0106 (Aegean Sea), excluding the Lisbon sample because only 4 individuals were sampled, which had identical haplotypes (Table 1).

Evolutionary relationships among haplotypes are shown in Fig. 1B as a minimum spanning network with the main phylogeographic groups indicated in different colours as described in the next paragraph. Two samples could not be confidently assigned to a phylogeographic group because of low sample sizes (Lisbon and Ionian Sea) and were removed from the network. Two of 5 alternative connections were resolved based on criteria derived from coalescent theory (Pfenninger & Posada 2002). We kept 2 alternative connections (dotted lines) resulting in loops connecting haplotypes 17 (H17) and H27 and related haplotypes to the 2 most common haplotypes H1 and H5. A third alternative connection was kept linking the highly divergent haplotype H34 to H41. Haplotype 1 (H1) is the most abundant haplotype in the NE Atlantic Ocean and Tyrrhenian Sea and is surrounded by several closely related unique or low frequency haplotypes resulting in a star-like structure. H5 is the central haplotype in the network and found in all geographic areas making it the most likely ancestral haplotype. It is also the most abundant haplotype in the Fjords and Black Sea samples. Haplotype 2 represents a divergent lineage (3 and 4 mutational steps away from H5 and H1, respectively) and is most commonly found in the more western Atlantic samples (Faroe Islands and Gijón) termed 'Oceanic inflow' group. Other area-specific haplotypes include H16 and H19, which are most commonly found in the Adriatic Sea, H31, which is unique to the Mljet population, and H34, which is on a long branch and unique to the Aegean Sea.

Estimates of genetic differentiation between samples (pairwise Φ_{ST} , Table 2) and hierarchical AMOVA (Table 3) indicated strong spatial structuring between as well as within basins. Of the total mitochondrial variance, 28.1% was due to genetic differentiation among 4 basins (NE Atlantic, W and E Mediterranean,

and Black Sea; $p < 0.001$) and 13.6% and 58.3% were due to variances among samples within basins and within samples, respectively (Table 3). Interestingly, the Tyrrhenian sample (located in the W Mediterranean basin) did not significantly differ from NE Atlantic samples but did show significant differentiation from all samples located in the E Mediterranean basin (Φ_{ST} between 0.236 and 0.580). Within the NE Atlantic, the 2 fjord samples were significantly differentiated from all other samples (Φ_{ST} between 0.171 and 0.401) but not from each other, and the Faroe and Gijón samples were marginally differentiated from other Atlantic samples as well (Φ_{ST} between 0.106 and 0.223). The Lisbon sample was neither significantly differentiated from Faroe and Gijón nor from other NE Atlantic samples. Within the E Mediterranean basin, N Adriatic and S Adriatic populations were not genetically differentiated from each other (Φ_{ST} ranging from negative values to 0.031). However, the populations from the marine lake of Mljet Island and the Aegean Sea were significantly differentiated from the Adriatic samples and from each other (Φ_{ST} between 0.209 and 0.368). The Ionian sample did not significantly differ from any of the E Mediterranean samples, probably because of low sample size. We obtained a total of 7 phylogeographic groups—Fjords, Oceanic Inflow, NE Atlantic/Tyrrhenian, Adriatic, Mljet, Aegean, and Black Sea—that explained 39.7% of the total genetic variation ($p < 0.001$, Table 3). Average genetic distance within phylogeographic groups ranged from 0.14% in the Black Sea to 1.06% in the Aegean Sea, and between phylogeographic groups ranged from 0.20 between Fjords and Black Sea to 1.01% between Mljet and Aegean Sea (Table A2). Average genetic distance between *Calanus helgolandicus* and *C. euxinus* sequences was 0.39%.

Demographic history and neutrality

Mismatch distributions together with the results of neutrality tests clearly indicated different genetic signatures for the 7 phylogeographic groups (Fig. 2). Unimodal distributions of pairwise differences with the largest proportion in the '0' category (meaning that most haplotypes were the same) and significant deviations from neutrality were found for the Fjords and NE Atlantic/Tyrrhenian samples. Such genetic signatures are indicative of a recent population expansion, population bottleneck, founder event, selective sweep or a combination of these factors. By contrast, multimodal mismatch distributions and nonsignificant Tajima's D and Fu's F_s were found for Oceanic Inflow, Mljet and Aegean Sea samples, suggesting more stable population sizes and/or mixing of divergent haplotypes. The

Table 2. *Calanus helgolandicus* and *C. euxinus*. Estimates of genetic differentiation between samples of *C. helgolandicus* (Atlantic and Mediterranean) and *C. euxinus* (Black Sea) based on 16S mitochondrial DNA sequence data (below diagonal: Φ_{ST} based on pairwise differences; above diagonal: p-values based on 10 000 permutations). Significant deviations of genetic homogeneity are shown in *italics* when $p < 0.05$, and in **bold** type when still remaining significant after sequential Bonferroni correction. Data outlined in boxes represent comparisons within each of 7 phylogeographic groups (see text)

Site (sample size)	Raune-fjord	Gulmar-fjord	Faroe	Gijón	Lisbon	Stonehaven	Dogger Bank	Helgoland	Plymouth	Roscoff	San Sebastián	Tyrrhenian	N Adriatic	Trieste	S Adriatic	Ionian	Mijet	Aegean	Black Sea
Raunefjord (19)		0.403	0.002	0.003	0.023	0.000	0.000	0.000	0.000	0.001	0.000	0.001	0.000	0.000	0.000	0.010	0.000	0.001	0.009
Gulmarfjord (18)	-0.008		0.006	0.005	0.011	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.022	0.000	0.014	0.058
Faroe (16)	0.209	0.171		0.752	0.335	0.043	0.026	0.050	0.005	0.100	0.028	0.066	0.000	0.000	0.000	0.011	0.000	0.026	0.000
Gijón (22)	0.226	0.183	-0.038		0.200	0.020	0.008	0.011	0.000	0.027	0.007	0.024	0.000	0.000	0.000	0.009	0.000	0.008	0.000
Lisbon (4)	0.404	0.365	0.069	0.137		1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.002	0.016	0.001	0.005	0.001	0.027	0.000
Stonehaven (13)	0.373	0.368	0.147	0.202	-0.130		0.839	0.925	0.822	0.932	0.844	0.554	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Dogger Bank (23)	0.300	0.314	0.136	0.183	-0.121	-0.031		0.765	0.730	0.859	1.000	0.261	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Helgoland (14)	0.354	0.358	0.139	0.206	-0.132	-0.027	-0.016		0.487	1.000	0.669	0.504	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Plymouth (59)	0.285	0.323	0.165	0.223	-0.103	-0.028	-0.013	-0.006		0.897	0.768	0.178	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Roscoff (14)	0.343	0.343	0.106	0.158	-0.132	-0.040	-0.035	-0.020	-0.031		0.663	0.556	0.000	0.000	0.000	0.000	0.000	0.000	0.000
San Sebastián (16)	0.409	0.401	0.178	0.228	-0.135	-0.047	-0.039	-0.019	-0.019	-0.032		0.323	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Tyrrhenian (12)	0.249	0.260	0.094	0.167	-0.103	-0.006	0.008	-0.036	0.014	-0.002	0.010		0.000	0.000	0.000	0.001	0.000	0.002	0.000
N Adriatic (14)	0.378	0.326	0.345	0.372	0.481	0.541	0.520	0.506	0.563	0.521	0.576	0.422		0.444	0.192	0.638	0.007	0.003	0.001
Trieste (14)	0.381	0.336	0.339	0.367	0.458	0.515	0.492	0.481	0.532	0.497	0.552	0.398	-0.014		0.230	0.781	0.001	0.000	0.000
S Adriatic (11)	0.599	0.532	0.479	0.498	0.688	0.715	0.666	0.666	0.690	0.687	0.744	0.580	0.031	0.021		0.126	0.001	0.000	0.000
Ionian (6)	0.257	0.192	0.228	0.264	0.430	0.504	0.439	0.438	0.475	0.461	0.554	0.312	-0.048	-0.066	0.099		0.055	0.168	0.031
Mijet (15)	0.279	0.226	0.294	0.310	0.388	0.463	0.463	0.452	0.523	0.449	0.499	0.376	0.209	0.269	0.368	0.151		0.004	0.001
Aegean (9)	0.249	0.172	0.148	0.188	0.210	0.345	0.356	0.325	0.455	0.333	0.381	0.236	0.221	0.252	0.334	0.082	0.212		0.003
Black Sea (17)	0.132	0.047	0.312	0.311	0.663	0.616	0.514	0.570	0.500	0.574	0.647	0.456	0.316	0.341	0.548	0.207	0.222	0.238	

Adriatic Sea and Black Sea groups have an intermediate distribution with mixed results for the 2 neutrality tests. The Adriatic Sea has a unimodal mismatch distribution, suggestive of a population expansion, but with a higher mean number of pairwise differences than Fjords and NE Atlantic/Tyrrhenian samples, suggesting that the population expansion may be older (assuming equal mutation rates among samples). The Black Sea has a bimodal mismatch distribution but with a very high proportion of identical sequences.

Morphometric diversity

Individual prosome and urosome lengths showed geographic as well as seasonal variation. The largest individuals were collected in the Black Sea (June, 2.46 ± 0.10 mm, average \pm SD) and Stonehaven (September, 2.55 ± 0.08 mm) and the smallest individuals were collected in the southern Adriatic Sea (June, 1.98 ± 0.05 mm) and Aegean Sea (May, 2.05 ± 0.08 mm) (data not shown). We also observed large seasonal variation in length from Plymouth samples collected every week in the year 2005 with the largest individuals collected in May (2.61 ± 0.12 mm) and the smallest individuals collected in September (2.11 ± 0.11 mm, data not shown). For each of the 7 genetically differentiated groups, we found a significant positive relationship between prosome and urosome length except for the Black Sea. Since the slopes and origins did not significantly differ between groups ($F_{5,200} > -6.64$, $p > 0.05$) we pooled all Atlantic and Mediterranean samples and obtained a single significant regression between prosome and urosome length ($r^2 = 0.38$, $p < 0.001$, Fig. 3). Also Black Sea samples (*Calanus euxinus*) have relatively longer prosomes compared with Atlantic and Mediterranean samples (Fig. 3). When prosome/urosome ratios are plotted against latitude we observed a weak gradient with decreasing ratios from north to south; however, this relationship was not significant (data not shown).

Geographic barriers

The strongest genetic barrier separates the E Mediterranean from the W Mediterranean and Atlantic samples (Fig. 4A). The second

Table 3. *Calanus helgolandicus* and *C. euxinus*. Hierarchical analyses of mitochondrial 16S rDNA molecular variance (AMOVA) of *C. helgolandicus* and *C. euxinus* amongst (A) 4 European basins (NE Atlantic, W Mediterranean, E Mediterranean, Black Sea) and (B) amongst 7 phylogeographic groups (Fjords, Oceanic inflow, NE Atlantic/Tyrrhenian, Adriatic, Mljet, Aegean, and Black Sea). Lisbon and Ionian samples were removed because of low sample sizes (see text). Associated with each hierarchical level are: percentage of total variance explained (%), Φ -statistics, df and associated significance levels: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ns: not significant

Source of variation	% of variance	Fixation index	Significance	df
(A) Four basins				
Among basins (17 sites, 4 groups)	28.1	$\Phi_{CT} = 0.28$	***	3
Among populations within basins	13.6	$\Phi_{SC} = 0.19$	***	13
Among populations within total	41.7	$\Phi_{ST} = 0.42$	***	289
Within populations	58.3			
(B) 7 phylogeographic groups				
Among groups (17 sites, 7 groups)	39.7	$\Phi_{CT} = 0.40$	***	6
Among populations within groups	-1.1	$\Phi_{SC} = -0.02$	ns	10
Among populations within total	38.6	$\Phi_{ST} = 0.39$	***	289
Within populations	61.4			

and third genetic barriers separate peripheral marine habitats from other samples, namely the 2 fjords from NE Atlantic samples (barrier 2) and the marine lake (Mljet) from Adriatic samples (barrier 3). Barriers 4, 5 and 6 represent marginally significant differentiation (Φ_{ST} between 0.147 and 0.238, Table 2) with barrier 4 separating the Black Sea from E Mediterranean samples and barriers 5 and 6 separating Oceanic Inflow samples (Faroe and Gijón) from other NE Atlantic samples. The strongest morphometric barriers are located in the E Mediterranean basin (Fig. 4B), with the first barrier separating Adriatic samples and the second barrier separating the Black Sea population (*Calanus euxinus*) from E Mediterranean samples. Morphometric barriers 3 and 4 probably represent

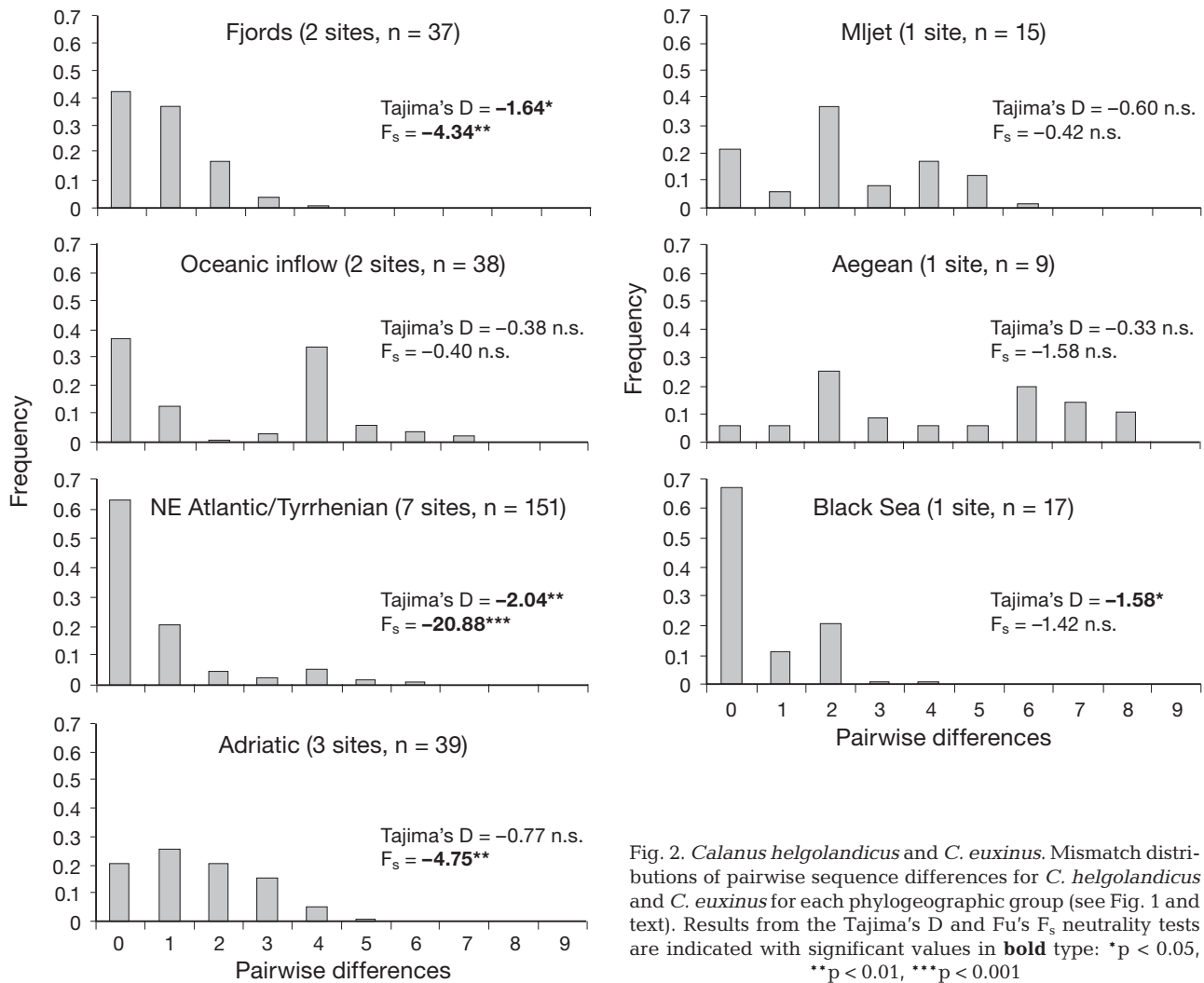


Fig. 2. *Calanus helgolandicus* and *C. euxinus*. Mismatch distributions of pairwise sequence differences for *C. helgolandicus* and *C. euxinus* for each phylogeographic group (see Fig. 1 and text). Results from the Tajima's D and Fu's F_s neutrality tests are indicated with significant values in bold type: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

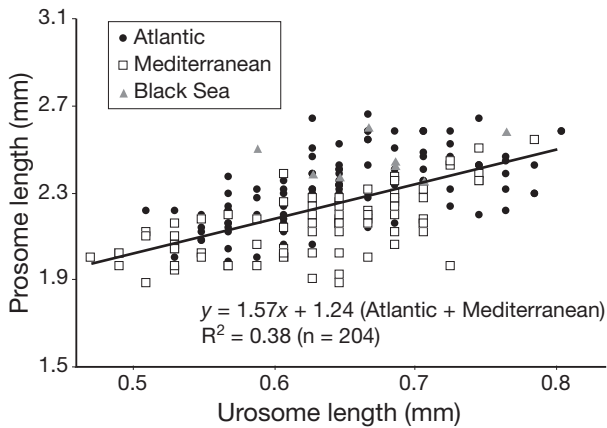


Fig. 3. *Calanus helgolandicus* and *C. euxinus*. Prosome length plotted against urosome length for *C. helgolandicus* (Atlantic and Mediterranean) and *C. euxinus* females (Black Sea). Prosome and urosome lengths were significantly positively related for each of the 7 phylogeographic groups except for the Black Sea. All Atlantic and Mediterranean samples were pooled because slopes and origins did not significantly differ between groups resulting in a single significant regression

latitudinal variation in the NE Atlantic since we observed a weak cline in prosome/urosome ratios. The fifth barrier separates the Aegean sample, which contained the smallest copepods. SST differed most strongly in the E Mediterranean (Fig. 4C), separating the Black Sea (first barrier) and the Adriatic Sea (second barrier) from the Aegean Sea similar to the steepest 2 morphometric clines. Additionally, the third and fifth SST barriers were congruent with morphometric barriers (3 and 5, respectively).

DISCUSSION

Barriers in the pelagic

In agreement with previous phylogeographic studies on the *Calanus helgolandicus*/*C. euxinus* species pair (Papadopoulos et al. 2005, Unal et al. 2006) we found highly significant genetic structuring of populations separated in different European basins. In the present study, however, we also uncovered significant spatial genetic structuring at the within-basin scale and obtained a better resolution of the geographic locations of barriers. In total, we found 7 phylogeographic groups: Fjords, Oceanic Inflow, NE Atlantic/Tyrrhenian, Adriatic Sea, Mljet Island, Aegean Sea and Black Sea. This high degree of genetic structuring suggests limited dispersal between as well as within basins which is remarkable given the fact that these copepods are holoplanktonic. The 16S is generally assumed to be a conserved mitochondrial gene (Simon et al. 1994); however, in our study we found comparable rates of

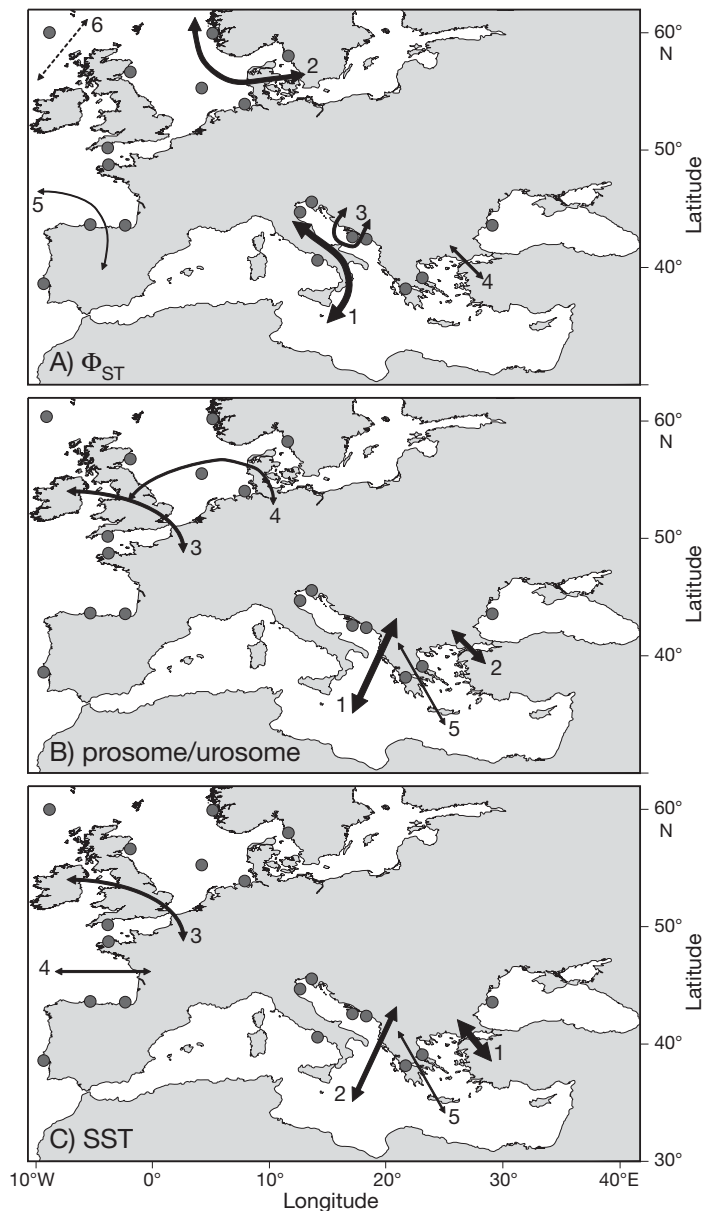


Fig. 4. *Calanus helgolandicus* and *C. euxinus*. Location of abrupt changes (barriers) in the (A) genetic (Φ_{ST}) and (B) morphometric (prosome/urosome length) composition of *C. helgolandicus* and *C. euxinus* samples, as well as (C) environmental barriers based on composite June and December SSTs. Thickness of barriers and numbers next to barriers indicate importance in decreasing order (see text for further explanations)

divergence between Atlantic, Adriatic, and Black Sea populations for 16S as for COI (average ratio of between-group comparisons for COI/16S is 1.01, see Papadopoulos et al. 2005). Similar to previous genetic studies of marine copepods we also found highly skewed haplotype distributions and rather low estimates of genetic diversity (e.g. Bucklin & Wiebe 1998, Bucklin et al. 2000a). A general limitation of this study

is that genetic inferences are based on a single mitochondrial marker, which has a large stochasticity associated with it (Edwards & Beerli 2000) and may not be neutrally or clock-like evolving (Ballard & Whitlock 2004, Galtier et al. 2009). Adding information of multiple (nuclear) loci would result in more precise estimates and may allow neutral and selective effects to be disentangled. Keeping these general limitations in mind, 16S nevertheless provided sufficient amounts of genetic variation to reveal present-day barriers to gene flow as well as signatures of past demographic events.

The most important genetic barrier in the *Calanus helgolandicus*/*C. euxinus* distribution range separates W and E Mediterranean populations. Gene flow between these populations is absent or severely limited because communication can probably only occur through the narrow Strait of Messina (3 km wide, <200 m deep) since the much wider Siculo-Tunisian Strait (160 km wide, 400 m deep) is located to the south of the species distribution range (Fleminger & Hulsemann 1987). Several other marine species show genetic differentiation between W and E Mediterranean basins (e.g. Bahri-Sfar et al. 2000, Sala-Bozano et al. 2009), but the exact locations of barriers are usually unknown because of insufficient geographic sampling and may actually differ depending on the species biology.

NE Atlantic and W Mediterranean (Tyrrhenian) populations are not genetically differentiated based on our 16S data even though the Strait of Gibraltar and the Almeria-Oran Front (AOF) are considered important phylogeographic barriers in many marine species (reviewed in Patarnello et al. 2007). Keeping in mind our limited sampling of the W Mediterranean (single Tyrrhenian sample, 12 individuals) there could be 2 explanations for this lack of structuring. (1) The NE Atlantic and Tyrrhenian *Calanus helgolandicus* populations are presently connected by high levels of dispersal. For instance, there is some evidence of transport of diapausing copepods across the Gibraltar Strait/AOF (Stöhr et al. 1996). (2) The populations are not or hardly connected, but our genetic data failed to reject homogeneity. There could be several explanations for this. Populations may not yet have reached mutation–drift equilibrium, which is plausible for extremely large zooplankton populations. Another possibility is that our genetic marker is not sensitive enough to reject homogeneity. However, it is difficult to explain that we found significant population structuring for other *Calanus* populations with the same genetic marker and similar sampling intensity. Another possibility is that a recent population bottleneck erased historical population structure and too little time has passed to allow for the accumulation of new genetic differences between NE Atlantic and Tyrrhenian populations.

The sharp genetic barrier separating W and E Mediterranean populations of *Calanus helgolandicus* is not congruent with the main morphometric barriers (prosoma/urosome length) that separate Adriatic and Black Sea (*C. euxinus*) populations from Ionian and Aegean populations. Morphometric barriers reflect to a large extent SST barriers (Fig. 4). In terms of environmental conditions (0 to 500 m depth layer) during seasons when *C. helgolandicus* prevails, the Black Sea and E Mediterranean are at opposite ends of the spectrum, with the warm (14 to 16°C) and saline (38.8 to 38.9 psu) E Mediterranean waters to the west, and the cool (7.6 to 14°C) and brackish (18 to 22 psu) Black Sea to the east (Fleminger & Hulsemann 1987). There are also important ecological, behavioural and metabolic differences between the Black Sea *C. euxinus* and Mediterranean *C. helgolandicus* (reviewed in Fleminger & Hulsemann 1987, Unal et al. 2006). This suggests that selection probably plays an important role in keeping these populations apart. Even though E Mediterranean and Black Sea populations share haplotypes there is probably hardly any contemporary gene flow between these basins because of the narrow connection through the straits of the Dardanelles, Sea of Marmara and the Bosphorus (3 km wide, <200 m deep), which results in a thousand times lower flow than through the Strait of Gibraltar (Sverdrup et al. 1942). Furthermore, the communication is essentially one way, from the Black Sea to the E Mediterranean, as the more saline E Mediterranean waters immediately sink into the deep anoxic waters of the Black Sea. We found average uncorrected sequence divergence of 16S between *C. euxinus* and *C. helgolandicus* of 0.39%, which is similar to estimates based on COI and the cytochrome *b* gene (CYTB) sequences (0.2 to 0.6%, Papadopoulos et al. 2005, Unal et al. 2006). These values are within the range typically observed for conspecific calanoid copepod populations (e.g. Bucklin et al. 1999, 2003).

An interesting finding of this study is the genetic isolation of peripheral populations, in particular the 2 fjord populations and the marine lake population of Mljet Island. It is possible that fjord populations are connected by the Norwegian Coastal current that flows northwards from the Baltic Sea to the Norwegian Sea along the coast. The warmer and more saline waters of the Atlantic Ocean flow in the opposite direction entering the northern North Sea and create a front (Otto et al. 1990). This front may prevent mixing of fjord and NE Atlantic populations. Similar haplotype frequencies for 4 to 5 different Norwegian fjords were also reported by Bucklin et al. (2000a) for *Calanus helgolandicus* and *C. finmarchicus*, but not for *Acartia clausi*. Bucklin et al. (2000a) concluded that *Calanus* spp. fjord populations represented drifting populations that may be replaced periodically, whereas *A. clausi*

populations were long-term residents in the fjords. An alternative explanation is that fjord populations are not connected by gene flow but share a recent common ancestor, and too little time has passed for genetic differences to have accumulated between fjord populations. Fjords are very young habitats and the fact that fjord populations of *C. helgolandicus* are characterized by high frequencies of the ancestral haplotype 5 support this explanation. The marine lake population of Mljet is also genetically isolated but shows a different picture. It contains a mixture of haplotypes including the ancestral H5, E Mediterranean haplotypes, and a high proportion of 2 related unique haplotypes (H30 and H31). This mixing of genetic lineages resulted in a higher estimate of genetic diversity than observed in the fjord populations even though this lake is also a young habitat (formed 4000 to 7000 yr ago, Schmidt 1993, Benovic et al. 2000). Ecologically and genetically differentiated assemblages in the Mljet lakes were also found for the moon jelly *Aurelia* sp. (Benovic et al. 2000, Schroth et al. 2002) and the planktonic chaetognath *Sagitta setosa* (K. Peijnenburg & M. Batistic unpubl. data). Strong stratification in summer differentiates the lakes, which are moderately eutrophic, from the oligotrophic surrounding S Adriatic and may result in the isolation of deeper layers (20 to 45 m) that have constantly lower temperatures (Benovic et al. 2000).

The W Bay of Biscay (Gijón sample) was genetically differentiated from NE Atlantic samples but not from Faroe and Lisbon samples. There is a fluctuating front between the general oceanic circulation (N Atlantic Central Water) and the slope current (Iberian Poleward Current) in the NW Iberian peninsula, which may be a strong hydrographic barrier isolating the W Bay of Biscay from the east and the north (Koutsikopoulos & Le Cann 1996). Furthermore, Bonnet et al. (2005) reported a mismatch in the reproductive cycles of *Calanus helgolandicus* females from the SW Bay of Biscay and Roscoff (NE Atlantic) populations lending further support to the presence of a significant barrier in this region. Oceanic Inflow samples were characterized by a large proportion of the H2 haplotype, which is at least 4 mutational steps away from the most common haplotype in the NE Atlantic. It may be that the H2 haplotype is more abundant in the mid- or W Atlantic and is transported to the NE Atlantic by the Gulf Stream. To test this, samples would be necessary from the more western part of the *C. helgolandicus* distributional range.

Biogeographic history

Many studies on both terrestrial (e.g. Hewitt 2004) and marine (e.g. Patarnello et al. 2007) faunas have

shown that the majority of contemporary genetic diversity in populations originated during the Pleistocene (1.8 million to 12 000 yr ago). Even though most studies are based on a single mitochondrial marker and estimates of population divergence times should be interpreted with caution, the congruence across many unrelated species suggests that climatic oscillations during the Pleistocene had a major impact on species distribution patterns and demographies. Hence, intra-specific genetic variation cannot be interpreted without reference to the biogeographical past. *Calanus helgolandicus* and *C. euxinus* are considered cold water or 'boreal' elements in the Mediterranean and Black Sea faunas (e.g. Bianchi & Morri 2000). In the Atlantic Ocean, *C. helgolandicus* is considered to be an indicator of more temperate waters (11.4 to 16.1°C) than is *C. finmarchicus* (6.0 to 10.6°C, Helaouët & Beaugrand 2007). It is also more typical of coastal areas (0 to 500 m) compared with *C. finmarchicus*, and is thus considered a pseudo-oceanic or distant neritic species (Bonnet et al. 2005, Helaouët & Beaugrand 2007). *C. helgolandicus* and *C. euxinus* occur where salinities are 35.7 to 38.9 psu and 18 to 22 psu (Fleminger & Hulsemann 1987), respectively, and are thus dependent on marine conditions and ocean currents for their survival and dispersal.

The dynamic geological and climatic history of the Atlantic Ocean, Mediterranean Sea and Black Sea (reviewed in Patarnello et al. 2007 and Luttkhuizen et al. 2008) have left significant genetic signatures in *Calanus helgolandicus* and *C. euxinus* populations (Fig. 2). We found the highest levels of genetic diversity in the Mediterranean basin (Aegean Sea), which may be explained by a relatively long uninterrupted marine history of up to 5.3 million yr in this basin following the Messinian Salinity Crisis. By contrast, the North Atlantic Ocean and Black Sea regions were greatly affected by the Pleistocene ice ages when temperatures and sea levels fell, resulting in sea ice conditions as far south as the Iberian Peninsula during the Last Glacial Maximum (LGM, ~18 000 yr ago) in the Atlantic Ocean, and freshwater to brackish conditions as recent as ~7000 yr ago in the Black Sea. High levels of diversity may also be explained by higher temperatures in the Mediterranean Sea. For example, latitudinal diversity gradients in marine bacteria and copepods were largely explained by temperature (Fuhrman et al. 2008, Rombouts et al. 2009). Other studies with similar geographic sampling have also reported highest levels of genetic diversity in Mediterranean populations, e.g. for shrimp (Luttkhuizen et al. 2008) and chaetognaths (Peijnenburg et al. 2004). However, for these species W Mediterranean populations had highest levels of diversity and were significantly differentiated from

Atlantic populations. Conversely, our W Mediterranean sample of *C. helgolandicus* was not significantly differentiated from Atlantic populations. Furthermore, NE Atlantic/Tyrrhenian sequences produced a star-like genealogy (Fig. 1b) and a typically L-shaped mismatch distribution, suggestive of a recent population bottleneck followed by expansion and/or selective sweep. We hypothesize that the most probable explanation for these observations is that the W Mediterranean was recently (re-)colonised from the Atlantic Ocean. However, more data are necessary to substantiate this hypothesis.

High levels of genetic diversity and multimodal mismatch distributions for the Oceanic Inflow, Mljet and Aegean populations are suggestive of more stable population sizes and/or mixing of divergent haplotypes in these areas. It may be that there were geographically separate refugia in the Atlantic and E Mediterranean basins where *Calanus helgolandicus* populations retracted and survived during glacial maxima. Pinpointing the exact locations of such refugia, however, would require much more geographic sampling and may remain elusive for such highly mobile populations as zooplankton.

Our results differ strikingly from a recent phylogeographic analysis of the congener *Calanus finmarchicus* sampled across the North Atlantic Ocean (Provan et al. 2009, but see Bucklin & Wiebe 1998, Bucklin et al. 2000b for a different result). Provan et al. (2009) concluded that *C. finmarchicus* is characterised by high levels of dispersal and has maintained a stable effective population size throughout Pleistocene climate changes. By contrast, *C. helgolandicus/C. euxinus* populations show strong genetic structuring even on small spatial scales and have been vulnerable to past climatic fluctuations as shown by strikingly different genetic signatures for phylogeographic groups. Furthermore, whereas *C. finmarchicus* probably persisted through the LGM as a single, continuous population (probably located in the NE Atlantic Ocean to the SW of the British Isles, Provan et al. 2009), *C. helgolandicus* probably survived in geographically separate refugia located in the Atlantic Ocean and E Mediterranean Sea because of the presence of distinct evolutionary lineages in both basins. If we assume similar evolutionary rates and comparable levels of selection on both species' mitochondrial DNA, the differences could be explained by the fact that distant neritic species (*C. helgolandicus*) may be more vulnerable to climate-induced range shifts and compressions than are the more widely distributed oceanic species (*C. finmarchicus*). Similarly contrasting results were found for intraspecific mitochondrial DNA variation between a neritic and oceanic chaetognath, *Sagitta setosa* and *S. elegans*, respectively (Peijnenburg et al. 2005).

Dramatic biogeographical shifts have been documented for copepod assemblages associated with recent climate warming (Beaugrand et al. 2002, Hays et al. 2005, Conversi et al. 2009). For instance, in the Gulf of Trieste (N Adriatic Sea), ecosystem-wide changes are reported that include the increase of species with more southern distributions and a decline and/or changes in phenology of cold-water species such as *Pseudocalanus elongatus* and *Calanus helgolandicus* (Conversi et al. 2009). The response of copepods to climate change will be of crucial importance because of their central roles in marine food webs. In the NE Atlantic Ocean, *C. helgolandicus* will probably continue to expand its distribution northwards at the expense of *C. finmarchicus*, even though the latter species has a wider ecological tolerance (Helaouët & Beaugrand 2007). This may result in the homogenization of the subtle population structure of Atlantic *C. helgolandicus* populations. In the Mediterranean and Black Sea basins, *C. helgolandicus/C. euxinus* populations may be particularly vulnerable to climate warming for several reasons. (1) Because the species are cold stenotherms, they are predicted to be gradually replaced by more thermotolerant species. (2) It will be impossible for species already trapped in the northernmost, coldest parts to migrate northwards. (3) The Mediterranean and Black seas are among the most impacted seas in the world, and climate change tends to interact synergistically with other disturbances (e.g. Oguz & Gilbert 2007, Lejeune et al. 2010). (4) Because we found ample evidence of population structuring and signs of local adaptation, it may not be possible for populations to simply 'track' changes in their habitat. We thus conclude that *C. helgolandicus* and *C. euxinus* are important species to monitor both ecologically as well as genetically.

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Appendix 1. Additional genetic data for *Calanus helgolandicus*/*C. euxinus*Table A1. *Calanus helgolandicus* and *C. euxinus*. Geographic distribution of 16S mitochondrial haplotypes (H) for both species. P indicates pool of unique haplotypes

Sample	H1	H2	H3	H5	H13	H16	H17	H19	H21	H26	H27	H31	H34	H38	H41	H42	P	Total
Raunefjord	4			12													2	19
Gulmarfjord	3			11			1		1								2	18
Faroe	10	5													1		0	16
Gijón	10	7	1	1													3	22
Lisbon	4																0	4
Stonehaven	11										1						1	13
Dogger Bank	17	1	1						1	1							2	23
Helgoland	12		1														1	14
Plymouth	45	2	1	3	2							1					5	59
Roscoff	12	1															1	14
San Sebastián	14									1	1						0	16
Tyrrhenian	8			1											1	2	0	12
N Adriatic				2				8						2			2	14
Trieste	3			1		3		5									2	14
S Adriatic				1		3		5									2	11
Ionian				3		1		1									1	6
Mljet		1		4				2				6					2	15
Aegean				2			1						2				4	9
Black Sea				14		1											2	17
Total	154	17	4	55	2	8	2	21	2	2	3	6	2	2	2	2	32	316

Table A2. *Calanus helgolandicus* and *C. euxinus*. Average genetic *p*-distances (in percentage) between and within (on the diagonal in **bold** text) 7 phylogeographic groups of *C. helgolandicus* (Atlantic and Mediterranean) and *C. euxinus* (Black Sea)

	n	Fjords inflow	Oceanic Tyrrhenian	NE Atlantic/	Adriatic	Mljet	Aegean	Black Sea
Fjords	37	0.20						
Oceanic Inflow	38	0.48	0.53					
NE Atlantic/Tyrrhenian	151	0.30	0.47	0.19				
Adriatic	39	0.50	0.81	0.64	0.39			
Mljet	15	0.52	0.82	0.69	0.66	0.57		
Aegean	9	0.73	0.95	0.85	0.95	1.01	1.06	
Black Sea	n	0.20	0.53	0.37	0.42	0.45	0.72	0.14

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