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ScienceDirect

Deep-Sea Research II 53 (2006) 1961–1975

DEEP-SEA RESEARCH
PART II

www.elsevier.com/locate/dsr2

Phylogeography of *Calanus helgolandicus* and the Black Sea copepod *Calanus euxinus*, with notes on *Pseudocalanus elongatus* (Copepoda, Calanoida)

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Received 1 August 2004; accepted 18 March 2006

Abstract

Calanus helgolandicus is a widespread epipelagic copepod species whose geographical range extends from the temperate Atlantic Ocean to the northern Mediterranean Sea. *Calanus euxinus*, recently designated as a distinct species though closely related to *C. helgolandicus*, occurs in the Black Sea. Very subtle morphological differences distinguish the two species. *Pseudocalanus elongatus* has a similar geographic range including North Atlantic Ocean, Mediterranean Sea and the Black Sea. In this study, population genetic variation of *C. helgolandicus*, *C. euxinus* and *P. elongatus* was investigated using DNA sequence variation of 540 base pair (*Calanus* spp.) and 575 base pair (*P. elongatus*) regions of mitochondrial cytochrome oxidase I (mtCOI) gene. *C. helgolandicus* was collected from the English Channel, the Adriatic Sea, and *C. euxinus* was collected from various regions of the Black Sea. *P. elongatus* was collected from the English Channel and the Black Sea.

Intraspecific differentiation in mtCOI was $<1\%$ for all species; mtCOI sequence variation between *C. helgolandicus* and *C. euxinus* was $<0.5\%$. The absence of substantial genetic differentiation between *C. helgolandicus* and *C. euxinus* is particularly striking in comparison to other close species pairs in these genera. Statistically significant haplotype frequency differences were determined for different locations of the Black Sea, English Channel, and Adriatic Sea *Calanus* populations ($\chi^2 = 3.94$, $P<0.0001$). The haplotype diversity was high for all species: *C. euxinus* ($h = 0.92$), *C. helgolandicus* ($h \leqslant 0.80$), *P. elongatus* ($h \leqslant 0.60$). No haplotype sharing was reported for different locations of *P. elongatus*, whereas the presence of haplotype sharing between *C. helgolandicus* and *C. euxinus* was remarkable. The size distribution in terms of prosome length measurements was found to be region-specific. The lack of phylogenetic differentiation between the *Calanus* species pair may suggest ancestral polymorphisms. The morphological and genetic similarities between *C. euxinus* and *C. helgolandicus* raise new questions about the status of *C. euxinus* as a different species.

Keywords: Black Sea; *Calanus euxinus*; *Calanus helgolandicus*; *Pseudocalanus elongatus*; Phylogeography; Cytochrome oxidase I

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doi:10.1016/j.dsr2.2006.03.017

1. Introduction

1.1. The Black Sea and its history

The Black Sea is a unique marine environment, representing the largest land-locked anoxic basin in the world. The basin consists of an oxygenated surface layer (~100 m depth, 13% of the sea volume) and anoxic deep water (100–2200 m). Its waters are almost completely isolated from the world ocean. There is restricted exchange with the Mediterranean Sea through the Bosphorus Strait, the Marmara Sea, and the Dardanelles Strait (Fig. 1). The surface mixed layer has low salinity values of 18 as a result of excess river run-off and precipitation (Murray et al., 1991; Oguz et al., 1992). A permanent halocline (50–200 m) separates the surface water from the deep water (Murray et al., 1991; Oguz et al., 1993; Ozsoy and Unluata, 1997). Dissolved oxygen decreases rapidly from saturated levels of 250–350 to 20–30 μM through the main oxycline, and becomes undetectable in anoxic waters below sigma-theta (σ_t)~16.15 kg m^{-3} density surface. The suboxic zone, with oxygen concentrations of $<10\,\mu$M and sulfide concentrations of $<1\,\mu$M between σ_t~15.65–16.15 kg m^{-3} (Basturk et al., 1997; Murray et al., 1989, 1995) is ecologically important since intense copepod aggregation occurs in this region (Vinogradov et al., 1992).

The Black Sea was a lake during the glacial period with a depressed water level of 90–100 m (Aksu et al., 1999). Following the disintegration of the Northern European ice sheets, the first post-glacial, two-way exchange between the Marmara Sea and the Black Sea occurred about 7200–7000 years BP (Aksu et al., 1999). The Black Sea zooplankton is basically a derivative of Atlantic plankton, which occurs in the Mediterranean Sea. Copepods are the most important group of zooplankton food for fish (Vinogradov et al., 1992; Unal, 2002) in the Black Sea, and hence the majority of the investigations have been on this group (Kideys et al., 2000; Kovalev et al., 1998, 2001). Even though the Black Sea ecosystem deteriorated significantly during the late 1980s and early 1990s due to the invasive ctenophore *Mnemiopsis leidyi*, there have been some signs of significant recovery since mid-1990s (Kideys, 2002).

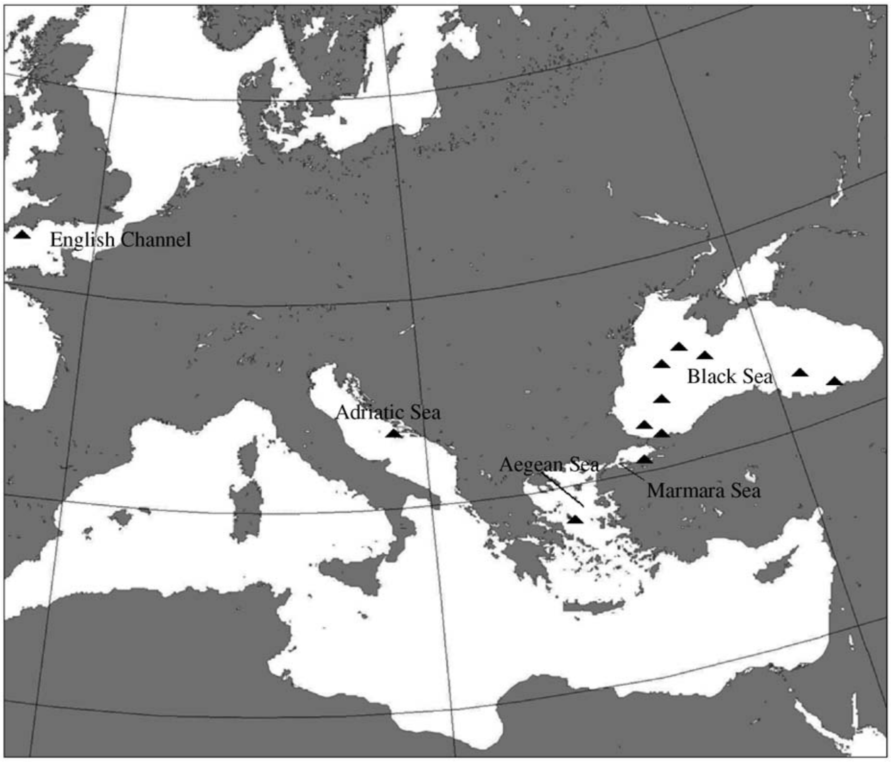

Fig. 1. The sampling locations for *Calanus euxinus, Calanus helgolandicus* and *Pseudocalanus elongatus*. Detailed information about the sampling locations is given in Table 1.

1.2. *Calanus euxinus* and *Calanus helgolandicus*

The genus *Calanus* is a subject of intense research throughout the temperate-boreal regions of the world oceans, usually dominating the mesozooplankton biomass, playing a prominent role in the carbon cycle, and constituting a major link in pelagic food webs. (Bradford, 1988; Fleminger and Hulsemann, 1977, 1987; Hulsemann, 1991). *C. helgolandicus* is an epipelagic copepod species with a geographical range extending continuously from shelf waters off eastern North America across the temperate North Atlantic to the northern Mediterranean Sea (Fleminger and Hulsemann, 1977). In the eastern Mediterranean, *C. helgolandicus* is abundant in the Adriatic Sea (Vucetic, 1965) and is present seasonally in the Aegean Sea (Apostolopoulou, 1985); it is also reported as far east as Lebanon's shelf waters (Lakkis, 1976), but appears to be absent throughout most parts of the southeastern Mediterranean.

The species of *Calanus* in the Black Sea was first identified as *C. finmarchicus* by Karavaev in 1894. Since Boeck had already erroneously synonomized *finmarchicus* with *helgolandicus*, Karavaev considered this to be the correct name (See Hulsemann, 1991, for review). Later, Sars pointed out the separate status of *C. finmarchicus* and *C. helgolandicus*; and Jashnov (1970) distinguished the Black Sea population as a subspecies *C. helgolandicus ponticus* based on its geographic distribution. Kovalev et al. (1975) presented further supporting evidence for the distinctiveness of the Black Sea specimens based on some morphometric characters, i.e. the body size. Fleminger and Hulsemann (1977, 1987) used the prosome to urosome length ratio and the differential distribution of supernumerary pores overlying integumental glands of the female urosome as diagnostic characters for the *Calanus* species. Studies on geographic analysis of the integumental pore signature patterns indicated the existence of two distinctive populations, one in the Black Sea, the other in the Mediterranean Sea and Atlantic Ocean (Fleminger and Hulsemann, 1987; Kovalev, 1975). Finally, a new name *C. euxinus* was given to this species as a replacement name for *C. ponticus* (Hulsemann, 1991). Though designated as a distinct species, the morphological differences are exceedingly subtle (Fleminger and Hulsemann, 1987).

The Black Sea *Calanus* population has been subject to different conditions than the Atlantic or Mediterranean Sea populations. Behaviorally, the diel vertical migration is sharply limited (Petipa et al., 1960; Vinogradov et al., 1985). *C. euxinus* cannot migrate vertically to diapausing depths of 300–500 m typical of *C. helgolandicus* elsewhere (Hirche, 1983) and has adapted to accommodate the lower salinity and temperature values of the Black Sea waters. *C. euxinus* has an important role in transferring organic matter from primary producers to higher taxa. It is also among the most preferred food for fish (Besiktepe, 1998). The copepodite stage V (CV) of *C. euxinus* undergoes seasonal (ontogenetic) migration during summer and early autumn in the Black Sea. During this time period, two behaviorally different groups of CV individuals have been observed, while one group is diapausing within the suboxic zone, the other group is still performing diel vertical migration (Arashkevich et al., 1998; Beşiktepe et al., 1998). Vinogradov (1990) observed that 60–75% of the CV have remained at the lower limit of the oxygenated layer during the night in August, while the rest migrated to the 10–50 m layer. They also reported that not more than 1/3 of the CV population was in diapause in October.

Svetlichny et al. (1998, 2002a) pointed out an important difference in behavior, stating that the strategy of daily vertical migrations in *C. euxinus* is strongly related to the hypoxic state of their tissues. Svetlichny et al. (2000) further showed that the total metabolism of *C. euxinus* in the Black Sea decreased up to 7.2-fold in hypoxic zones, resulting in a significant metabolic advantage during diel vertical migrations. No such relationship has been observed elsewhere in the geographical range of *C. helgolandicus*. Moreover, while *C. helgolandicus* typically inhabits the saline waters of world oceans with salinity values of 32–39, *C. euxinus* has adapted to the lower salinity values of the Black Sea (~18). In fact, the Black Sea is the only location where *Calanus* is found to live in such low salinities.

Even though there is a two-way flow between the Aegean and Black Seas, H_2S conditions in the Black Sea usually prevent survival of introduced Aegean Sea stocks. Hence, contact between the two populations probably has been confined to the Aegean and Marmara Seas. Within the Marmara Sea, *C. euxinus* is found to reproduce in a thin layer of surface water (up to 25 m) originated from the Black Sea (Unal et al., 2000), but no adequate sampling has been carried out to verify the presence of *C. helgolandicus* in deeper Mediterranean waters of the same basin.

One sampling location for *C. helgolandicus* is the marine lake located in Mljet National Park within the Adriatic Sea. The Island of Mljet harbors two natural salt lakes (Veliko and Malo Jezero) connected by narrow and shallow channels to the Adriatic Sea. The sampling location, Veliko Jezero (Large Lake), is 46 m deep and has connection with the open sea through the Soline Channel. The formation of the lakes has been dated back to about 8400 years B.P. in the early Holocene and the marine ingression into Veliko Jezero through the Soline Channel took place about 5000–4000 years B.P. (Govorcin et al., 2001; Wunsam et al., 1999).

1.3. Pseudocalanus elongatus

P. elongatus is a cold-water species with an upper temperature limit of occurrence of about 13 °C. In general, *Pseudocalanus* species suspend development during winter and may enter resting stages during summer. Genus *Pseudocalanus* is found to possess a high level of morphological similarity and a number of sibling species (Frost, 1989). The life cycle variability of the genus in semi-enclosed bodies of water, including UK waters is reviewed in detail by Corkett and McLaren (1978). The genus *Pseudocalanus* also has been a focus of genetic research throughout the northern hemisphere (Bucklin et al., 1998, 1999, 2003; Sevigny et al., 1989).

The population dynamics of *P. elongatus* in the English Channel has been studied in detail by Green et al. (1993). *P. elongatus* is an abundant zooplankton species and is an important component of the diet of a number of different species of fish in the English Channel and adjacent waters (Last, 1978a, b). *P. elongatus* is also very abundant throughout the Black Sea (Niermann and Greve, 1997). In fact, *P. elongatus* usually dominates the copepod composition within the Black Sea and numerically makes up to 38% of the zooplankton through the autumn within the offshore regions (Unal, 2002). This species is also present within the Marmara Sea where the two water masses from the Black and Mediterranean Seas overlap (Kovalev et al., 1998; Unal et al., 2000). *P. elongatus* also has been observed in the Alboran Sea (south of Spain) in the western Mediterranean basin (Vives et al., 1981), but has not been reported from the eastern Mediterranean region.

1.4. Goals

In this study, a partial DNA sequence of mtCOI gene has been used to describe the population genetic variation of *C. helgolandicus, C. euxinus*, and *P. elongatus* obtained from different geographic locations. Prosome length (PL) measurements of *Calanus* and *Pseudocalanus* species have been performed from all the sampling locations. The main goals are: (1) to determine the level of genetic differentiation and structuring among the two closely related species, *C. helgolandicus* and *C. euxinus*, (2) to investigate the intraspecific differentiation within each of the *C. euxinus* and *C. helgolandicus* populations inhabiting different geographic locations, (3) to investigate the comparative population genetic structure of *P. elongatus* from the Black Sea and the English Channel, and (4) to define the body size (i.e. PL measurements) distributions within the context of their usage as a diagnostic character among different populations (notably between *C. helgolandicus* and *C. euxinus*).

2. Methods

2.1. Sample collection

Samples of *C. euxinus* (Table 1, Fig. 1) were collected from the Black and Marmara Seas during cruises of the R/V *Bilim* 2000 (METU Institute of Marine Sciences, Turkey) and R/V *Knorr* 2001 (UW School of Oceanography, US). Closing Nansen nets of 112 and 200 μm mesh sizes were used. During Black Sea sampling, wherever possible, two vertical net hauls were made from each station at nighttime in order to sample the oxic and the suboxic zones. (Table 1) The Black Sea samples were preserved in 95% ethanol, and the Marmara Sea samples were preserved in formaldehyde.

C. helgolandicus samples were obtained from the English Channel (Plymouth Marine Laboratory, UK) in July 2000; from a marine lake (Veliko Jezero) on the Island of Mljet in the Adriatic Sea (Laboratory of Plankton Ecology, Dubrovnik, Croatia) in September 2000; and from the northern Aegean Sea (Athens, Greece) in September 2000 (Table 1, Fig. 1).

P. elongatus samples were obtained from the same sampling station as *C. helgolandicus* off Plymouth (UK) in July 2002, and from the Black Sea at the center of western cyclonic gyre (Station Knorr-2) in

Table 1
Sampling information for *C. euxinus*, *C. helgolandicus* and *Pseudocalanus elongatus* (see Fig. 1 for the map locations)

Species	Location	Station	Date	Time	Latitude and longitude	Sampling depth (m)	# of ind. for genetic analysis	# of ind. for PL analysis
C. euxinus	Black Sea	Knorr-2	June 3, 2001	0:30 0:45	42°30.00′N 30°46.00′E	140–0 65–0	8	52
C. euxinus	Black Sea	Knorr-3	June 4, 2001	8:00	44°07.50′N 30°55.00′E	170–0	6	—
C. euxinus	Black Sea	Knorr-10	June 6, 2001	16:50	44°26.20′N 31°31.00′E	150–0	4	—
C. euxinus	Black Sea	Knorr-12	June 7, 2001	12:20	44°17.90′N 32°16.09′E	170–0	5	—
C. euxinus	Black Sea	Knorr-14	June 9, 2001	12:40 0:30	41°27.50′N 30°15.75′E	170–0 100–0	2	—
C. euxinus	Black Sea	Bilim-1	September 30, 2000	20:00	42°10′N 29°41′E	50–0 120–92	13	—
C. euxinus	Black Sea	Bilim-2	October 10, 2000	01:30	42°00′N 37°00′E	50–0 140–100	7	—
C. euxinus	Black Sea	Bilim-3	October 14, 2000	20:20	41°23′N 39°50′E	50–0 165–115	4	—
Calanus sp.	Marmara Sea	Bilim-4	October 20, 2000	14:10	40°46′N 29°00′E	200–0	—	27
C. helgolandicus	English Channel	Plymouth	July, 2002	—	50°15′N 4°13′W	—	22	31
C. helgolandicus	Adriatic Sea	Island of Mljet	September 8, 2000	—	42°46.02′N 17°22.02E	—	28	10
C. helgolandicus	Aegean Sea	Athens, Greece	September 19, 2000	—	39°25′N 25°44′E	—	—	4
P. elongatus	Black Sea	Knorr-2	June 3, 2001	0:30 0:45	42°30.00′N 30°46.00′E	140–0 65–0	15	63
P. elongatus	English Channel	Plymouth	July, 2002?	—	50°15′N 4°13′W	—	15	63

The *Calanus* samples obtained from the Marmara and Aegean Seas were only used for prosome length (PL) measurements and were not included in the genetic analysis. The *Pseudocalanus* individuals were sorted out from the same samples as *Calanus* individuals, but for two locations only, the Black Sea and the English Channel. The identification of the Black Sea copepods used in the study was done by Bruce W. Frost.

June, 2001 (Table 1, Fig. 1). *Calanus* and *Pseudocalanus* individuals were sorted out and preserved in 95% ethanol.

The PL measurements were carried out for different geographic locations for all the species and size-frequency distributions were calculated. PL was taken from the anterior margin of the cephalosome to the posterior margin of the last thoracic somite.

2.2. DNA amplification and sequencing

DNA amplifications were carried out from whole-copepod homogenizations without initial purification of DNA. Individual copepods were sorted from the ethanol preserved samples and re-hydrated in 500 μl of distilled water for 10–24 h. The individual copepods were then incubated at 65 °C for 3 h in 20 μl of lysis buffer (PCR buffer/

Proteinase K solution in 50:1 ratio), and then at 95 °C for 15 min.

Initial polymerase chain reactions (PCRs) to amplify a 658 bp region of mtCOI gene were carried out using the consensus primers HCO2198 and LCO1490 (Folmer et al., 1994). A single PCR tube (20 μl total) contained 2 μl DNA solution in 10X PCR buffer, 2 μl of 2 mM dNTP, 1.5 mM $MgCl_2$ final solution, 0.2 μl of 10 μM forward and reverse primers, only 0.16 μl *Taq* polymerase [5 U/μl]. The PCR protocol used was as follows: 5 cycles of 94 °C for 1 min, 45 °C for 1 min, 72 °C for 90 s, 27 cycles of 94 °C for 15 s, 50 °C for 15 s, 72 °C for 30 s; 1 cycle of 72 °C for 5 min.

Only the PCR products that gave strong and specific bands were used for direct sequencing. The PCR products (with universal primers) yielding non-specific bands were prepared for cloning reactions by using TOPO-TA Cloning Kit. The sequencing was subsequently carried out with both forward and reverse primers by using a MegaBACE 1000 Automated Sequencer (Amsterdam Biosciences). Since these universal primers did not consistently amplify the desired 658 long fragment successfully, the available sequences were then used to design species-specific primers, LCO1537 (GCGTACTCAGGAATAATCGGTA) and HCO2121 (GTCGTATTTAGGTTTC GGTCTG), to amplify routinely a 540 bp region of the mtCOI gene for *Calanus* species. For *P. elongatus*, the species-specific primers designed to amplify the 575 bp region of the gene were Ps1F (AATTGGTACAGGGTTGAGAATG) and Ps2R (CCTCCCCCTACATCATAAAAG). The PCR protocol used for species-specific primers were: 1 cycle of 90 °C for 1 min, 45 °C for 1 min; 35 cycles of 90 °C for 15 s, 57 °C for 30 s, 72 °C for 45 s, 1 cycle of 72 °C for 5 min. The direct sequencing of the PCR products was performed by using these primers for both species.

2.3. Phylogeographic analysis

The nucleotide sequences obtained for partial mtCOI gene for *C. euxinus*, *C. helgolandicus* and *P. elongatus* were analyzed by using Sequencher V. 4.0.5 (Gene Codes, Ann Arbor, MI, USA) and Bioedit Sequence Alignment Editor (Hall, 1999) software. For each set of sequence from a given location, the most common haplotype was chosen as the consensus sequence. Pairwise nucleotide differences were calculated by using BioEdit Sequence Alignment Editor for the consensus sequences obtained from each location in comparison with the published sequences of *C. helgolandicus* from the Northeastern Atlantic (GenBank Accession # AF332761) and *P. elongatus* from the Dutch Wadden Sea (GenBank Accession # AY144467). *C. australis* sequence from the Southwest Pacific (GenBank Accession # AF332766) and *P. newmani* sequence from NW Atlantic (GenBank Accession # AF332796) were included as outgroups.

A χ^2 test using Monte Carlo simulation (Roff and Bentzen, 1989) was used to evaluate the statistical differences in the haplotype frequencies. For the phylogenetic analysis performed, molecular evolutionary genetic analysis (MEGA, Kumar et al., 2001) package was used and Tamura/Nei gamma distances were calculated. Neighbor Joining trees were constructed for both *Calanus* and *Pseudocalanus* species by using Tamura Nei distances (Tamura and Nei, 1993). However, due to the low bootstrap values, these trees are not included in the current study. Analysis of Molecular Variance (AMOVA) analysis was carried out by using Arlequin software (Schneider et al., 2000) in order to estimate the significance of differences in population pairwise fixation indices (F_{ST} values) among different regions.

3. Results

3.1. Calanus euxinus and Calanus helgolandicus

The nucleotide sequence of 658 base-pair region of the mtCOI gene was determined for *Calanus* individuals. GenBank Accession Numbers were obtained for the two most frequent haplotypes of *C. euxinus* in the Black Sea (AY604518, AY604519), for *C. helgolandicus* in the English Channel (AY604520), and for *C. helgolandicus* in the Adriatic Sea (AY604521). However, for the phylogeographic analysis of the *Calanus* species, only 540 bp region of the mtCOI is used since most of the sequence was obtained by using the newly designed species-specific primers (HCO2121 and LCO1537). 49 *C. euxinus* individuals from the Black Sea, 22 *C. helgolandicus* individuals from the English Channel, and 28 *C. helgolandicus* individuals from the Adriatic Sea were sequenced. The sequence of the most common haplotype for each geographic location was chosen as the consensus sequence. The sequence identity matrix was established

Table 2
Sequence identity matrix table for the consensus sequence (the most common haplotype) of 540 bp region of mtCOI gene for *C. euxinus* from the Black Sea (Ceux_BS) and *C. helgolandicus* from the Adriatic Sea (Chel_AD) and English Channel (Chel_UK)

	Ceux_BS	Chel_AD	Chel_UK	Chel-NE	Caus
C. euxinus_BS	1.000	0.998	0.996	0.996	0.807
C. helgolandicus_AD	—	1.000	0.994	0.994	0.809
C. helgolandicus_UK	—	—	1.000	1.000	0.807
C. helgolandicus-NE	—	—	—	1.000	0.807
C. australis	—	—	—	—	1.000

These sequences are compared with the published sequence of *C. helgolandicus* from the Northeastern Atlantic (Chel-NE, GenBank # AF332761). Published sequence of *C. australis* from Southwest Pacific (Caus, GenBank # AF332766) is included as an out-group.

Table 3
Haplotype numbers according to geographic locations defined by a 540 bp region of mtCOI gene for *C. helgolandicus* and *C. euxinus* (see Fig. 2 for corresponding haplotype frequency diagram)

Locations	H1	H2	H3	H4	H5	H6	H7	H8	H9	Unique haplotype #	Total ind #	Total haplotype #
BS	10	1	2	5	0	3	3	6	1	18	49	26
UK	0	0	0	0	1	0	0	0	9	12	22	14
AD	2	1	0	0	16	0	0	0	0	9	28	12
Total	12	2	2	5	17	3	3	6	10	39	99	52

Only the haplotypes that possess multiple individuals are labeled (H1–H9). The haplotypes with only one individual are called as 'unique haplotypes'. The geographic locations are abbreviated as BS for Black Sea (*C. euxinus*), UK for English Channel (*C. helgolandicus*) and AD for Adriatic Sea (*C. helgolandicus*).

between the consensus and published sequences to show the proportion of identical residues in the alignment in a two-dimensional matrix table (Table 2).

The possible differentiation among the diapausing and migrating *C. euxinus* was investigated for 20 CV individuals obtained from the Black Sea during R/V *Bilim* 2000 cruise (Stations 1–3 in Table 1) during their diapausing period. The consensus sequences obtained from diapausing and migrating *C. euxinus* groups were identical. In order to determine the intraspecific differentiation within the Black Sea (see Table 1 for station locations), a total of 49 *C. euxinus* individuals were sequenced and low levels of within species variation (0.2–0.9%) were found. The comparison of the sequences obtained from different locations (eastern, western and middle parts) of the southern Black Sea revealed a 0.2% divergence.

Pairwise comparisons carried out between the *C. euxinus* and *C. helgolandicus* individuals indicated a very low value of sequence divergence (0.2–0.4%) regardless of the geographical location and extent of isolation (Table 2). This low level of divergence of *C. euxinus* corresponds to only one or two base pair changes out of 540 bp region of mtCOI gene. On the other hand, the selected out-group sequence provided for comparison, *C. australis* revealed about 20% nucleotide difference within the same portion of the gene. The previously published *C. helgolandicus* sequence from the Northeast Atlantic (GenBank Accession No. AF332761) was found to be the same as the most frequent haplotype (H9) sequence obtained out of the individuals from the English Channel (Table 2).

The haplotype frequencies were determined for different localities of *Calanus* populations and the statistical differences in the haplotype frequencies are evaluated by a χ^2 test using Monte Carlo simulation (Roff and Bentzen, 1989). Highly significant differences were identified among the Black Sea, English Channel, Adriatic Sea *Calanus* populations ($\chi^2 = 3.94$, $P < 0.0001$). AMOVA analysis resulted in significant genetic structuring among different regions ($F_{ST} = 0.369$, $P < 0.0001$). The pairwise F_{ST} values were the smallest for Black Sea and Adriatic Sea populations ($F_{ST} = 0.316$), and the largest for English Channel and Adriatic Sea populations ($F_{ST} = 0.509$).

A total of 52 haplotypes were identified for 99 individuals of *C. helgolandicus* and *C. euxinus* across t0he geographic range sampled (Table 3). However,

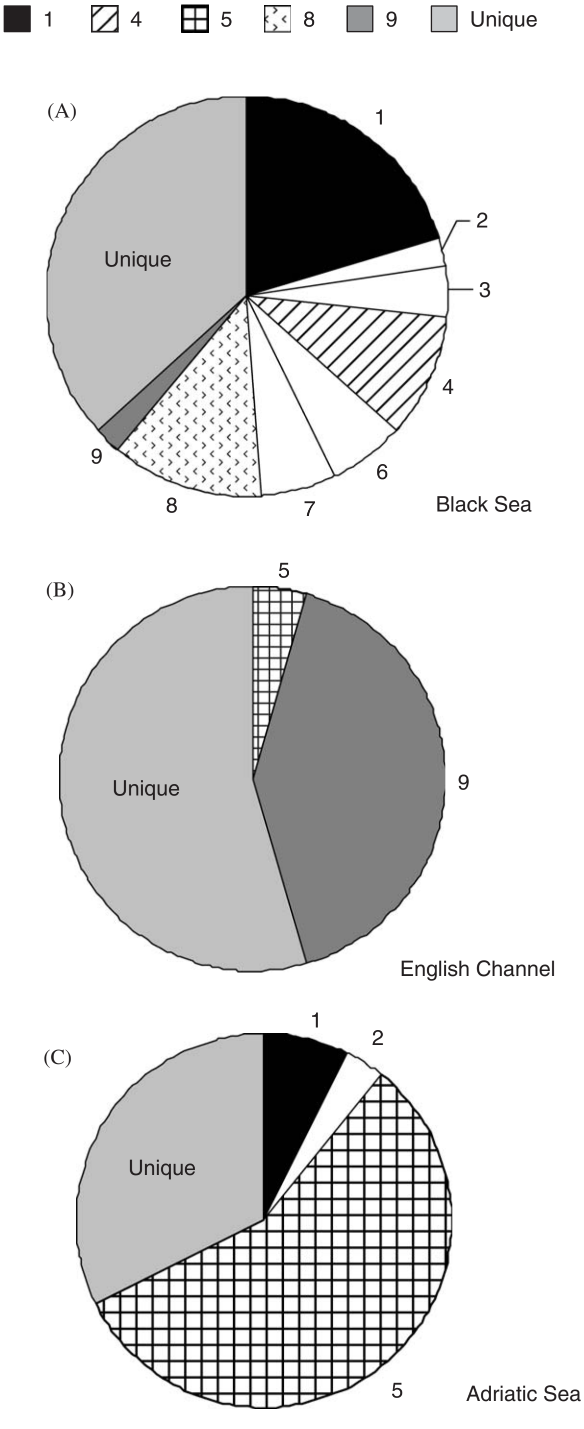

Fig. 2. Haplotype frequency diagrams defined by a 540 bp region of mtCOI gene for *Calanus* spp. sampling locations. The numbers around the pie represent the common haplotype numbers (H1–9) and the unique haplotypes represented by single individuals were collapsed in one pie section labeled as 'unique' (see Table 3 for the detailed haplotype information). (A) *C. euxinus* in the Black Sea (49 individuals, eight common and 18 unique haplotypes), (B) *C. helgolandicus* in the English Channel (22 individuals, two common and 12 unique haplotypes), (C) *C. helgolandicus* in the Adriatic Sea (28 individuals, three common and nine unique haplotypes).

39 of these were unique haplotypes represented by single individuals. Only nine haplotypes were found to be represented by multiple individuals (H1–H9) (Table 3, Fig. 2). The presence of haplotype sharing between two different species, *C. helgolandicus* and *C. euxinus* was remarkable.

The greatest haplotype diversity was identified within the Black Sea for *C. euxinus* ($h = 0.92$) where 26 haplotypes were present (Table 3). Black Sea seems to contain all the common haplotypes except for H5. The haplotype diversity for the English Channel was also high ($h = 0.80$), with 14 haplotypes identified. On the other hand, the Adriatic Sea revealed a smaller haplotype diversity value ($h = 0.66$), with 12 haplotypes identified (Table 3) and most of the sequences being identical.

The most frequent haplotype found in the Black Sea (H1) did not appear among the English Channel individuals; however two representatives were identified within the Adriatic Sea (Table 3, Fig. 2B). The most frequent haplotype from the English Channel (H9) had a single representative in the Black Sea. H5 was the most frequent one for the Adriatic Sea, and a single individual from the English Channel was found to possess this haplotype. The Black Sea shared a single haplotype (H9) with the English Channel and three haplotypes (H1, H2, H5) with the Adriatic Sea. The English Channel shared a single haplotype with the Adriatic Sea (H5). The number of unique haplotypes was the highest for the Black Sea (18), and lower for the English Channel and the Adriatic Sea (12 and 9, respectively).

PL measurements were determined for female *Calanus* individuals obtained from the Black Sea (Station Knorr 2), Marmara Sea, Aegean Sea, Adriatic Sea and the English Channel (Fig. 4). The largest individuals belonged to the Black Sea, the mean PL being 2.61 ± 0.02 SE (52 individuals) (Fig. 4A). The 27 Marmara Sea individuals were found to be much smaller with the mean PL of 1.97 ± 0.02 SE. Only four PL measurements were performed for the Aegean Sea with the mean PL of

2.09 ± 0.08 SE, being similar to the Marmara Sea individuals. The 10 Adriatic Sea individuals had a mean PL of 2.44 ± 0.03, and the 31 English Channel individuals had a slightly smaller mean PL of 2.29 ± 0.02 SE (Fig. 4B). Non-parametric Wilcoxon two-sample test (Sokal and Rohlf, 1981) was used to analyze the significance of the PL differences for *Calanus* locations. All of the comparisons revealed highly significant size frequency differences ($t_s = 3.33$ to 7.47, $P < 0.001$). The highest statistics obtained were for Black Sea-Marmara and Black Sea-UK comparisons.

3.2. *Pseudocalanus elongatus*

The nucleotide sequence of 575 base pair region of the mtCOI gene was determined for *P. elongatus* individuals. GenBank Accession Numbers were obtained for the most common haplotypes of *P. elongatus* in the Black Sea (AY604522) and in the English Channel (AY604523).

The intraspecific genetic variation between the two *P. elongatus* populations inhabiting the Black Sea and the English Channel was investigated based on 15 individuals from each location (a total of 30 sequences). *P. elongatus* consensus sequences obtained from the Black Sea and from the English Channel yielded in 0.2% base sequence difference. The intraspecific variation for *P. elongatus* within the Black Sea was low (up to 0.4%), with most of the sequences being identical. *P. elongatus* individuals within English Channel showed a larger intraspecific variation, values reaching up to 0.7%. The comparison made with the only published sequence of *P. elongatus* from the Dutch Wadden Sea (Bucklin et al., 2003) yielded in less than 0.6% genetic difference, whereas the sequence divergence with the published sequence of *P. newmani* (Bucklin et al., 2003) was 20%.

P. elongatus showed a different pattern of population genetic structure (Table 4, Fig. 3). Out of a total of eight haplotypes identified, none of them was shared between the two localities, and a fixed base pair difference was present. Moreover, the Black Sea individuals showed a much lower haplotype diversity ($h = 0.32$) than the English Channel individuals ($h = 0.60$). Interestingly, no unique haplotypes were determined for the Black Sea, whereas English Channel possessed four unique haplotypes. The haplotype frequency difference between the Black Sea and English Channel populations of *P. elongatus* was highly significant ($\chi^2 = 12.0$, $P < 0.0001$). The F_{ST} value was also significantly high among these regions ($F_{ST} = 0.561$, $P < 0.0001$).

The PL measurements were carried out for 63 female *P. elongatus* individuals from each of the Black Sea and the English Channel locations (Fig. 5). The mean PLs were 1.75 ± 0.004 SE and 1.81 ± 0.006 for the Black Sea and the English Channel individuals, respectively. The two locations were found to differ highly significantly in terms of their mean PL measurements (Student's *t*-test, $t = 7.36$; $P < 0.001$ for a two tailed *t*-test).

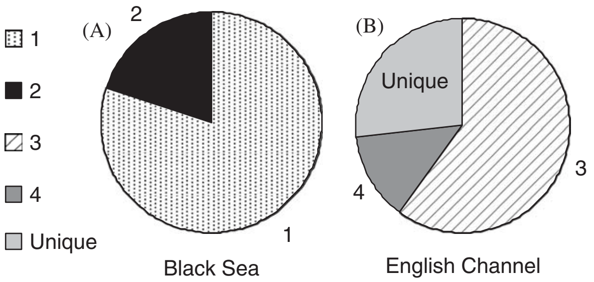

Fig. 3. Haplotype frequency diagrams defined by a 575 bp region of mtCOI gene for *Pseudocalanus elongatus* sampling locations. The numbers around the pie represent the common haplotype numbers (H1–4) and unique haplotypes represented by single individuals were collapsed in one pie section (see Table 4 for the detailed haplotype information) (A) Black Sea (15 individuals, two common haplotypes), (B) English Channel (15 individuals, two common and four unique haplotypes).

Table 4
Haplotype numbers according to geographic locations defined by a 575 bp region of mtCOI gene for *Pseudocalanus elongatus* (see Fig. 3 for corresponding haplotype frequency diagram)

Locations	H1	H2	H3	H4	Unique haplotype #	Total ind #	Total haplotype #
BS	12	3	0	0	0	15	2
UK	0	0	9	2	4	15	6
Total	12	3	9	2	4	30	8

Only the haplotypes that possess multiple individuals are labeled (H1–H4). The haplotypes with only one individual are called as 'unique'. The geographic locations are abbreviated as BS for Black Sea and UK for English Channel.

4. Discussion

Mitochondrial cytochrome oxidase I (mtCOI) gene has been shown to be very useful to resolve evolutionary relationships among closely related species groups for a wide range of taxa (Avise, 1994), especially for calanoid copepods and euphausiids (Bucklin et al., 1998, 1999, 2001, 2003). For most species, variation of mtCOI within a species is far less than variation between species, making the gene a diagnostic molecular systematic character. While intraspecific mtCOI sequence variation ranges from 0.5% to 2%, interspecific variation generally ranges from 10% to 20% (Bucklin et al., 2003).

4.1. *Calanus euxinus* and *Calanus helgolandicus*

Calanus is a target genus for taxonomic genomics and appears to represent genetically well differentiated evolutionary lineages within the family Calanidae (Bucklin et al. 1992, 1995, 1999; Hill et al., 2001; Lindeque et al., 1999), but contains several putative species and subspecies of uncertain status. The interspecific differentiation for the genus *Calanus* is reported to be 7–25% (Hill et al., 2001).

C. helgolandicus occurs in the North Atlantic and the Mediterranean Sea. *C. euxinus* occurs in the Black Sea and has been proposed as a distinct species derived from *C. helgolandicus* (Hulsemann, 1991). However, our new results indicate that there is a low level of genetic differentiation (0.2–0.4%) between the *C. helgolandicus*—*C. euxinus* species pair based on mtCOI partial sequence. These results are not comparable with the previously obtained range of 7–25% nucleotide differences between *Calanus* species (Hill et al., 2001), but are closer to the range of mtCOI sequence variation of 0.5–2% within a species (Bucklin et al., 1999).

Highly significant differences in the haplotype frequencies of populations from three geographic locations (Black Sea, the English Channel and the Adriatic Sea), indicates a high level of population genetic heterogeneity. High haplotype diversities have been identified, as well as many unique haplotypes. Out of a total of 52 haplotypes identified, half were found in the Black Sea (Fig. 2A). A distance-based mtCOI gene tree was constructed by Neighbor Joining algorithm using Tamura-Nei distances (Tamura and Nei, 1993). The tree for *Calanus* species (not shown) clustered the English Channel and Adriatic Sea individuals, but failed to resolve the Black Sea individuals. The *C. euxinus* individuals from the Black Sea were found to be dispersed throughout the gene tree, but the bootstrap value between the clades was low ($<50\%$). Given the low bootstrap values for the gene tree, it was not shown here.

The Aegean and Black Seas are in close proximity and *C. helgolandicus* and *C. euxinus* possibly co-occur in the Marmara Sea (Svetlichny et al., 2002b) (Fig. 1), where two different water masses are in contact with each other without significant mixing (Tugrul et al., 1992). Haplotype sharing between the two species might suggest a gene flow between Mediterranean and Black Seas. The presence of a two-way exchange between the Aegean Sea and the Black Sea might enable such introductions. However, it is very unlikely for the Mediterranean copepod species to migrate to the Black Sea due to the sinking of saltier Mediterranean water into the deep anoxic Black Sea water. Unfortunately, very few individuals have been successfully sequenced from the Aegean Sea, and DNA sequencing from the Marmara Sea was not possible because the samples were fixed in formaldehyde. Therefore, these two regions have not been included in this population genetic analysis.

This low genetic differentiation between these two species is somewhat expected since the Black Sea became a marine environment only about 7200 years ago. It is usual to have low variation between species that have recently separated from each other. Since there is a lack of phylogenetic differentiation between these two species, the genetic variation observed in between could be due to ancestral polymorphism.

C. euxinus and *C. helgolandicus* are morphologically separated by the differential distribution of the supernumerary pores, apart from the body size differences. This requires a labor intensive procedure, which is not routinely carried out even by the experts. Even so, the frequency differences in supernumerary pores are considered to be diagnostic rather than fixed differences between populations. It is also not possible to identify the juvenile stages of the two species. Therefore, within the Turkish Straits including the Marmara Sea, the identification of *C. euxinus* and *C. helgolandicus* is basically done using sample location and body size, *C. euxinus* being much larger than *C. helgolandicus*. However, all the individuals belonging to the '*helgolandicus* group' within the Marmara Sea are thought to migrate from the Black Sea and are regarded as *C. euxinus*, even though there are two different and distinct size classes (Svetlichny et al., 2002b).

In this study, The PL measurements are carried out to infer the differentiation of the body size among different locations. PL measurements obtained from the Marmara Sea *Calanus* individuals (1.84–2.33 mm) are close to the range obtained for the Aegean Sea individuals (1.94–2.26 mm). The specimens from the Adriatic Sea (2.24–2.56 mm) and the English Channel (2.10–2.48 mm) are larger than the Aegean and Marmara Sea individuals, but smaller than the Black Sea specimens (2.36–2.84 mm). All of these ranges are comparable with the ranges previously published from the Marmara Sea (Svetlichny et al., 2002b), and from the Black Sea, Aegean Sea and English Channel (Fleminger and Hulsemann, 1987).

Body size in planktonic calanoid copepods is strongly affected by the temperature and feeding conditions during their development (Corkett and McLaren, 1978; Frost, 1980). Within the UK waters, *Calanus* individuals overlapping completely with the size range of the Black Sea individuals have been observed previously (B.W. Frost, unpublished). Under conditions of high temperature and low food, it is quite possible to obtain individuals of *C. helgolandicus* close to the lower range as in the Aegean and Marmara Seas. In fact, considering the entire year, the PL of *C. helgolandicus* ranges from 1.93 to 3.06 mm (Bogorov, 1934), including nearly the entire range of PL measurements in the present data. The same seasonal range also was observed for the population of *C. helgolandicus* in the Celtic Sea (Bottrell and Robins, 1984). However, *C. euxinus* in the Black Sea shows a much narrower range of seasonal variation in its PL, being especially unnoticeable in adult stages (Svetlichny et al., 2004). In any case, the differences in size classes according to the locations were found to be highly significant for all comparisons, indicating that the size distributions are region- and/or season-specific, and cannot be used as a diagnostic character (Fig. 4).

4.2. *Pseudocalanus elongatus*

The range of intraspecific and interspecific mtCOI sequence divergence for *Pseudocalanus* species in general is found to be about 2–3% and 18%,

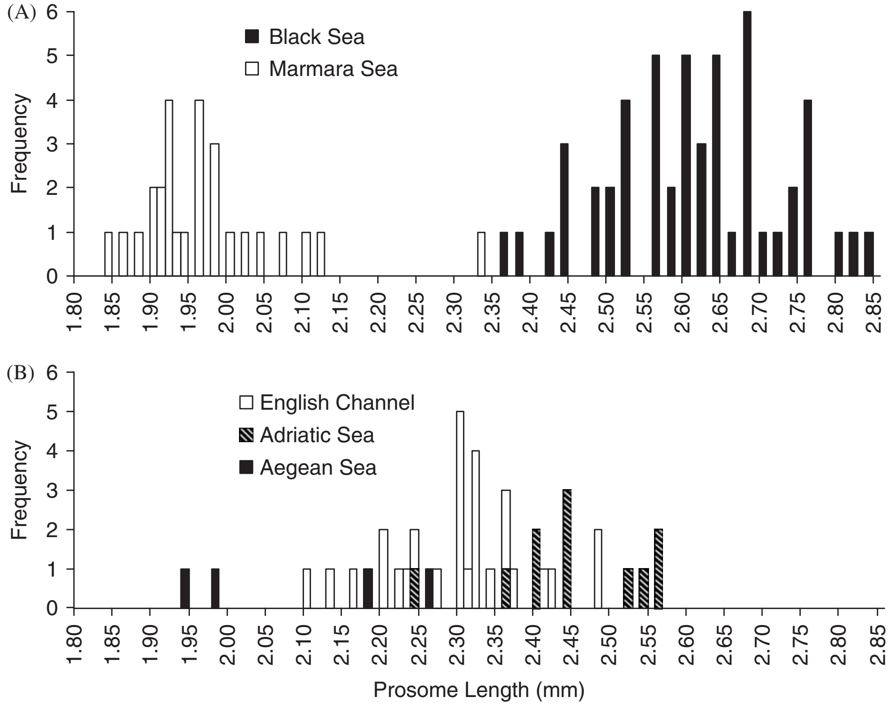

Fig. 4. Size frequency distributions of female PL (mm) for *Calanus euxinus* and *Calanus helgolandicus* individuals obtained from (A) Black Sea and Marmara Sea, (B) English Channel, (C) Adriatic Sea and Aegean Sea. PL measurements are taken for 52 , 27, 31, 10 and 4 individuals from the Black Sea, Marmara Sea, English Channel, Adriatic Sea and Aegean Sea, respectively.

respectively, which are typical genetic differentiation values for copepods for the mtCOI gene (Bucklin et al., 2003). In this study, the *P. elongatus* sequence differentiation is also low (0.2%) between the Black Sea and the English Channel, and the comparison with the published *P. elongatus* sequence yielded up to 0.6% divergence. These values are found to be lower than the published values of 2–3% for intraspecific variation (Bucklin et al., 2003) of *Pseudocalanus*. However, the single base sequence difference was fixed, i.e. the same base change was observed between two populations in every individual sequenced. The sequence divergence with the published *P. newmani* sequence (20%) was in consistent with the previously published values of about 18%.

The haplotype frequencies are found to be highly significantly different and the individual haplotypes are not shared between the two locations (Fig. 3). Besides, no unique haplotypes are identified for the Black Sea *P. elongatus* and the haplotype diversity was low ($h = 0.32$). The Neighbor Joining tree constructed for the *Pseudocalanus* individuals clearly formed distinct clusters of the two locations within the gene tree (not shown), but since the bootstrap value between the clades was low ($<50\%$), the tree was not included in this study.

Fixed nucleotide differences may be used as diagnostic characters for species identification together with the morphological evidence; however, in our case the number of individuals is not large enough (15 individuals each) to make a definitive statement. It is therefore sufficient to say that there is a low level of sequence differentiation among these two geographically distant populations of *P. elongatus*.

In terms of the PL analysis, the Black Sea specimens are found to be significantly smaller than the English Channel specimens (Fig. 5). However, body size in *P. elongatus* also shows large seasonal variation at Plymouth (UK); according to Digby (1950), where PL varies seasonally from a minimum of 0.7 mm to a maximum of 1.2 mm, covering the entire range of values that we observed from our sampling locations.

5. Conclusion and future work

It is especially difficult to resolve the systematic significance of genetic differences within a species (or between closely related species) inhabiting different geographical locations. Little to no genetic divergence can be detected if the two species (or subspecies) were very recently isolated. Though designated as a distinct species, the morphological and genetic differences between *C. euxinus* and *C. helgolandicus* are exceedingly subtle and the status of *C. euxinus* as a different species is questionable.

The current results verify that body size is not a good indicator of relationships among populations of a copepod species. Even though there are significant differences in the body size, they cannot be used as a diagnostic character due to the overlapping distribution. In our opinion, the taxonomic analysis of *C. helgolandicus* by Fleminger and Hulsemann (1987) is not fully representative of the entire range of sizes, which can vary in both temporal and spatial scales. Another morphological comparison needs to be made on similar-sized specimens for different locations to clarify the status of *C. euxinus*.

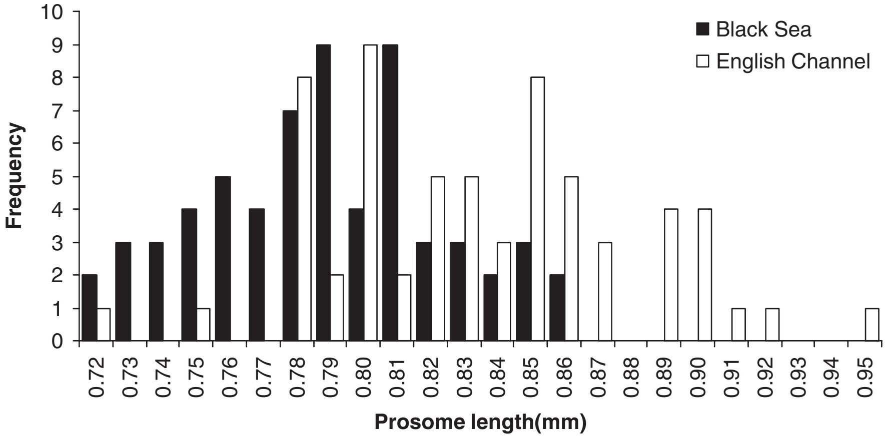

Fig. 5. Size frequency distributions of female PL (mm) for *Pseudocalanus elongatus* individuals obtained from the Black Sea (Knorr 2) and the English Channel (Plymouth, UK). Sixty-three individuals from each location are measured.

Future work should include genetic analysis of *C. euxinus* and *C. helgolandicus* specimens ($N = 30-40$ from each species) along with the observations of the integumental pores on the ventral surface of the urosome and PL measurements on the same individuals. It is crucial to be certain that variation of morphological characters between the two species as described by Fleminger and Hulsemann (1987) are in fact in consistent with the genetic data.

Moreover, the analysis should be carried out using additional neutral population genetic markers to resolve better the population genetic diversity and dispersal of *Calanus* species. In addition, the other widely distributed copepod species inhabiting the Black Sea (i.e. *Acartia clausi* and *tonsa*, *Paracalanus parvus*, *Oithona similis*) should be investigated genetically.

The populations inhabiting the Black, Marmara and Aegean Seas provide a unique opportunity to study a speciation event that is in its earliest stages. This system can be used to learn more about the speciation processes. Marmara Sea is especially interesting since the coexistence of Mediterranean and Black Sea waters provides an ecological barrier without geographical isolation. This region is a point of contact for many copepod species. Therefore, stratified sampling needs to be carried out at multiple stations to reveal the possible occurrence and any partitioning of these two *Calanus* species.

Acknowledgments

We are most grateful to Prof. Ann Bucklin for her guidance in phylogeographic analyses and kindly accepting to review this manuscript. We acknowledge the METU R/V Bilim crew and R/V Knorr crew for their valuable help in the Black Sea cruises. We thank the Plymouth Marine Laboratory (UK), to the Laboratory of Plankton Ecology in Dubrovnik (Croatia), and to the National Center for Marine Research in Athens (Greece) for providing the copepod samples. We acknowledge the graduate students and technicians in Marine Molecular Biotechnology Laboratory (MMBL), UW, for their generous help in laboratory. We also thank P.H. Wiebe (Woods Hole Oceanographic Institution) for his assistance. E.U. thanks Mike Canino and Winnie Lau, who have provided valuable assistance at the MMBL. This study was supported by UW School of Oceanography (US), METU Institute of Marine Sciences (Turkey), and NSF-Tubitak US-Turkey/Ukraine Cooperative Research: Research in the Black Sea (Award#: 0137601).

All the Black Sea sampling was done as a part of the M.Sc. research of the first author (E. Unal) through the Institute of Marine Sciences, Middle East Technical University, Turkey. The genetic analyses were carried out in Marine Molecular Biotechnology Laboratory, School of Oceanography, University of Washington.

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