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Self-maintaining or continuously refreshed? The genetic structure of *Euphausia lucens* populations in the Benguela upwelling ecosystem

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Abstract

Populations of *Euphausia lucens* over the shelf of the southern Benguela upwelling region could be self-maintaining. Alternatively, they could be continually refreshed by expatriates from the SW Atlantic that enter the system via South Atlantic Central Water in the south, before developing and then being lost through advection off Namibia. These two hypotheses are investigated here by examining geographic heterogeneity and molecular variation (cox1 and ND1) of the species across its distributional range in the Southern Hemisphere. Comparisons are made with *E. vallentini*, which is assumed to show panmixia associated with its circumglobal distribution between 50 and 60°S. Phylogenetic analysis with mitochondrial 16S ribosomal RNA and cytochrome oxidase 1 (cox1) confirmed that *E. lucens* and *E. vallentini*represent sister taxa. Strong geographic structuring of cox1 and ND1 mtDNA genetic variation by ocean basin was recorded in *E. lucens*, indicating that *neritic* populations off South Africa are likely self-maintaining. This contrasts with the results for *E. vallentini*, which appears to occur as a single panmictic population across its distributional range. These differences are likely related to the habitats (neritic, *E. lucens*; oceanic, *E. vallentini*) occupied by each species. The results of the neutrality tests are consistent with demographic processes and suggest growth in *E. lucens* and equilibrium or shrinkage in *E. vallentini*. Although purifying selection cannot be ruled out in the former, the very few haplotypes recovered from *E. vallentini* could indicate that any population expansion following a crash is not yet reflected in the relatively slowly evolving mtDNA markers used here. Further work using other methods is recommended.

Euphausia lucens Hansen 1905 is the dominant species of krill in nearshore waters of the southern Benguela upwelling region off the west coast of South Africa. Its regional distribution extends eastwards to Port Elizabeth on the South coast, and northwards into southern Namibia, where it is replaced by *Nyctiphanes capensis* Hansen 1911 inshore and by *E. hanseni* Zimmer 1915 further offshore (see map in Pillar *et al.*, 1992; Gibbons, 1995). It is thought to be an upwelling specialist (Gibbons and Hutchings, 1996) that has a suite of dietary and behavioural characteristics allowing it to persist in the Benguela upwelling region throughout the year. *E. lucens* is an omnivore that can match its diet to the ambient food environment (Pillar *et al.*, 1992), being largely herbivorous when phytoplankton is abundant

(near the surface and during upwelling conditions) and switching to zooplankton if phytoplankton is scarce (in deeper water and during quiescent/downwelling conditions).

Like other upwelling specialists in the area (Verheye *et al.*, 1991), *E. lucens*displays pronounced ontogentic diel vertical migration (DVM; Pillar *et al.*, 1992). Adult populations reach greatest numbers in the phytoplankton-rich waters close to shore off St Helena Bay, and eggs are released that float into the near-surface waters. These are then moved offshore in the Ekman layer, where they hatch into calyptopes. As these early life history stages are transported further offshore and northwards along the west coast, so they and their migratory abilities develop. This eventually enables individuals to take advantage of onshoreward (upwelling) compensation currents, which return them to the more productive nearshore environments and they can then move back southwards in the subsurface, poleward flowing counter currents (Shannon, 1985). Ontogenetic DVM, therefore, would allow populations of *E. lucens* to maintain themselves within the region, and prevent them from being advected into the South Atlantic off Namibia.

However, *E. lucens* is not confined to the Benguela upwelling ecosystem, and is found over continental shelves across the Southern Hemisphere between 40 and 50°S (see maps in Mauchline and Fisher, 1969; Mauchline, 1980), being common off Australia and New Zealand (Bartle, 1976) as well as South America (Tarling *et al.*, 1995). Given that the source water that is upwelled along the west coast of South Africa is of South Atlantic Central Water (SACW) origin, a potential mechanism exists whereby South African populations of *E. lucens* could be seeded by expatriates from the West Atlantic. If re-seeding of populations in the East Atlantic occurs whenever upwelling-favourable winds blow along the South African west coast, there is a constant supply of new individuals into local populations that then "boom" in the productive waters of the southern Benguela, only to go "bust" when they get entrained into offshore flows off southern Namibia. Although this latter scenario implies a high level of gene flow between western and eastern populations, different subpopulations of *M. norvegica* (M. Sars 1857) in the North Atlantic have been identified and matched to basin scale circulation patterns there (Papetti *et al.*, 2005). That said, no such clear current closure systems are apparent in the South Atlantic, so it does not follow that significant genetic structure would be apparent within populations of *E. lucens* in the region.

Here, we set out to test the genetic integrity of South African populations of *E. lucens* using a variety of mitochondrial [16S rRNA, cytochrome oxidase 1 (cox1) and NADH dehydrogenase 1 (ND1)] and nuclear internal transcribed spacer 1 (ITS-1) molecular markers, with a view to explicitly testing the extent of genetic mixing between populations across the Southern Hemisphere. These DNA fragments have been successfully applied to the study of phylogenetic relationships, geographical structure and demographic processes of krill species (Patarnello *et al.*, 1996; Papetti *et al.*, 2005; Bucklin *et al.*, 2007; Goodall-Copestake *et al.*, 2010; Bortolotto *et al.*, 2011).

We additionally contrast the patterns observed for *E. lucens* with those for its putative sister taxon (Zane and Patarnello, 2000), *E. vallentini* Stebbing 1900. *Euphausia vallentini* is

abundant between 50 and 60°S and because it is associated with the Antarctic Polar Frontal, it is distributed more or less continuously across the Southern Hemisphere (see maps in Mauchline and Fisher, 1969; Mauchline, 1980). Only in the SE Pacific and the SW Atlantic do both species occur together over the shelf (Ramirez and Dato, 1983; Curtolo *et al.,* 1990; Tarling *et al.,* 1995; Palma and Silva, 2004), otherwise *E. vallentini*fails to penetrate the continental coastal waters in either the SE Atlantic or the SW Pacific. Given its distribution, we predict that *E. vallentini* should show little evidence of separate breeding pools across its distributional range, as has been observed for *E. superba* (e.g. Bortolotto *et al*., 2011).

Finally, Jarman *et al.* (Jarman *et al*., 2000) hypothesize that *E. lucens* and *E. vallentini* have been incorrectly classified as separate species. We investigate this hypothesis here and predict that if *E. vallentini* and *E. lucens* indeed represent separate species, fixed differences will be observed with both mitochondrial and nuclear genetic markers.

Method

The project was designed with the main objective of obtaining population genetics and historical demographic information for *E. lucens* and *E. vallentini*, with a preliminary step of validating their phylogenetic affinities. These objectives were achieved by utilizing a range of DNA fragments with known different mutation rates. Both cox1 and 16S have been extensively used in phylogenetic studies of plankton. For this purpose, a few individuals per species are normally sufficient and frequently only one specimen per species is used (e.g. Bucklin *et al*., 2007); cox1 has been recently adopted as the barcoding gene for species identification (Hebert *et al*., 2003). The ND1 gene is thought to have a higher evolutionary rate than 16S and cox1 (Saccone *et al*., 1999), which justifies a major effort on our part to obtain population information for this fragment, especially as it has been successfully applied to other krill micro-evolutionary studies (Zane *et al*., 1998; Papetti *et al*., 2005; Bortolotto *et al*., 2011). ITS-1 is a nuclear marker and was incorporated as an additional source of information here.

Sampling and DNA isolation

Five hundred and ninety-three specimens of *E. lucens* and two hundred and fifty-two *E. vallentini* were collected from various global localities (Table I). Species were identified following the identification key of Baker *et al.* (Baker *et al*., 1990). DNA extraction was achieved using the CTAB method of Corach (Corach, 1991), modified using 5 µg/mL of proteinase K (Harkins, 2007).

Table I: ND1 summary statistics of diversity for the Euphausia lucens *and* E. vallentini *samples along with the geographic coordinates of sampling locations*

	n	*h* (SD)	π (SD)	Geo coordinates
Euphausia lucens				
SE Atlantic 2000				
1	15	0.167 (0.0180)	0.00107 (0.00086)	32.5014S, 18.1681E
2	52	0.076 (0.0500)	0.00049 (0.00033)	34.3208S, 17.8569E
3	40	0.099 (0.0640)	0.00064 (0.00042)	34.1083S, 17.9722E
4	16	0.242 (0.1351)	0.00161 (0.00093)	34.2167S, 18.1389E
5	12	0.318 (0.1640)	0.00214 (0.00116)	29.5347S, 15.1225E
6	29	0.069 (0.0630)	0.00044 (0.00040)	34.0714S, 17.7878E
7	29	0.131 (0.0820)	0.00085 (0.00055)	34.7333S, 24.6167E
8	12	n/a	n/a	33.9142S, 18.4217E
SE Atlantic 2001				
9	18	0.490 (0.1420)	0.00427 (0.00156)	30.9669S, 15.8836E
10	36	0.162 (0.0820)	0.00142 (0.00079)	29.1019S, 15.4514E
11	36	0.056 (0.0520)	0.00036 (0.00033)	33.3833S, 17.7333E
12	50	0.040 (0.0380)	0.00026 (0.00024)	33.4347S, 17.5344E
13	28	0.140 (0.0870)	0.00092 (0.00058)	33.4844S, 17.3342E
14	29	0.069 (0.0630)	0.00044 (0.00040)	34.2683S, 18.135E
15	36	n/a	n/a	29.0181S, 15.8186E
SE Atlantic pooled	438	0.128 (0.0220)	0.00090 (0.00017)	
SW Atlantic 2002				
16	50	0.594 (0.0740)	0.00524 (0.00099)	49.2733S, 66.3564W
17	50	0.522 (0.0800)	0.00473 (0.00101)	51.1525S, 65.1844W
18	52	0.731 (0.0610)	0.00737 (0.00119)	47.5217S, 64.0406W
SW Atlantic pooled	152	0.619 (0.0430)	0.00579 (0.00063)	
SW Pacific 2002				
19	2	1.000 (0.5000)	0.01923 (0.00962)	46.635S, 171.3839E
Euphausia vallentini				
SubAntarctica				
1	54	0.338 (0.0690)	0.00236 (0.00053)	55.5014S, 57.8347W
2	35	0.363 (0.0780)	0.00233 (0.00050)	56.0839S, 59.1678W
3	36	0.322 (0.0820)	0.00207 (0.00052)	56.9842S, 59.6672W
SW Atlantic	62	0.565 (0.0442)	0.00448 (0.00048)	51.8683S, 65.0333W
Marion Island	54	0.481 (0.036)	0.00308 (0.00023)	46.7544S, 37.8781E
SW Pacific	11	0.509 (0.101)	0.00326 (0.00065)	45.8681S, 175.8192E

n, sample size; *h*, haplotype diversity; SD, standard deviation; π, nucleotide diversity.
Samples 8 and 15 each contained identical haplotypes.

Polymerase chain reaction and sequencing conditions

Inter-species variation was studied using ITS-1, 16S rRNA and cox1, using a subset of specimens from each species collected from a range of geographically distant sampling locations. A region of ~500 base pair (bp) spanning the nuclear ITS-1 region was amplified with the primers SP-1-5′ and Sp-1-3′ (Chu *et al.*, 2001). An ~570 bp region of the 16S rRNA mitochondrial gene was amplified using the primers 16Sa and 16Sb (Palumbi *et al.*, 1991). A region of subunit 1 of the cox1 gene 640 bp in length was amplified using the primers LCO and HCO of Folmer *et al.* (Folmer *et al.*, 1994).

Population structure and demographic history were investigated utilizing the polymorphisms in cox1 and a 156 bp region coding for subunit 1 of the mitochondrial ND1 gene, using the primers ND1f and ND1r (Zane *et al.*, 1998). Failure of these primers to amplify was further investigated by amplifying a 600 bp fragment with the primers ND1-F-lu (5′-TCCTTATTATTTGTCTCCTG-3′) (Harkins, 2007) and CbMnl3 (Zane *et al.*, 1998). DNA sequencing confirmed substitutions in the priming sites for SWA *E. lucens*, thus the primers *GH*1f (5′-TTTTTTCTATGTTGTACAAGATT-3′) and GH2r (5′-ACAATCTCGCTGATATAATGA -3′) (Harkins, 2007) were designed for these samples using the software program Oligo (Rychlick, 1992).

All PCRs were performed in a total volume of 15 μL containing 20–40 ng of purified DNA from one individual krill as template, 0.1 U of Taq polymerase (Promega) and final concentrations of 1× Mg^{2+}- free buffer, 2.5 mM $MgCl_2$, 0.24 mM deoxynucleotides (dNTPs) and 0.3 μM of each primer. PCRs were run in a Perkin Elmer 9600 Gene Amp. Cycling conditions were: 94°C (2 min): 94°C (30 s), annealing temperature (30 s): 72°C (1 min) for 37 cycles, final extension 5 min at 72°C. Annealing temperatures were 50°C for 16S rRNA, cox1, and ND1 and 55°C for ITS-1.

Big Dye Terminator v. 3.1 (Applied Biosystems) was used to cycle sequence PCR products under the conditions recommended by the manufacturer. All amplicons were sequenced in both directions. Sequencing products were run in an ABI3100.

SSCP screening of mtDNA variation

ND1 haplotype variants were screened using SSCP (single strand conformational polymorphism) analysis (Orita *et al.*, 1989; Hayashi, 1991). For each individual, 2 μL of the ND1f—ND1r PCR product, was added to an equal volume of formamide loading dye (98% formamide, 2 mM EDTA, Bromphenol Blue), heat denatured and loaded on a 10% acrylamide gel (37.5:1 acrylamide/bisacrylamide) with 5% glycerol. Runs were performed at 4°C at 135 V for 16 h in a 20-cm high vertical apparatus. Gels were silver stained (Sambrook *et al.*, 1989). Accuracy of scoring was confirmed by sequencing randomly chosen individuals displaying each of the observed SSCP patterns. All distinct ND1 SSCP profiles were sequenced in both directions more than once.

Phylogenetic analysis

For the euphausiid phylogeny reconstructions, we have augmented the information generated in this study with that from a further 13 species (15 sequences) for 16S rRNA and 16 species (21 sequences) for cox1. GenBank accession numbers are provided in Supplementary data, Table SI. The cox1 and 16S gene fragments were analysed separately. Sequences for subsequent analysis with PAUP (see below) were aligned using Bioedit (Hall, 1999), while those for MrBayes were aligned using Sequencher 5.1 (GeneCodes). Nucleotide substitution models for use in PAUP were assessed using MODELTEST v3.06 (Posada and Crandall, 1998), while in the case of MrBayes, jModelTest 0.1.1 (Guindon and Gascuel, 2003; Darriba *et al.*, 2012) was used. The numbers of variable and parsimony informative sites were calculated with DnaSP v5 (Librado and Rozas, 2009). For the 16S and cox1 data, the best fitting model was the general time reversible model with gamma distributed rate variation and a proportion of invariable sites (GTR + G + I), and for the ND1 data this was the Hasegawa, Kishino and Yano model with gamma distributed rate variation and a proportion of invariable sites (HKY + G + I).

The evolutionary relationships among species (rooted on *Meganyctiphanes norvegica*) were investigated using neighbour-joining (NJ), maximum likelihood and maximum parsimony, as well as Bayesian phylogenetic methods with PAUP and MrBayes 3.2.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003), respectively. In the case of PAUP, statistical confidence in the stability of tree nodes was calculated by non-parametric

bootstrap (Felsenstein, 1985) with 1000 replications. In the case of MrBayes, the model was run for 1 million generations, with three attempted swaps each iteration and with the temperature set at 0.1: two independent runs were analysed, each with one hot and three cold chains.

Genetic variation within species: geographical and temporal heterogeneity

The extent of the genetic diversity was evaluated using summary statistics estimates of haplotype diversity (h), nucleotide diversity (π) of Nei (Nei, 1987), and the number of segregating sites (S) calculated using DnaSP v5 (Librado and Rozas, 2009).

AMOVA (Excoffier *et al.*, 1992) using Arlequin (Excoffier *et al.*, 2005) was used to evaluate spatial and temporal structure in ND1 haplotype heterogeneity of *E. lucens* samples (Table I), as well as geographic structure of *E. vallentini*samples. AMOVA computations for *E. vallentini* haplotypes were performed using a *p*-distance matrix, but in the case of *E. lucens* standard haplotype frequencies AMOVA were applied. Significance was tested with 10 000 permutations. The degree of population structure was further examined from a parsimony network analysis of ND1 mtDNA haplotypes within species using the Median-Joining algorithm, with parameter ε set to 0, using Network v.4.5 (Bandelt *et al.*, 1999). To account for multiple testing, *P*-values (0.05) were adjusted using the Bonferroni correction (Quinn and Keough, 2002).

Gene flow among the four regions was further evaluated for *E. vallentini* using Migrate 3.4.2, using a Bayesian coalescent framework (Beerli and Felsenstein, 2001; Beerli, 2006). Final run conditions were determined after inspecting the effective sample size (all above 1000) and the lowest Ln[Prob(D|G)] for all parameters M ($M = 2N_{ef}\, m$), where $2N_{ef}$ is the effective population size of females and M is the number of migrant females per generation. Final run conditions were summarized over five replicate runs of 1 short chain with 50 000 recorded steps with 100 steps increments after 50 000 burn-in steps, and four chains with bounded adaptive heating scheme of 1, 3, 10 and 100 000.

Historical demographic processes were investigated for both species using neutrality tests. Departures from neutral expectations were investigated with two different tests implemented in DnaSP v 5.0 (Librado and Rozas, 2009): Tajima's D (Tajima, 1989) and Fu's Fs (Fu, 1997). The former uses information from the mutation (segregating site) frequency and is based on the difference between two alternative estimates of θ, while the latter uses information from the haplotype distribution and is based on Ewen's (Ewens, 1972) sampling distribution. Fu's F_s test is more sensitive to demographic changes. An excess of rare alleles indicates demographic expansion, whereas an excess of common alleles is indicative of population shrinkage. The significance of $F_s$was evaluated using 1000 random permutations with DnaSP (Librado and Rozas, 2009).

The relative magnitude of demographic changes through time was evaluated from different estimates of the population-scaled mutation rate theta ($\theta = 2Ne\mu$). These methods have varying degrees of sophistication and utilize different properties of the data. We used the

non-genealogical summary statistic methods of Watterson (Watterson, 1975) and Tajima (Tajima, 1983), and a genealogical estimator based on generalized linear models, UPBLUE (Fu, 1994). A long-term history of effective population size was obtained using Watterson's (Watterson, 1975) estimate θ_1 (Fu and Li, 1993), which excludes the influence of singletons.

Genealogical and non-genealogical estimators of θ put different weights on mutations occurring in different time periods (Crandall *et al.*, 1999; Su *et al.*, 2001). Fu'sUPBLUE estimator (Fu, 1994) places heavy emphasis on young mutations revealing relatively recent population processes, whereas Tajima's (Tajima, 1983) estimate places heavy emphasis on older mutations reflecting ancient population events (Fu, 1997). The comparison between these two estimators has been used to gain insights with respect to population size changes over time.

Results

Genetic variation between species in 16SrDNA, cox1, ND1 and ITS-1

We generated 16S sequence information for two *E. lucens* and two *E. vallentini*specimens. The 446 bp 16S rRNA data set for these species had a total of eight variable sites seven of which were fixed between species (Supplementary data, Table SIIa). We obtained DNA sequence information for 640 bp of cox1 for eight *E. lucens* and 14 *E. vallentini* specimens, with a total of 64 variable sites of which 55 were parsimony informative and 39 were fixed between species (Supplementary data, Table SIIb).

The 16S rRNA MrBayes tree is shown in Supplementary data, Fig. S1a. Although its topology in the context of *E. lucens* and *E. vallentini* is congruent with that of the NJ tree (data not shown) and in general agreement with the maximum likelihood and maximum parsimony trees, the latter were unable to resolve clearly the relationships between *E. lucens, E. vallentini* and *E. frigida*Hansen 1911 (data not shown). Notably, the sequence previously identified as *E. lucens* (AF177177.1) (Jarman *et al.*, 2000) groups within a monophyletic clade comprising all of the other *E. vallentini* samples with high bootstrap support indicating that this specimen has been misidentified (Supplementary data, Fig. S1a). Congruent topologies for *E. lucens* and *E. vallentini* were observed using cox1 in all four analyses, where the sequence AF177185.1 that was previously identified as *E. lucens* by Jarman *et al.* (Jarman *et al.*, 2000) groups within the *E. vallentini* clade with high bootstrap support (Supplementary data, Fig. S1b). The estimated mean pairwise distance for *E. lucens* and *E. vallentini*was 7.6% using cox1 and 1.4% for 16S rRNA. For *E. lucens* a clear geographical pattern can be superimposed onto the cox1 phylogenetic trees with two distinct reciprocally monophyletic *E. lucens* clades corresponding to the separate SEA and SWA ocean basins (Supplementary data, Fig. S1b).

The nuclear marker, ITS subunit 1, was sequenced in three individual *E. lucens*(GQ890562.1-GQ890564.1), *E. vallentini* (GQ890567.1- GQ890569.1) and two *E. recurva* specimens (GQ890565.1, GQ890566.1). These included specimens sampled from each of the regional locations (SEA and SWA) that were previously identified as belonging to distinct clades in the mtDNA phylogenetic analyses. No intra-species variation in ITS-1 was detected in either

species and two fixed substitutions and four indels separated the two species (Supplementary data, Table SIII).

Thirty-eight distinct ND1 haplotypes were identified among the 593 individuals collected from 19 *E. lucens* samples, with the highest number found in the SWA followed by SEA and the SWP (Supplementary data, Table SIV). In *E. vallentini* in contrast, only four ND1 haplotypes were detected among the 252 individuals sampled in the SEA, SWA, SWP and subAntarctic waters (Supplementary data, Table SIV).

Despite the initial expectation that the putative sister species would be very similar, they differed substantially in their respective levels of intra-specific ND1 genetic diversity. Nucleotide diversities (π) ranged between 2.6×10^{-4} and 7×10^{-3} substitutions/site among sampling localities (Table I) and the average percentage ND1 bp difference between these species was 19.27% with a net divergence of 17.79%. The highest haplotype diversity (h), nucleotide diversity (π) and number of segregating sites (S) (Table I) was exhibited in the SWA *E. lucens* population, where this pattern was consistently observed in all of the separate samples from this area. The high and dominant frequency of haplotype A1 (Supplementary data, Table SIV) in the SEA samples was responsible for the relatively low haplotype and nucleotide diversities.

Geographic patterns of population genetic diversity

The patterns of population structure inferred from the analyses of cox1 differed substantially between the sister species. Whereas *E. lucens* was characterized by strong genetic structure at the ocean-basin scale with genetically differentiated populations in the SWA and SEA ($\Phi st = 0.71$, $P < 0.05$), *E. vallentini* showed no evidence of geographical heterogeneity across its global range (Supplementary data, Fig. S1b). The AMOVA analysis of *E. vallentini* ND1 data showed that most variation is contained within samples (96.63%), indicating homogeneity across samples ($\Phi st = 0.033$, $P > 0.05$).

Modal values of the Migrate parameters (θ and M) and their 95% CI are shown for *E. vallentini* in Supplementary data, Table SV and Figure 1. High levels of long-term gene flow among regions and asymmetrical migration among most regions were detected. Although M from SWP to SEA is apparently restricted (CI encompasses 0), this site experiences high migration rates to and from all other regions. The SWP sample is the smallest in size and these values should therefore be taken with caution.

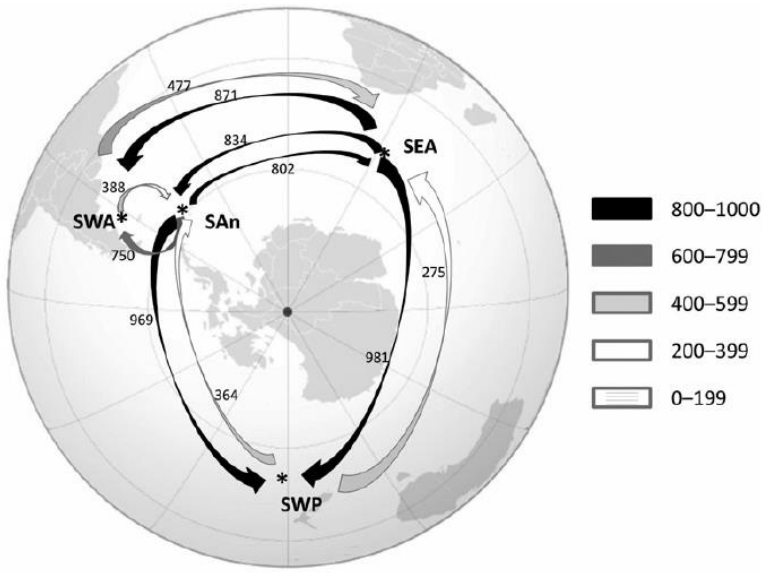

Fig. 1. Relative migration rates (number of females per generation) of *Euphausia vallentini* between sampling regions, as estimated with Migrate. *, sample locations (Table I).

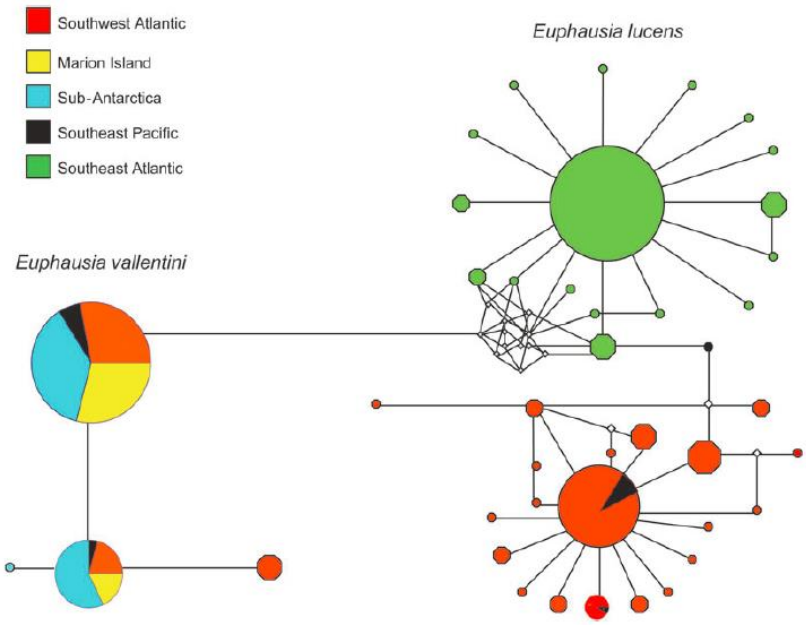

Fig. 2. Median-joining network depicting the phylogenetic relationships among, and the geographical assignment of, all *Euphausia lucens* and *E. vallentini* mtDNA ND1 haplotypes. The size of each circle is proportional to the corresponding haplotype frequency and the white diamonds indicate possible missing intermediates.

A ND1 median-joining network (Fig. 2) confirmed the results obtained using cox1, with *E. vallentini* and *E. lucens* forming reciprocally monophyletic clades in the network (Supplementary data, Fig. S1b). For the *E. lucens* subclade, an identical geographical pattern to that inferred from the cox1 phylogeny was observed with two distinct reciprocally monophyletic clades corresponding to the SEA and SWA basin populations (Supplementary data, Fig. S1b).

The high-frequency ND1 haplotypes were internal to the network, with rare or singleton haplotypes generally being derived from these. No evidence of any further geographic subdivision was apparent among the three SWA *E. lucens*samples, which also displayed a high number of singleton haplotypes (Harkins, 2007). Two haplotypes (B1 and BC20) were shared between the SWP and the SWA where, in the latter region, haplotype B1 occurred at the highest frequency in all of the local samples (Fig. 2).

A strikingly different pattern was observed in *E. vallentini* with only four distinct ND1 haplotypes detected. Haplotype (III) was found only in the SWA Continental Shelf sample while another haplotype (II) was recovered from a single subAntarctica sample (Supplementary data, Table SIV). The remaining two haplotypes (I and IV) were found in all the samples, whereas the latter was always the most frequent (Supplementary data, Table SIV).

Temporal heterogeneity within species: long-term and short-term genetic variation

For *E. lucens*, a significant departure from neutrality was detected for all samples (Table II). This is due to the more elevated proportion of rare alleles present in the samples (see also Supplementary data, Table SII and Fig. 2). The deviations from neutrality were more pronounced for Fu's F_s test, arguing that demographic processes associated with expansion are more plausible explanations for the patterns observed than those linked to selection.

Table II: Results of neutrality tests for the Euphausia lucens *and* E. vallentini *ND1 data sets: significance levels in parentheses*

Samples	*n*	Tajima's D	Fu's Fs
E. lucens			
SEA (Total)	438	−2.245 ($P < 0.01$)	−35.719 ($P < 0.0001$)
SEA (2000)	202	−1.929 ($P < 0.05$)	−13.104 ($P < 0.0300$)
SEA (2001)	236	−2.125 ($P < 0.01$)	−17.475 ($P < 0.0001$)
SWA	152	−2.004 ($P < 0.05$)	−18.929 ($P < 0.0010$)
E. vallentini			
SAn	125	−0.088 ($P > 0.10$)	0.086 ($P > 0.10$)
SWA	62	1.278 ($P > 0.10$)	1.086 ($P > 0.10$)
SEA	50	2.026 ($P > 0.10$)	0.530 ($P > 0.10$)
SWP	11	1.550 ($P > 0.10$)	1.854 ($P > 0.10$)

Table III: Estimation of θ for Euphausia lucens *and* E. vallentini *ND1 data*

	E. lucens		*E. vallentini*
Methods	θ SEA	θ SWA	θ
Tajima θ_π	2.081	2.632	1.5
Watterson θ_S	4.596	6.169	1.59
Fu's $_{UPBLUE}$	9.095	12.113	2.095
Wattersons θ^*	2.315	2.518	–

SWA, South West Atlantic; SEA, South East Atlantic. Watterson's θ^* was calculated excluding singleton mutations.

In contrast to the results for *E. lucens,* positive but non-significant values were obtained for *E. vallentini* data, indicative of a relative excess of common alleles in all the samples (Table II).

Genetic variation at short temporal scale

No significant inter-annual variation in diversity was observed in the temporal comparisons between *E. lucens* samples collected from the SEA in 2000 and 2001, with 8 and 11 ND1 haplotypes recovered, respectively, in these years. The most frequently observed haplotype was the same in both years and sample-specific haplotypes were common due to 72.22% of haplotypes occurring as singletons. Of the 17 ND1 haplotypes recorded for the SEA, only three were shared between years (A1, A2 and A14). Of the remainder, 14 occurred at very low frequencies with 11 being singletons (Supplementary data, Table SIV). The absence of significant temporal or spatial genetic structuring within the SEA population permitted combining of samples to achieve increased statistical power. Conventional AMOVA using haplotype frequencies (Excoffier *et al.*, 1992), failed to detect differentiation between the temporal samples. All variation was contained within samples and consequently, F_{st} was non-significant ($\Phi st = -0.0006$, $P > 0.05$).

Magnitude of the temporal genetic variation

E. lucens neutrality tests indicated significant departures from mutation-drift equilibrium for this species (Table II). Deviations from equilibrium can stem from the effects of selection or demographic processes of population size change. The highest deviations from mutation-drift equilibrium were recorded with Fu's F_s test, which is the most sensitive of the different tests employed here to detect demographic changes. We, therefore, assumed a demographic process was the most likely explanation for these results and proceeded to estimate the magnitude of historical population size change.

For *E. vallentini*, all of the neutrality tests for the combined ND1 data set failed to detect any significant deviation from equilibrium (Table II). Assuming that the site-specific nucleotide substitution rate for ND1 is similar for both these sister species, and given the observed ratio of 0.31 for the $\theta\pi$ (*E. lucens*)/$\theta\pi$ (*E. vallentini*) comparison, then this would imply that the effective population size of *E. lucens* was at least three times the size of *E. vallentini*. Even greater differences in Ne between these sister species were observed with the θ_{UPBLUE} estimator (Table III).

In *E. lucens*, the highest θ values were exhibited in the SWA samples (Table III). Estimates of Waterson's θ are influenced by segregating site numbers which are in turn influenced more by sample size than by haplotype frequencies. Tajima's estimate is based on nucleotide diversity which is relatively low in both of the south Atlantic basins reflecting the fact that most *E. lucens* haplotypes differ from the most-frequently occurring haplotype by a single nucleotide substitution (Table I). As expected, Tajima's θ results were slightly higher for the SWA (Table III).

The estimates of Watterson's θ^* (Table III) for separate subsamples are lower for both of the south Atlantic populations reflecting the relative contribution of singleton mutations to these statistics. Note that all of θ estimators vary consistently for the two subsamples. For example, the θ_{UPBLUE} estimate is four times as large as $\theta\pi$, for the separate SWA (θ_{UPBLUE} = 12.113/$\theta\pi$ = 2.632), and SEA (θ_{UPBLUE} = 9.09/$\theta\pi$ = 2.081) Atlantic populations (Table III).

Discussion

Here, we have shown that fixed differences between *E. lucens* and *E. vallentini*exist in both the nuclear and mitochondrial genomes, demonstrating that they represent valid taxonomic species. This settles some debate in the literature implied by the observations of Jarman *et al.* (Jarman *et al.*, 2000), and has implications for the systematics of Southern Hemisphere euphausiids (Zane and Patarnello, 2000).

There is strong geographical structuring of genetic variation in *E. lucens*populations in the South Atlantic. There is also a general absence of inter-annual differences in genetic structure of SEA populations, indicating that the differences between east and west are real and not the result of chance sampling. This suggests that the populations of *E. lucens* in the SEA are not seeded by individuals transported from the SWA in upwelled SACW, as provocatively proposed by Gibbons and Hutchings (Gibbons and Hutchings, 1996). Rather, the diel and ontogenetic vertical migration behaviours shown by *E. lucens* in the SEA (Pillar *et al.,* 1992) are likely effective in maintaining populations over the shelf.

Although there are no other studies from the South Atlantic that can be used to compare our results with (Patarnello *et al.*, 2010) building largely on the work of Papetti *et al.* (Papetti *et al.*, 2005), noted that population structure of *Meganyctiphanes norvegica* in the North Atlantic was closely tied to basin-wide circulation patterns. Thus, aside from the Mediterranean population, three geographically distinct oceanic populations were identified linked to the three regional gyres. Broadly, similar observations have also been noted for the chaetognath *Sagitta setosa* (Peijnenburg *et al.*, 2005). That distinct populations in the South Atlantic are similarly observed then should come as no surprise, especially as *E. lucens* is regarded as a shelf species and few specimens have been recorded from the open ocean (Mauchline and Fisher, 1969; Mauchline, 1980), while *M. norvegica* is a shelf-slope species occurring at depths >100 m. Interestingly, Papetti *et al.* (Papetti *et al.*, 2005) also noted a lack of a significant temporal signal within krill populations *M. norvegica* (Papetti *et al.*, 2005), implying high genetic stability over short periods of time.

This situation contrasts with that of *E. vallentini*, which, using the same markers employed here for *E. lucens*, appears to be panmictic across the distribution range investigated. These results are similar to those observed for another widely distributed austral species of oceanic krill, *E. superba,*populations of which show no evidence of continental-level and regional geographical structure (Goodall-Copestake *et al.*, 2010; Bortolotto *et al.*, 2011), although subregional differences attributed to recruitment process have recently been observed (Batta-Lona *et al.*, 2011). Panmixia has also been observed for *Clausocalanus arcuicornis* (Blanco-Bercial *et al.*, 2011).

An excess of rare and common ND1 alleles was observed in *E. lucens* and *E. vallentini*, respectively, and, consequently, all of the neutrality tests for ND1 returned significantly negative results for the former species and positive (albeit not significantly so) results for the latter. These results are consistent with population growth, sweepstake recruitment or purifying selection in *E. lucens* and population decline or selective sweeps in *E. vallentini*. In *E. lucens*which displays a shallow multifurcating genealogy, an excess of low frequency alleles and large differences between estimates of effective and census population sizes mirrors the expected patterns generated by sweepstakes recruitment (Árnason, 2004). These organisms reproduce under spatially and temporally varying oceanographic conditions that may affect their sexual maturation, choice of mate, fertilization success, survival of larvae and recruitment (Hedgecock, 1994), all of which are processes about which we are almost completely ignorant.

In the case of *E. vallentini*, the presence of the two high-frequency ND1 haplotypes could have resulted from successive selective sweeps and incomplete fixation, perhaps linked to historic changes in the oceanography of the Southern Hemisphere (e.g. Zane and Patarnello, 2000; Rabassa *et al*., 2005).

Alternatively, the observed patterns of variation could be driven by demographic processes. The neutrality tests indicated substantial deviations from neutrality in almost all the separate *E. lucens* samples consistent with a recent population size increase. Consistent with this hypothesis the θestimators that incorporate genealogy information return larger values than the summary statistic estimators (Crandall *et al*., 1999; Su *et al*., 2001). In contrast, for *E. vallentini* similar θ values were obtained both among and between samples with both types of estimators and, generally, deviation from a constant-population size model was in the direction of population shrinkage (Tajima, 1989; Fu and Li, 1993; Fu, 1995). That said, the very few haplotypes recovered from *E. vallentini*, which should have a large census population size, may indicate that it has undergone a major population crash during its evolutionary history, and the subsequent population expansion is not yet reflected in the relatively slowly evolving mtDNA markers used here. It should be realized that the small size of the fragments used and the low levels of variation observed are insufficient to make unequivocal statements about the respective roles of demography and selection in shaping the genetic variation in these species.

The differences observed in the patterns of population genetic structure of *E. lucens* and *E. vallentini* most likely reflect differences in both their habitats, geographical ranges and the hydrographic current regimes that characterize the areas they inhabit (Zane and Patarnello, 2000). By strongly influencing these regimes, historical climatic and concomitant hydrological events must have played an important role in shaping the spatial distribution of their genetic variation. We consider that the estimators that incorporate genealogical information provide strong evidence for underlying demographic processes to explain the patterns of variation in these two krill species, but full confirmation should be obtained from the analysis of multiple loci.

Supplementary data

Supplementary data can be found online at http://plankt.oxfordjournals.org.

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