SOURCES OF VARIANCE IN STUDIES OF KRILL POPULATION GENETICS

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Abstract

There has been a long-term interest in the population genetics of Antarctic krill species because of their ecological and economic importance. The possibility that there are distinct genetic stocks of these species would affect the design of management strategies for their conservation. A recent resurgence of interest in identifying distinct stocks of swarming krill species has been driven by the development of genetic technologies that are more sensitive to subtle population structure than older methods. Previous studies of the population genetics of the Antarctic species *Euphausia crystallorophias* and *E. superba* and the boreal species *Meganyctiphanes norvegica* that used allozymes found no evidence of genetic population structure. More recent investigations using sequence variation in mitochondrial DNA (mtDNA) have exposed genetic differentiation between samples taken from different parts of each species’ range. However, the underlying assumption of these studies that differentiation between samples is caused primarily by restricted gene flow between widely separated sampling sites may be incorrect. Our recent study of *E. crystallorophias* mtDNA variation has indicated that there is significant genetic differentiation between samples taken within one region. This has important implications for the design of future studies of krill population genetics, which must be able to accommodate this sympatric variance component as well as variance attributable to differences between regions. Genetic differentiation between stocks of krill in different regions therefore can not be adequately assessed unless multiple samples are taken from each region.

Résumé

La génétique des populations des espèces de krill antarctique engendre depuis longtemps de l’intérêt du fait de son importance tant écologique qu’économique. S’il se révélait qu’il existait, dans ces espèces, des stocks génétiques distincts, la conception des stratégies de gestion de leur conservation en serait affectée. On assiste depuis peu à un renouveau d’intérêt pour l’identification des divers stocks des espèces de krill grégaires, engendré par la mise au point de techniques génétiques qui sont plus sensibles à la structure subtile des populations que les anciennes méthodes. Les anciennes études de la génétique des populations des espèces antarctiques *Euphausia crystallorophias* et *E. superba*, ainsi que de l’espèce boréale *Meganyctiphanes norvegica*, qui étaient fondées sur les allozymes n’ont pas mis en évidence de structure génétique des populations. Des études plus récentes fondées sur la variation de la séquence de l’ADN de la mitochondrie (mtDNA) ont révélé des différences génétiques entre les échantillons provenant de divers secteurs de la répartition géographique de chaque espèce. Toutefois, l’hypothèse à la base de ces études, selon laquelle la différence entre les échantillons est principalement due au flux restreint de gènes entre des sites d’échantillonnage très espacés pourrait s’avérer erronée. Notre étude récente de la variation de la mtDNA de *E. crystallorophias* révèle l’existence de différences génétiques importantes entre les échantillons prélevés dans une même région. Ceci pourrait s’avérer important pour la conception des prochaines études de la génétique des populations de krill, lesquelles devront tenir compte de cet élément de variance sympatrique ainsi que de la variance attributable aux différences entre les régions. Les différences génétiques entre les stocks de krill des diverses régions ne peuvent donc être évaluées correctement tant que de nombreux échantillons ne seront pas prélevés de chaque région.

Резюме

Продолжающаяся заинтересованность в популяционной генетике видов антарктического криля объясняется их экологическим и экономическим значением. Возможность существования генетически обособленных запасов этих видов может сказаться на разработке стратегий управления, направленных на их сохранение.
INTRODUCTION

The stock structure of Antarctic krill (Euphausia superba) has been the subject of many studies, yet there is still no consensus on whether krill form a single interbreeding population or whether there are distinct stocks in the waters around the Antarctic continent. There are regions around the Antarctic where there are thought to be quasi-permanent, large-scale concentrations of krill (Lubimova et al., 1985; Mackintosh, 1972, 1973). Many of these concentrations are related to environmental features, but there may also be some biological separation of stocks within the Southern Ocean. There is some evidence that in certain areas there are persistent populations of krill that exhibit demographic continuity between seasons (Lascara et al., 1999). However, the scale of connectedness of these local populations with those in other areas remains to be explored.

Resumen

Hace ya mucho tiempo que el estudio de la genética de las poblaciones de las especies de kril antártico está despertando interés, debido a su importancia tanto para la ecología como para los intereses económicos relacionados con el producto. La posible existencia de stocks genéticamente distintos de estas especies afectaría el diseño de las estrategias de ordenación para su conservación. El desarrollo de técnicas genéticas más sensivas que las técnicas antiguas para detectar las sutiles diferencias en la estructura demográfica ha causado un renovado interés en la identificación de los distintos stocks de especies de kril agrupados en concentraciones. Los estudios anteriores de la genética de las poblaciones de las especies antárticas Euphausia crystallophorias y E. superba y las especies árticas Meganyctiphanes norvegica mediante alóenzimas no encontraron pruebas de que las poblaciones estuviesen estructuradas de acuerdo a sus características genéticas. Investigaciones más recientes, que estudiaron la variación de la secuencia del ADN mitocondrial (ADNmt), han descubierto que existen diferencias genéticas entre las muestras tomadas de distintas partes de la zona de distribución de cada especie. Sin embargo, es posible que la suposición subyacente de estos estudios (que las diferencias entre las muestras es causada principalmente por un flujo genético restringido entre las poblaciones de lugares distantes) sea incorrecta. Nuestro estudio reciente de la variación de la secuencia del ADN mitocondrial (ADNmt) de E. crystallophorias indicó que hay diferencias genéticas significativas entre las muestras tomadas dentro de una región. Esto tiene repercusiones importantes para el diseño de las futuras investigaciones de la genética de las poblaciones de kril, que debe tener en cuenta tanto este componente simpático de la varianza como la varianza atribuible a las diferencias entre las regiones. Por lo tanto, la diferenciación genética entre los stocks de kril de distintas regiones no puede ser evaluada de manera adecuada a no ser que se tomen muestras múltiples de cada región.

Keywords: krill, DNA, sample, genetics, population, CCAMLR
Early allozyme research did not identify distinct populations of Antarctic krill (Fevolden, 1988; Fevolden and Schnepfennheim, 1988, 1989; Mac-Donald et al., 1986). A more recent study using mtDNA sequence analysis by Zane et al. (1998) reported significant differences between krill collected at different sites around the continent. This study indicated significant genetic differences between krill collected from the Weddell Sea and South Georgia but revealed no differences between krill collected from the Ross Sea, South Georgia or the Bellingshausen Sea. Genetic differentiation within krill species identified by mtDNA analysis, but not previously by allozymes, has recently been found in two other krill species, Meganyctiphanes norvegica and Euphausia crystallorophias. A summary of population genetics studies of these three krill species is given in Table 1.

This identification of genetic differentiation in several krill species (Table 1) is consistent with other evidence that distinct populations of pelagic organisms can arise, despite the presumed homogenising effect of ocean currents, e.g. copepods (Bucklin et al., 1998) and pelagic fish (Miya and Nishida, 1996, 1997). Moreover, other oceanic and even truly holoplanktonic organisms can display genetic heterogeneity between populations (Ayoama et al., 1999; Bucklin and Wiebe, 1986), site fidelity (Swearer et al., 1999) and self-recruitment (Jones et al., 1999). Thus, the separation of krill into distinct local populations in the Southern Ocean is not easy to dismiss without direct evidence to the contrary (Ayala and Valentine, 1979). It should be emphasised that studies that have been unable to demonstrate differences between populations may merely have used techniques which are not sensitive enough or sampling strategies which do not sufficiently take into account local variation. We have recently conducted an mtDNA-based study on genetic variation in the Antarctic coastal krill E. crystallorophias (Jarman et al., 2002). This study has provided some insight into the sampling density needed to determine whether detectable differences between populations of krill are due to geographic separation.

Materials and Methods

Sample Collection

Samples of E. crystallorophias were collected from four locations during 1999 (Figure 1). One sample (n = 61) was taken from the West Antarctic Peninsula region (66º53’S 68º55’W) in January 1999 (austral summer). Two samples (n = 59, 47) were collected from the Mertz Glacier Polynya (66º23’S 145º54’E; 66º29’S 146º1’E) during September 1999 (winter). One sample (n = 65) was collected from near the Shackleton Ice Shelf in the Davis Sea (65º32’S 107º0’E) during December 1999. Each sample is the result of a single, short (<10 min) net tow and is assumed to be from one krill swarm or a small number of adjacent aggregations.

Amplification of Mitochondrial DNA

A 616 base pair region of the mitochondrial COI region was amplified with the polymerase chain reaction (PCR) using ‘universal’ primer HCO (5’ – taaacttcagggtgaccaaaaaatca – 3’) (Folmer et al., 1994) and the species-specific primer EcLCO (5’ – gggtcgtgagctgggatagtggg – 3’). EcLCO was designed using the sequence previously reported for a slightly longer segment of the E. crystallorophias mtCOI by Jarman et al. (2000) as GenBank accession AF177183.

Each 20 μL PCR contained ~100 ng purified E. crystallorophias DNA from one individual krill as a template. The reaction buffer was 50 mM KCl, 10 mM Tris-HCl pH 8.9, 2.5 mM MgCl2, 0.1% Triton X-100. 0.25 mM of each dNTP, 2 units Taq DNA polymerase (Promega) and 10 pmol of each primer were included. The reactions had an initial incubation at 94°C for 2 min to denature template DNA, followed by thermal cycling at 94°C 20 s, 43°C 1 min and 72°C 1 min for 40 cycles. A 6 min incubation at 72°C followed to finish synthesis of incomplete strands.

Single Strand Conformational Polymorphism (SSCP) Analysis of mtDNA Haplotypes

SSCP analysis (Orita et al., 1989) of mtDNA COI haplotypes was used to avoid the cost of sequencing the COI region for each individual. 10 μL of each completed PCR amplification was restriction digested at 37°C for 4 h with endonuclease Rsa I (New England Biolabs). The following components were added for this reaction: 1.2 μL 100 mM Bis Tris propane-HCl, 100 mM MgCl2, 10 mM dithiothreitol (New England Biolabs 10 x buffer 1), 0.2 μL Rsa I (2 units) and 0.6 μL H2O. This cut the 665 bp PCR product (616 bp of amplified krill DNA + 49 bp of primers) into two fragments of 303 and 362 bp. 2 μL of the restriction-digested DNA solution was then added to 10 μL of 95% formamide, 0.25% xylene cyanol, 0.25% bromophenol blue, 10 mM NaOH. These mixtures were placed in a 95°C heating block for 3 min to denature the DNA and then quickly placed on ice.
Table 1: Summary of methods and sampling strategies used in population genetics studies of three krill species. All studies to date use either different combinations of polymorphic enzymes or variable nucleotide sites in mitochondrial DNA. The sample sizes for allozyme studies represent the maximum number of scorable individuals for any one enzyme system. In most cases, fewer individuals were scored for the other enzyme systems used.

<table>
<thead>
<tr>
<th>Study</th>
<th>Variation</th>
<th>Sample Sizes</th>
<th>Conclusion</th>
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<tr>
<td><em>Euphausia crystallorophias</em></td>
<td></td>
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<td></td>
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<tr>
<td>Kühl and Schneppenheim, 1986</td>
<td>6 allozyme loci</td>
<td>149, 164, 104, 195</td>
<td>No differentiation</td>
</tr>
<tr>
<td>Jarman et al., 2002</td>
<td>82 mtDNA haplotypes</td>
<td>65, 61, 59, 47</td>
<td>Differentiation</td>
</tr>
<tr>
<td><em>Euphausia superba</em></td>
<td></td>
<td></td>
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<tr>
<td>Fevolden and Ayala, 1981</td>
<td>21 allozyme loci</td>
<td>62, 60, 60</td>
<td>No differentiation</td>
</tr>
<tr>
<td>Schneppenheim and MacDonald, 1984</td>
<td>7 allozyme loci</td>
<td>295, 101, 232, 93, 359, 343, 342, 675, 149, 191, 165, 369, 287, 338</td>
<td>No differentiation</td>
</tr>
<tr>
<td>Kühl and Schneppenheim, 1986</td>
<td>8 allozyme loci</td>
<td>284, 247, 162, 300, 50</td>
<td>No differentiation</td>
</tr>
<tr>
<td>MacDonald et al., 1986</td>
<td>4 allozyme loci</td>
<td>202, 190, 197, 196, 189, 198</td>
<td>No differentiation</td>
</tr>
<tr>
<td>Fevolden and Schneppenheim, 1989</td>
<td>8 allozyme loci</td>
<td>400, 240, 240</td>
<td>No differentiation</td>
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<tr>
<td>Zane et al., 1998</td>
<td>63 mtDNA haplotypes</td>
<td>70, 68, 63, 48</td>
<td>Differentiation</td>
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<td><em>Meganyctiphanes norvegica</em></td>
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<tr>
<td>Sundt and Fevolden, 1996</td>
<td>5 allozyme loci</td>
<td>144, 144, 144, 93, 96, 144, 96, 182</td>
<td>No differentiation</td>
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<tr>
<td>Bucklin et al., 1997</td>
<td>75 mtDNA haplotypes*</td>
<td>14, 12, 14, 13, 16, 18</td>
<td>Differentiation</td>
</tr>
<tr>
<td>Zane et al., 1999</td>
<td>12 mtDNA haplotypes</td>
<td>46, 47, 46, 50, 49, 50, 50, 47</td>
<td>Differentiation</td>
</tr>
</tbody>
</table>

* Based on analysis of combined COI and CytB datasets.
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Figure 1: Significant ($\alpha = 0.05$) pairwise $\Phi_{st}$ values found between four samples of *Euphausia crystallorophias*. #The difference between Mertz Glacier Polynya 1 and Davis Sea was not significant with Bonferroni correction (corrected $\alpha = 0.0083$). No significant $\Phi_{st}$ values were found for comparisons between samples from the Davis Sea and West Antarctic Peninsula.
The samples were then loaded onto a 200 x 170 x 0.75 mm gel composed of 0.5 x MDE poly-acrylamide solution (FMC Bioproducts), 0.6 x TBE, 0.1% TEMED and 0.1% ammonium persulfate. SSCP were resolved on this gel by electrophoresis in 0.6 x TBE at 4 W for 14 h while the gel was kept at 4°C by circulation of chilled water through a chamber adjacent to the gel (BioRad Protean IIxi). Twenty-five samples were run on each gel, with one sample being a reference that was also run on another gel to allow comparisons between gels.

After electrophoresis, one glass plate was removed and a 30 mL 1 x SyBr Gold (Molecular Probes) nucleic acid stain was poured onto the gel. This was left for 20 min and the gel transferred onto a UV transilluminator. The gel was photographed through a SyBr Gold/Green filter (Molecular Probes) with Polaroid 655 film by exposure for 4 min at F8 while transilluminated with 300 nm UV.

Haplotype Scoring

Photonegatives of SSCP gels were used to assign haplotypes for the large and small COI fragments to each krill. Most haplotypes could be assigned easily by eye. Samples not run in adjacent lanes were sometimes compared on an image file taken from the photonegative using NIH image 1.62 software to measure the relative migration of the fragments. If there was doubt about scoring, the sample was run again alongside different samples.

Haplotype Sequencing

Each observed haplotype was sequenced at least once. Any haplotype that occurred more than once was sequenced at least twice to check that the scoring of haplotypes was consistent and that each SSCP represented a single DNA sequence. mtCOI haplotypes to be sequenced were selected from their SSCP. The PCR products for individuals that had lost the Rsa I site used in the SSCP assay, so that one fragment remained after restriction digestion, were also sequenced.

10 μL of PCR amplified DNA that was not restriction digested was separated from the unincorporated primers and other PCR components using the Qiagen Quick Spin PCR purification procedure. 30 ng of the purified PCR product was used as a template in Big Dye Terminator (ABI) sequencing reactions. The reactions were separated on an ABI 377 automated sequencer.

Data Analysis

The haplotype sequences for each fragment of the COI region and the number of times each individual sequence occurred in each population were the basis for all analyses. A composite haplotype for the entire 616 bp region was assigned to each individual either from its small and large fragment SSCP or from direct sequencing.

The variance in mtDNA haplotype distributions among four E. crystallorophias populations was calculated by Analysis of Molecular Variance (AMOVA) (Excoffier et al., 1992) using the program Arlequin version 2.0 (Schneider et al., 2000). This is a modification of a hierarchical ANOVA that accounts for relationships between the genotypes as well as the variance in their frequency. The variance is partitioned into within-population and between-population components. Pairwise Φst calculations (Excoffier et al., 1992) were then used to determine which populations were responsible for the genetic differentiation that was identified in the overall population pool.

Six pairwise Φst calculations (m = 6) were made using Arlequin between all combinations of the four populations analysed to assess how much of the between-sample variation was attributable to each sample. The same model of DNA substitution used for the AMOVA was applied. Tests for the significance (α = 0.05) of these values were made by generating 1 000 pseudoreplicate datasets and using these as a null distribution to see how likely it was that the Φst result arose by chance. A Bonferroni correction (α = α / m = 0.05 / 6 = 0.0083) was applied to the significance calculations to reduce the chance of making false positive observations (Everitt, 1998).

RESULTS

Our AMOVA analysis of krill haplotype distribution demonstrated that a significant proportion (3.68%) of the variance in distribution was due to between-population differences. Pairwise Φst calculations were then used to make an estimate of the degree of genetic differentiation between each pair of krill populations. The results of the pairwise Φst calculations are shown in Figure 1. A more comprehensive analysis of these results is given in Jarman et al. (2002).

This study of E. crystallorophias population genetics was conducted with the same original assumptions as all previous population genetics studies of krill. A major assumption of these
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studies is that any genetic differentiation identified is likely to be caused by restricted gene flow between resident krill populations in each area. Our samples of *E. crystallorophias* were taken from three widely separated locations in the Southern Ocean. From one site in the Mertz Glacier Polynya, krill had been captured in two net tows at locations 16 km apart. This allowed us to analyse four populations of *E. crystallorophias* and to make a preliminary assessment of the effect of degree of geographic separation between samples based on the degree of genetic differentiation between samples. Figure 1 shows that the degree of genetic differentiation between populations did not correlate with the degree of geographic separation of the sampling sites.

**DISCUSSION**

By using DNA-based techniques, it is now possible to identify genetic differentiation between samples of krill. The causes of the genetic differentiation identified so far have not been properly assessed. Our study of *E. crystallorophias* population genetics found significant genetic differentiation between samples collected only 16 km apart, but not between samples collected 5000 km apart. This suggests that there is considerable variance in mtDNA haplotype distribution within large-scale ocean features. A previous study of *E. superba* population genetics by Zane et al. (1998) also only found significant genetic differences between krill from the two closest sites in the study, although these samples were still separated by a considerable distance. In their study, the closest sampling sites were also separated by major oceanographic features, especially the Weddell Gyre and Weddell–Scotia Confluence. The authors suggested that these features may be restricting gene flow between the two sites. This may be true, but it is also possible that the differentiation detected between these two populations is simply part of the variance that can be found between any two krill populations regardless of their geographic separation.

There are good reasons to believe that there will be variance in genetic parameters between krill samples taken from one region. Variation in other features of krill caught in one area has already been demonstrated for *E. superba*. Watkins et al. (1986, 1990) found significant differences in weight, sex-ratio and length of *E. superba* caught in different net tows in the vicinity of South Georgia. Watkins et al. (1990) estimated the number of trawl hauls necessary to get an unbiased estimate of these population parameters for krill from one region and found that a minimum of 14 hauls containing 100 individuals were needed. This strongly suggests that single hauls of krill cannot adequately assess genetic parameters for whole regions of the Southern Ocean either.

**CONCLUSION**

If we are to use population genetics to identify krill ‘stocks’ for management purposes, then we must be able to distinguish between genetic differentiation between and within regions. Future population genetics studies should assume that there is within-region variance and include the extra variance component of within-region genetic differentiation in the experimental design and statistical analysis. The extent of within-region variance in krill genotype frequencies is unknown, so these studies should compare sufficient krill samples from each of the major Southern Ocean regions to be sure that within-region variance can be assessed. Watkins et al. (1990) suggested that a minimum of 14 samples of krill are necessary to characterise morphological characters of krill from one region. Given that variance in selectively neutral genetic characters is likely to be lower as they are not subject to environmental variation, a minimum of 10 samples of at least 100 individuals from each region should be analysed. An intensive sampling project like this is essential to determine the causes of genetic differentiation within Southern Ocean krill species.

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Tableau 1: Résumé des méthodes et des stratégies d’échantillonnage utilisées dans les études génétiques des populations de trois espèces de krill. À ce jour, toutes les études utilisent soit diverses combinaisons d’enzymes polymorphiques ou de sites de nucléotides variables dans l’ADN de la mitochondrie. La taille des échantillons prélevés pour les études de l’allozyme représente le nombre maximum d’individus à pointer pour chaque système d’enzymes. Dans la plupart des cas, moins d’individus ont été pointés pour les autres systèmes d’enzymes utilisés.

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Figure 1: Valeurs relativement importantes ($\alpha = 0.05$) de $\Phi_{st}$ par paire trouvées sur quatre échantillons d’Euphausia crystallorophias. #La différence entre la polynie 1 du glacier Mertz et la mer de Davis n’était pas importante après une correction de Bonferroni ($\alpha$ corrigé = 0,0083). Aucune valeur importante de $\Phi_{st}$ n’a été trouvée pour les comparaisons entre les échantillons de la mer de Davis et la péninsule de l’ouest de l’Antarctique.

Список таблиц

Табл. 1: Методы и стратегии взятия проб, применявшиеся в исследованиях популяционной генетики 3 видов криля. Во всех проведенных на сегодня исследованиях использовались либо различные комбинации полиморфных ферментов, либо различные участки нуклеотидов митохондриальной ДНК. Размеры выборки при исследованиях аллозимов – это максимальное число идентифицируемых особей для одной системы ферментов. В большинстве случаев было идентифицировано меньше особей для других использовавшихся систем ферментов.

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Рис. 1: Значимые ($\alpha = 0.05$) попарные значения $\Phi_{st}$, обнаруженные между 4 образцами Euphausia crystallorophias. #Разница между Польньей 1 Ледника Мерц и морем Дейвиса была несущественной с поправкой Бонферрони (откорректированное $\alpha = 0.0083$). При сравнении образцов из моря Дейвиса и с запада Антарктического п-ова существенных значений $\Phi_{st}$ найдено не было.
Tabla 1: Resumen de los métodos y estrategias de muestreo utilizados en los estudios genéticos de las poblaciones de tres especies de kril. A la fecha, todos los estudios utilizan ya sea combinaciones diferentes de enzimas polimórficas o diversas secuencias de nucleótidos del ADN mitocondrial. El tamaño de las muestras requerido por los estudios que utilizan alloenzimas representan el máximo número de individuos que se pueden identificar mediante un sistema de una sola enzima. En la mayoría de los casos, se identificaron menos individuos al utilizar los otros sistemas enzimáticos.

Figura 1: Valores emparejados significativos ($\alpha = 0,05$) de $\Phi_{st}$ encontrados entre cuatro muestras de Euphausia crystallorophias. # La diferencia entre la polinia 1 de Mertz Galcier y el mar de Davis no fue significativa con la corrección de Bonferroni ($\alpha$ corregido = 0,0083). No se encontraron valores significativos de $\Phi_{st}$ para las comparaciones entre las muestras del mar de Davis y del oeste de la Península Antártica.