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Molecular evidence for genetic subdivision of Antarctic krill (*Euphausia superba* Dana) populations

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Antarctic krill (*Euphausia superba* Dana) is a key species in the Antarctic food web and occurs on a circum-continental scale. Population genetic structure of this species was investigated by sequence analysis of the *ND1* mitochondrial gene in four population samples collected at different geographical localities around the Antarctic continent. Results indicate the existence of significant genetic differences between samples, and we suggest that oceanographic barriers could be sufficiently strong and temporally stable to restrict gene flow between distinct areas. Moreover, our data indicate that Antarctic krill is not at mutation–drift equilibrium and that the species possibly has a low effective population size as compared to the census size.

Keywords: mtDNA; *ND1*; *Euphausia superba*; mismatch distribution; population genetics; Antarctic Ocean

1. INTRODUCTION

Antarctic krill (*Euphausia superba* Dana) is regarded as the key trophic resource in the Southern Ocean, being the main (or only) food source for the majority of higher-level heterotrophs, including Antarctic vertebrates such as penguins, whales and seals. The remarkable adaptive success of krill in this extreme environment is evident from its extraordinarily high abundance of at least 500 million tons (Miller & Hampton 1989), possibly the largest for any multicellular species on the planet (Hempel 1994). Antarctic krill also sustain the biggest single species crustacean fishery of the world (Nicol & Endo 1997), with a harvest regulated by the Convention for the Conservation of Antarctic Marine Living Resources (CCAMLR), which sets annual catch limits for Atlantic and Indian sectors of the Southern Ocean (CCAMLR 1996). Recent reports have provided evidence of a possible decline in krill abundance (perhaps linked to the long-term global warming trend (Loeb *et al.* 1997)), accentuating the need for careful management of this important resource.

Krill is a swarming crustacean whose discontinuous distribution is intriguing (Marr 1962; Spiridonov 1996). Areas of higher concentration of adult krill are associated mainly with major oceanic gyres, which are formed by the two main surface currents of the Antarctic Ocean (figure 1). This association suggested that gyres may generate a retention mechanism that promotes the formation of separate, self-supporting stocks of krill (Mackintosh 1973).

However, population genetic investigations of Antarctic krill populations, based on allozyme data, suggest genetic homogeneity for the species; in fact, despite an early report of genetic differentiation between two samples collected from each side of the Antarctic Peninsula (Fevolden & Ayala 1981), further examination of a larger data set, which also included these two populations, showed that krill apparently behave as a single breeding unit (Fevolden & Schneppenheim 1989).

The resolution of protein polymorphism analyses, however, might be too low to detect small differences at the intraspecific level, and investigations at the DNA level are perhaps more appropriate for addressing this matter (Avise 1991).

Here, we report a study of Antarctic krill population genetics based on DNA sequence analysis of a portion of the gene coding for subunit 1 of the mitochondrial NADH dehydrogenase gene (*ND1*).

Four population samples were collected from the East Weddell Sea, Bellingshausen Sea, South Georgia Island and Ross Sea (figure 1, table 1). Weddell and Bellingshausen waters support two of the largest aggregations of *E. superba* (Witek *et al.* 1988). The pattern of surface currents occurring in the Weddell–Bellingshausen–South Georgia region is characterized by two major features: the Weddell gyre, which separates a large portion of the Weddell Sea from the surrounding water mass, and the clockwise-flowing West Wind Drift (WWD), which connects Bellingshausen Sea to South Georgia through the Antarctic Peninsula region. The interaction between these two currents results in the Weddell–Scotia Confluence, which defines the Western boundary of the Weddell Sea (figure 1). The Ross Sea, which also supports a large krill concentration, is separated from the Weddell–Bellingshausen–South Georgia system by a considerable distance (at least 3000 km). Interestingly, despite such a geographic separation and the very scarce presence of krill reported within the waters dividing the Ross region from the other 'krill-rich' areas, the question of whether

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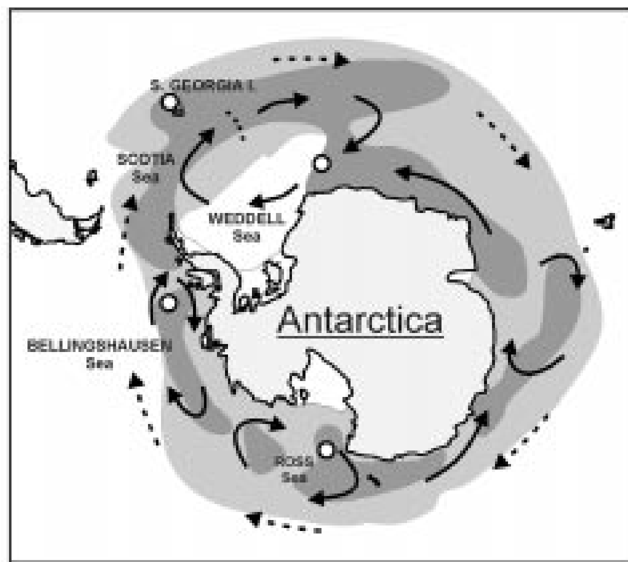

Figure 1. Krill distribution, underlying pattern of circulation, and sampling sites. Light and dark grey represent overall krill distribution and areas of higher concentrations, respectively. Dashed arrows represent the clockwise-flowing surface current West Wind Drift (WWD). Solid arrows represent the counter-clockwise-flowing Coastal Current (CC) and the gyres formed by the interaction of the surface currents. Circles indicate the sampling sites.

krill swarms of the Ross Sea may be not differentiated from other populations remains open. In fact the WWD and the counter-clockwise-flowing Coastal Current (CC) could promote the exchange of individuals, and may homogenize the krill populations on a circum-continental scale.

2. METHODS

Population samples of *Euphausia superba* were collected from the Bellingshausen, Weddell, and Ross Seas, and from South Georgia Island in the period 1992–1995. Sample sizes ranged between 48 and 70 individuals (figure 1, table 1). Specimens were deep-frozen and stored at $-40\,^{\circ}\mathrm{C}$ or fixed in 100% ethanol until analysed.

Total DNA was extracted from a few milligrams of muscle, using a Chelex protocol (Walsh *et al.* 1991). Target DNA was PCR-amplified with primers ND1f (5′-TTTTTCTTGTGTT-GTACTAGTTTAGG-3′) and ND1ar (5′-GGCAAAAATCTT-TTCCAGGCTAAGTA-3′). Sequence information of 154 bp was obtained by cycle sequencing using the internal primer ND1r (5′-ACAATCACGCTGATAAAGAGAAAAT-3′), end-labelled with $\gamma\ ^{33}$P.

Genetic homogeneity between populations was tested by the ϕ_{ST} statistic (Excoffier *et al.* 1992), which is analogous to the Wright HF_{ST} statistic, but modified to take account of sequence divergence between alleles (estimated using Tamura–Nei distance (Tamura & Nei 1993)). The statistical significance of each ϕ_{ST} was assessed under the null hypothesis of panmixia, performing 10 000 permutations of the original data set, in which individuals were randomly reallocated to each population (implemented in the program Arlequin v. 1.1; Schneider *et al.* 1997). A Fisher correction for multiple tests was used to assess the statistical significance of the distribution of the ϕ_{ST} values, as described in Manly (1984). The null hypothesis tested by this procedure is that the probabilities associated with the ϕ_{ST} are drawn from a uniform distribution with interval (0,1), as expected for a homogeneous sample.

Table 1. *Sampling sites, year of collection, and number of specimens analysed for the present study*

population	year of collection	sample size
Bellingshausen	1992	63
Ross Sea	1995	48
S. Georgia	1994	70
Weddell Sea	1992	68

The female effective population size (N_e) was estimated (under the assumption of population equilibrium) from the coalescence time (t_d) needed to produce the observed average number of differences per nucleotide site (π), using the equation

$$t_d = \pi/\mu, \tag{1}$$

where μ is the divergence rate per nucleotide per year. The coalescence time for the observed haplotypes can also be expressed as the time in generations (t_g) by simply dividing t_d by the generation time of the organism under investigation. This value represents exactly N_e for haploid genomes, being the reciprocal of the chance of fixation under the neutral theory (Hartl & Clark 1989).

Mutation–drift equilibrium and the neutrality hypothesis were investigated both at the species and at the single population level, using three different tests.

(1) The Tajima test of neutrality (Tajima 1989) assesses the departure from zero of the standardized difference (D) between two estimates of M ($2Nu$), where N is the effective size of the population, and u is the mutation rate per sequence per generation; M is calculated from the average number of pairwise differences and from the number of segregating sites. Selective or other non-equilibrium processes are expected to shift the value of D toward positive or negative values.
(2) The Ewens–Watterson (Ewens 1972; Watterson 1978) test of neutrality, which compares the observed level of homozygosity (for the mtDNA this is the gene identity) with the one expected under the neutral hypothesis, for a sample having the same size and the same number of alleles.
(3) The Chakraborty test (Chakraborty 1990), which compares the observed and expected number of alleles of the sample. The latter value is calculated from the observed homozygosity, under the neutral hypothesis.

The observed distribution of the number of pairwise differences between sequences was fitted to (i) the theoretical expectation for a population with stable effective size, and to (ii) the theoretical expectation for a population experiencing a sudden expansion (Rogers & Harpending 1992). These authors have shown that the time at which population expansion started can be estimated from the peak of the theoretical curve. The time is expressed as τ, or units of $0.5u$ generations (where u is the mutation rate per locus per generation). The relationship between absolute time (t_e), in years, and τ is:

$$t_e = \tau/2u_y, \tag{2}$$

where u_y is now the mutation rate per locus per year. Once an estimate for u_y is available, expansion time estimation is not dependent on the organism generation time.

3. RESULTS AND DISCUSSION

Sequence analysis of a 154 bp portion of the mitochondrial *ND1* region in 249 specimens revealed a high degree of polymorphism (63 mitotypes, haplotype diversity 0.85). Genetic homogeneity was tested by ϕ_{ST} analysis (see table 2). Pairwise comparisons reveal a significant heterogeneity between the samples of Weddell Sea and South Georgia ($\phi_{ST}=0.021$, $p<0.05$); this differentiation is statistically significant after the Fisher correction for multiple tests ($p<0.05$ that the distribution of the estimated probabilities is uniform, calculated after pooling the homogeneous Ross and Bellingshausen Sea samples) (Manly 1984).

Our results are in contrast with the current view of krill as a huge, unstructured population. The use of mitochondrial DNA (mtDNA) sequence analysis probably enabled us to reveal population genetic differences that were previously 'invisible' to the less powerful allozyme technique.

Interestingly, the S. Georgia and Weddell populations are geographically the closest of all the samples analysed here, suggesting that a simple model of 'isolation by distance' cannot be invoked to explain the observed genetic divergence. Oceanographic barriers, such as the Weddell gyre and/or the Weddell–Scotia Confluence could, however, be responsible for the reduced gene flow between the Weddell Sea and S. Georgia populations. This result may account for a major role of Antarctic Ocean currents in shaping krill population structure, and indicates that the present-day ocean circulation pattern must have been stable for a period of time long enough to prevent mixing of genetically differentiated populations. A similar role in promoting species differentiation was also suggested for the Antarctic Convergence, which represents the northern boundary of the Southern Ocean: the temporal stability of this barrier could explain the separation between Sub-Antarctic and Antarctic euphausiid species (Patarnello *et al.* 1996). However, considering that our samples were collected in different years, we cannot exclude the possibility that the genetic differentiation we have observed between krill populations is the result of temporal (rather than geographical) variability. A multi-year analysis of populations from the same geographic locations would help to clarify whether such genetic differentiation is geographically and temporally stable. This is especially important in light of the recognized inter-annual variability in krill abundance at some geographical locations (including S. Georgia; Brierley *et al.* 1997), that has been explained in terms of recruitment variability (Siegel & Loeb 1995). If recruitment involved only small numbers of individuals, or individuals that were closely related, then this led to localized shifts in allelic frequencies. In this regard, it is worth noting that effective population size (N_e) of female krill, estimated under the simplified model of population equilibrium, appears to be relatively small, especially if compared with the census size. As described in § 2, $N_e=t_g=t_d$/generation time (where $t_d=\pi/\mu$). From equation (1), using values for

Table 2. *Pairwise ϕ_{ST} between populations, based on Tamura–Nei distances*

(Values significant at the 95% level are represented by *).

sample	Bellingshausen Sea	S. Georgia	Weddell Sea
S. Georgia	−0.0020	—	—
Weddell Sea	0.0050	0.0213*	—
Ross Sea	−0.0053	0.0116	−0.00924

π and μ, respectively, of 0.0138 and 1.66–2.6% per million years (these mutation rates were proposed for crustacean mtDNA coding sequences; Knowlton *et al.* 1993; Schubart *et al.* 1998), the effective population size of krill females ranges between 264 000 and 415 000 individuals. This is a much smaller value as compared to the census size, which is *ca.* 5×10^{14} individuals (as roughly calculated from the biomass divided by an individual weight of 1 g). This estimation of N_e is based on a generation time of two years, whereas using a three-year generation time, as suggested in the literature (Siegel 1987; Hosie *et al.* 1988), N_e is even smaller, emphasizing the discrepancy between the effective population size and the actual census size. Large differences between these two values have also been observed in other organisms (Frankham 1995), and are highest in marine species for which discrepancies of 10^2–10^5 are reported (Hedgecock 1994). In turn, given the reduced N_e of Antarctic krill, we cannot exclude that stochastic events (such as genetic drift associated with high variance in reproductive success) may play a role in temporally differentiating krill populations.

Our mtDNA data set also enabled us to test mutation–drift equilibrium in Antarctic krill, that is, to test whether the observed distribution of mitotypes in *E. superba* fits the assumption of genetic 'equilibrium' (no selection, no reduction/expansion of population). In the case of significant departures from the equilibrium (as revealed by means of specific tests, namely, the Tajima, Ewens–Watterson, and Chakraborty tests), mitotype selection and/or variations of the population size can be hypothesized. In order to test the equilibrium in *E. superba* (at the species level), we pooled all samples irrespective of their geographical origin. Results of each of the three tests indicated unambiguously significant departures from the equilibrium hypothesis (see table 3). The same pattern was obtained when the tests were performed on each population separately: in this case, however, the values were not always statistically significant due to the low sample size (see table 1). These results are due mostly to an (observed) excess of the predominant mitotypes when compared with the values expected for a sample of the same size and the same number of alleles. Selection acting on mtDNA haplotypes could cause departure from the equilibrium. However, in our data set only 1 out of 249 individuals showed non-synonymous mutation (leading to an amino-acid change), whereas all other nucleotide substitutions were synonymous. Thus, it seems unlikely that selection can be at work on this region, though we cannot exclude that the overrepresented *ND1* mitotype may be

Table 3. *Neutrality tests*

(D is the Tajima D-statistic; F_{obs} and F_{exp} are the observed and expected homozygosity, respectively; N_{obs} and N_{exp} are the observed and expected number of alleles, respectively. Significant departures from the equilibrium–neutrality hypothesis at the 95% and 99% levels are represented by * and **, respectively.)

		Ewens–Watterson		Chakraborty	
samples	Tajima D	F_{obs}	F_{exp}	N_{obs}	N_{exp}
Weddell Sea	−1.0846	0.12413	0.09321	22	16.74
S. Georgia	−1.4066	0.18857	0.07128**	27	11.65**
Bellingshausen	−1.6090*	0.16100	0.09054**	22	13.09**
Ross Sea	−1.2737	0.16927	0.10610*	18	11.64*
populations pool	−1.7403*	0.15063	0.03937**	63	19.40**

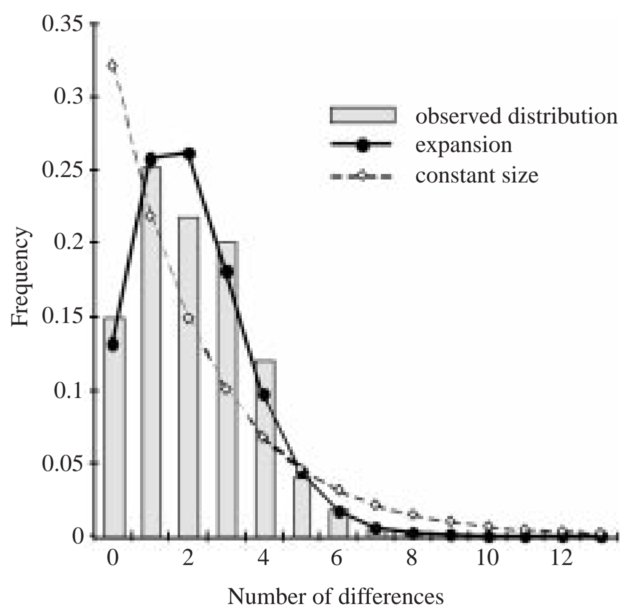

Figure 2. Observed distribution of pairwise differences. The dashed line shows the expected curve for an equilibrium population of constant size; the continuous line is the curve predicted for a population that has undergone a demographic expansion in the past.

linked to another mitochondrial region, that is under selection, since the entire mtDNA behaves as a single locus.

Alternatively, the non-equilibrium condition of *E. superba* could be explained by variations of population size. In fact, the observed distribution of pairwise differences between mitotypes (figure 2) fits better the distribution predicted for a population that has experienced a demographic expansion than the distribution expected for a stable population (Rogers 1995). The peak of the theoretical distribution of pairwise differences can be related to the time (t_e) at which the population expansion started if an estimation of u_y is available (see § 2). Using a 'per nucleotide' divergence rate μ, ranging between 1.66% and 2.6% per million years (as we did for N_e, see above), and considering the sequence length of 154 bp, u_y ranges between 2.56×10^{-6} and 4.04×10^{-6}. From equation (2), and using our t=1.66, krill population expansion started between 205 000 and 324 000 years ago. If this time estimate is realistic, a major demographic expansion (or a reduction followed by expansion) should have occurred much earlier than the last maximal glaciation (16 000 years ago), which is also thought to have produced a significant effect on krill population size (Spiridonov 1996). This would mean that *E. superba* may have experienced remarkable demographic fluctuation over a period ranging between 16 000 and 324 000 years ago, from which the extant krill population is still recovering. Glaciological studies have reported significant fluctuations in the ice volume during the late Pliocene (which may be due to climate change) on a time-scale in the range of 23 000–400 000 years ago (Dingle *et al.* 1997). Although the impact of these events on marine fauna remains speculative, the present data support the possibility of a link between climatic changes and biomass fluctuation of *E. superba*. This hypothesized historic abundance−climate link gains support in the light of a recent study, which suggests that krill is presently experiencing a rapid biomass decline, possibly due to reduction in ice extent caused by climatic warming (Loeb *et al.* 1997).

In summary, our results indicate that *E. superba* may be subdivided into at least two discrete genetic units and seems to have a low effective population size compared to census size. The present-day population decline and local over-fishing could then have a considerable impact on Antarctic krill, which might be prone to the risk of genetic erosion.

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