SCI. MAR., 68 (Suppl. 1): 121-128 SCIENTIA MARINA 2004

BIOLOGICAL OCEANOGRAPHY AT THE TURN OF THE MILLENIUM. *J.D. ROS, T.T. PACKARD, J.M. GILI, J.L. PRETUS and D. BLASCO (eds.)*

# Distribution of *Calanus* spp. as determined using a genetic identification system*

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SUMMARY: The morphological similarity of *Calanus* species necessitates that the only unambiguous way of discriminating between the different species at any developmental stage is with molecular tools. We have developed a simple molecular technique to distinguish between the four species of *Calanus* copepods found in the North Atlantic (*Calanus helgolandicus, C. finmarchicus*, *C. glacialis* and *C. hyperboreus*) at any life stage. This system involves the PCR amplification of a region of the 16S rRNA gene, followed by Restriction Fragment Length Polymorphism (RFLP) analysis of the amplified product. This paper describes the application of the technique to a number of *Calanus* samples taken from a wide geographical range within the North Atlantic. Samples were acquired from sites ranging in latitude from the English Channel (50°N) to Tromsø (69°N), including many regions of co-occurrence. A comparison has been made between the distribution of *Calanus* as determined by molecular techniques and distributions established using traditional morphological identification. The molecular analysis has clearly shown extended areas of distribution and co-occurrence and has confirmed the intraspecific conservation of the restriction sites and therefore the reliability of the technique.

*Key words: Calanus*, 16S rRNA, mtDNA, RFLP, genetic identification, distribution.

RESUMEN: DISTRIBUCIÓN DE *CALANUS* SPP. DETERMINADA UTILIZANDO UN SISTEMA DE IDENTIFICACIÓN GENÉTICA. – La semejanza morfológica de las especies de *Calanus* obliga a que la única manera clara de discriminar entre las distintas especies en cualquier estadio de desarrollo sea mediante herramientas moleculares. Hemos desarrollado una técnica molecular simple para distinguir las cuatro especies de copépodos del género *Calanus* que se encuentran en el Atlántico Norte (*Calanus helgolandicus, C. finmarchicus*, *C. glacialis* and *C. hyperboreus*), sea cual sea el estadio de su ciclo biológico. Este sistema implica la amplificación mediante PCR de una región del gen 16S rRNA, seguida de análisis de Polimorfismo de Longitud de Fragmentos de Restricción de (RFLP) del producto amplificado. Este trabajo describe la aplicación de la técnica a varias muestras de *Calanus* procedentes de una amplia región geográfica en el Atlántico Norte. Se obtuvieron muestras de localidades que iban desde el canal de la Mancha (50°N) a Tromsø (69°N), incluyendo muchas regiones de presencia conjunta. Se ha hecho una comparación entre la distribución de *Calanus*, determinada por las técnicas moleculares, y las distribuciones sobre la base de la identificación morfológica tradicional. El análisis molecular ha demostrado claramente áreas extendidas de distribución y ocurrencia conjunta y ha confirmado la conservación intraspecífica de los lugares de restricción, con lo que se ha confirmado la fiabilidad de la técnica.

*Palabras clave*: *Calanus*, 16S rRNA, mtDNA, RFLP, identificación genética, distribución.

## INTRODUCTION

Knowledge of the pelagic food web is fundamental to the understanding of marine ecosystem function. Zooplankton communities, forming central and important links in this food web, are often dominated by copepods that represent up to 90% of the biomass of shelf areas (Jaschnov, 1970). In the North Atlantic copepods of the genus *Calanus* form a significant proportion of the zooplankton biomass

*Received September 20, 2001. Accepted November 19, 2002.

and play an important role as secondary producers in the marine food web, grazing extensively on phytoplankton and representing significant prey species for larvae, juveniles and adults of commercial fish (Dickson and Brander, 1993; Conover *et al.*, 1995; Runge and Lafontaine, 1996).

One of the persistent problems when investigating marine zooplankton communities, especially copepod communities, relates to the correct identification of the different species within a genus. The four major North Atlantic *Calanus* species have very similar morphologies, with diagnostic features being restricted to size or minor variations in secondary sex characteristics. This presents a persistent problem for the identification of individuals to species level, with immature animals being the most problematic. For the identification of such juvenile stages the geographic location of collection is, at least partially, relied upon as an indicator of species identity.

It is important to distinguish between *Calanus* species because they have different vertical distributions and temperature affinities, yet they co-occur and compete interspecifically. For a better understanding of how each species differs in its life strategy and response to environmental conditions, we need to accurately describe their spatial and temporal pattern, and to do this we must have a reliable means of discriminating between the species.

Despite morphological similarity, species of *Calanus* exhibit considerable base sequence divergence within their genomes (Bucklin *et al.*, 1995). In the mitochondrial genome, the gene encoding the large subunit of ribosomal RNA (the 16S rRNA gene or 16S rDNA) has a sequence which varies sufficiently to discriminate closely-related species but that has an intraspecific variation which is generally low. Based on this gene a simple molecular technique to differentiate between the North Atlantic *Calanus* species (*C. helgolandicus, C. finmarchicus, C. glacialis* and *C. hyperboreus*) at any developmental stage has been developed (Lindeque *et al.*, 1999). Using the polymerase chain-reaction (PCR), a region of the mitochondrial 16S rRNA gene can be amplified from individual copepods preserved in ethanol. Subsequent digestion of the amplified products with the restriction enzymes *Dde* I and *Vsp* I, followed by electrophoretic separation in 2% Metaphor agarose gel, produces a characteristic 'fingerprint' for each species. Using this system, it is possible to identify to species level not only whole animals at any developmental stage from egg to adult, but also individual body parts.

This paper describes the application of the diagnostic technique to a number of ethanol preserved animals taken from a wide geographical range within the North Atlantic, testing comprehensively the accuracy and reproducibility of the technique. The 16S rDNA primers (16SAR and 16SB2R) used for the amplification are complementary to conserved regions of the 16S gene, ensuring amplification from all four of the *Calanus* species studied. The restriction sites of the chosen enzymes *Dde* I and *Vsp* I are present at regions of variable sequence between the *Calanus* species under study and are therefore suitably diagnostic. From detailed comparisons of *Calanus* sequence data from geographically diverse conspecific populations these restriction sites show no intraspecific variation. However, a true test of the intraspecific conservation of the restriction sites, and therefore the method, is the application of the technique to *Calanus* samples collected from a wide geographical range.

The need for an unambiguous method to discriminate between *Calanus* species was illustrated clearly in a study of *Calanus* populations in Lurefjorden (western coast of Norway). It is suspected that in Lurefjorden, the populations of *Calanus* are not composed of a single species; however, adult *Calanus* are of a similar size and therefore harder to separate into species than in other systems (Ketil Eiane, 1999 and pers. comm.). *Calanus* collected from the fjord were attributed to species level, using the morphological characteristics of the curvature of the coxa of the fifth pair of swimming legs. The identification was therefore restricted to CV stages and adult females. For a non-taxonomist this method is both time consuming and ambiguous, so it was decided to test the morphological identification of the *Calanus* species against molecular identification in a blind test of the samples.

Finally, the distribution of *Calanus* in the North Atlantic determined by molecular techniques is compared with traditional distributions established using morphological discriminators.

## METHODS

### Collection and preservation of samples

Individuals of *Calanus spp.* (adults and late copepodite stages) were collected from a wide range

TABLE 1. – Location, method and depth of sample collections to which the molecular method of *Calanus* identification was applied. Samples collected by P. Lindeque[1+6], S.R. Gonzalez and S.S. Oosterhuis[2], E. Bagoien and K. Eiane[3], E. Gaard[4+5], X. Irigoien[7], K. Olsen[8], B. Hansen[9+10].

| Location | Method of collection | Depth of collection |
|---|---|---|
| L4, English Channel [1] | Oblique tow | 50-0 m |
| North Sea [2] | Vertical haul | 70-0 m |
| Lurefjorden [3] | Vertical haul | 400-0 m |
| Faroe shelf [4] | Vertical haul | 50-0 m |
| Faroe off-shelf [5] | Vertical haul | 50-0 m |
| Oban, West Scotland [6] | Vertical haul | 50-0 m |
| Weathership M [7] | Vertical haul | 100-0 m |
| Saltenfjord [8] | Vertical haul | 380-0 m |
| Bergen [9] | Vertical haul | 100-0 m |
| Tromsø [10] | Vertical haul | 100-0 m |

of geographical locations in the North Atlantic with 200 µm WP-2 plankton nets. The source, location and method of collection of the *Calanus* samples are presented in Table 1. Samples collected from the English Channel, Lurefjorden, East Scotland, Weathership M, Saltenfjord, Bergen and Tromsø were stored in absolute ethanol with approximately 1 animal per 1 mL of ethanol. Samples collected from the North Sea were stored at a higher concentration (approximately 10-20 animals per 1 mL ethanol) and, for those samples collected around the Faroes, animals were stored at a significantly higher concentration (approximately 100 individuals per 1 mL of ethanol).

### Molecular identification of *Calanus* individuals

The technique for molecular identification of *Calanus* individuals described by Lindeque *et al.* (1999) has been modified to reduce the cost of analysis. Modifications include the use of *Taq* DNA polymerase (Promega UK Ltd.) in preference to DynazymeTM (Flowgen Instruments Ltd.), and a reduction in the amount of restriction enzyme used for each reaction. The final technique is described below:

Animals were rehydrated in 0.5 mL of MilliQ water in a microcentrifuge tube for 6-12 h at room temperature. After rehydration, the water was removed and replaced with 34 µL MilliQ water and 5 µL 10 x *Taq* DNA polymerase buffer (Promega UK Ltd.). Copepods were homogenised using a pellet pestle homogeniser (Anachem Ltd.) and incubated at 4°C overnight. After incubation, the homogenate was transferred to a 0.7 mL tube and the remaining reaction components were added [5 µL 2 mM dNTPs, 2.5 µL each of primers 16SAR and 16SB2R (100 ng $\mu L^{-1}$), and 2 U of *Taq* DNA polymerase (Promega UK Ltd.)]. The amplification primers used were 16SAR (5'-CGCCTGTTTAACAAAAACAT-3'; Palumbi and Benzie 1991) and 16SB2R (5'-ATTCAACATCGAGGTCACAAAC-3'; custom designed from existing *Calanus* sequences). Amplification was performed in a thermal cycler (PTC-100™, MJ Research, Inc). The cycling parameters included an initial denaturation step of 94°C (5 min) followed by 40 cycles of 45°C (2 min), 72°C (1 min), and 94°C (1 min). A final annealing phase at 45°C (2 min) was followed by an extension phase at 72°C (3 min) and storage at 4°C until use. Aliquots (10 µL) of the amplification reactions were analysed by agarose gel electrophoresis (1.5%) to check amplification efficiency.

### Restriction Fragment Length Polymorphism analysis

Restriction digests were performed on a 15 µL aliquot from each amplification by the addition of 0.5 µL 5 M NaCl, 2 µL bovine serum albumin (1 mg $mL^{-1}$) and 2.5 U of each restriction enzyme (*Dde* I and *Vsp* I). Incubations were performed at 37°C for 1 h. The digestion products were separated by electrophoresis through a 2% Metaphor agarose gel, prechilled for 30 min at 4°C to improve resolution. The gels were observed and photographed by UV transillumination.

### Comparison of morphological and molecular identification of *Calanus* species from Lurefjorden

Using the morphological characteristics of the coxa on the fifth pair of swimming legs, Espen Bagoien (Norway) identified 154 *Calanus* collected from Lurefjorden. The ethanol-preserved animals were then individually subjected to molecular identification with no prior knowledge of the morphological results.

### Molecular identification of *Calanus* from a wide geographical range in the North Atlantic

From *Calanus* samples obtained from each of the geographical areas shown in Table 1, between 20 and 30 individual animals were picked randomly from storage in ethanol. Each individual was identified using the technique described above.

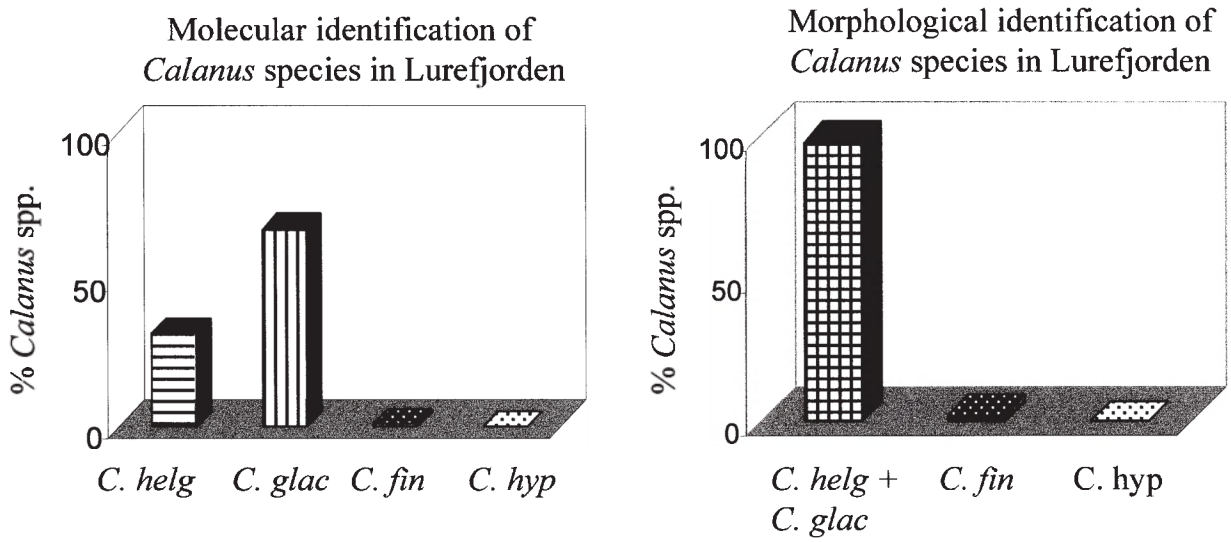


FIG. 1. – Species identification of *Calanus* from Lurefjorden. Comparison of morphological and molecular identification techniques. *C. helg* = *Calanus helgolandicus*, *C. glac* = *C. glacialis*, *C. fin* = *C. finmarchicus*, *C. hyp* = *C. hyperboreous* (n=153).

## RESULTS

### Preservation of samples

Animals stored at concentrations of 1-20 individuals per 1 mL absolute ethanol were preserved successfully, and proved suitable for DNA amplification. Many samples of *Calanus* stored at a higher density (approximately 100 animals per 1 mL ethanol) appeared contaminated with copepod debris, possibly because of physical degradation during transportation. However, intact animals within these samples, following thorough washing in MilliQ water to remove any contaminating fragments, were still suitable for amplification.

### Molecular identification of *Calanus* individuals

Over 99% of copepods analysed produced a PCR product of the correct size when amplified with the 16SAR and 16SB2R primers. Less than 1% of individual *Calanus* failed to produce any amplification product. All amplified products were digested successfully with *Dde* I and *Vsp* I to give a characteristic restriction profile for *Calanus helgolandicus*, *C. finmarchicus*, *C. glacialis* or *C. hyperboreus*. No aberrant restriction profiles were obtained.

### Comparison of morphological and molecular identification of *Calanus* species from Lurefjorden

*Calanus* collected from Lurefjorden, following morphological identification in Norway, were characterised to species level using the developed molecular technique. Out of 154 animals, 153 were identified successfully and 1 failed to produce an amplification product. The molecular results showed that 67% of the *Calanus* collected were *C. glacialis*, 32% were *C. helgolandicus*, and 1% were *C. finmarchicus*. No *C. hyperboreous* were found (Fig. 1). In comparison with determination of species by molecular techniques, 98% of *Calanus* were attributed to *C. helgolandicus* and/or *C. glacialis* species and 2% to *C. finmarchicus* species based on morphological identification (Fig. 1). Morphological identification was restricted to stage V copepodites and adult females. It appeared that the criteria used for the identification did not allow the separation of *C. helgolandicus* and *C. glacialis* species, and no convincing morphological trait was found to classify males to species level. The morphological methods of identification proved ambiguous and even after detailed examination of the morphological characteristics it was noted that many individuals were identified 'with doubt'.

A comparison between the molecular and morphological identification of *Calanus* highlights the limitations of morphological identification methods, and demonstrates clearly the potential of the molecular technique. The morphological identification was so limited that it precluded a direct comparison of species identification of individuals by morphological and molecular means. The results of the molecular technique showed that most *Calanus* were identified as either *C. helgolandicus* or *C. glacialis*, whereas by morphological means no such differentiation was possible between these two species.

Molecular analysis also determined that 1% of the animals were *C. finmarchicus*, compared with 2% based on morphological identification. Only one *Calanus* individual was identified as *C. finmarchicus* by both morphological and molecular means.

### Distribution of *Calanus* species in the North Atlantic determined by the molecular technique

Between 20 and 30 *Calanus* individuals (154 for Lurefjorden) were characterised to species level from each of 18 different geographical locations within the North Atlantic region (Fig. 2).

Molecular identification showed that *C. helgolandicus* was distributed predominantly in the south of the area studied, being the sole representative of the genus in the English Channel sample. At eight of the locations sampled, *C. helgolandicus* co-occurred with *C. finmarchicus*, and at four of these locations, *C. helgolandicus* and *C. finmarchicus* co-occurred with *C. glacialis*. *Calanus finmarchicus* showed the greatest area of distribution, ranging from the North Sea (55°N), to Tromsø (69°N) and dominated the species composition in the Faroe Shetland channel and the North Atlantic basin (Fig. 2). At the most southerly edge of the range, *C. finmarchicus* co-occurred extensively with *C. helgolandicus*, and at the northern extreme with *C. glacialis* and/or *C. hyperboreus*. Although having a wide area of distribution (Oban, 56°N to Tromsø, 69°N), *C. glacialis* contributed only a small percentage of the *Calanus* species composition. The only location where *C. glacialis* dominated was in Lurefjorden (Norway). *Calanus hyperboreus* was found in the most northern

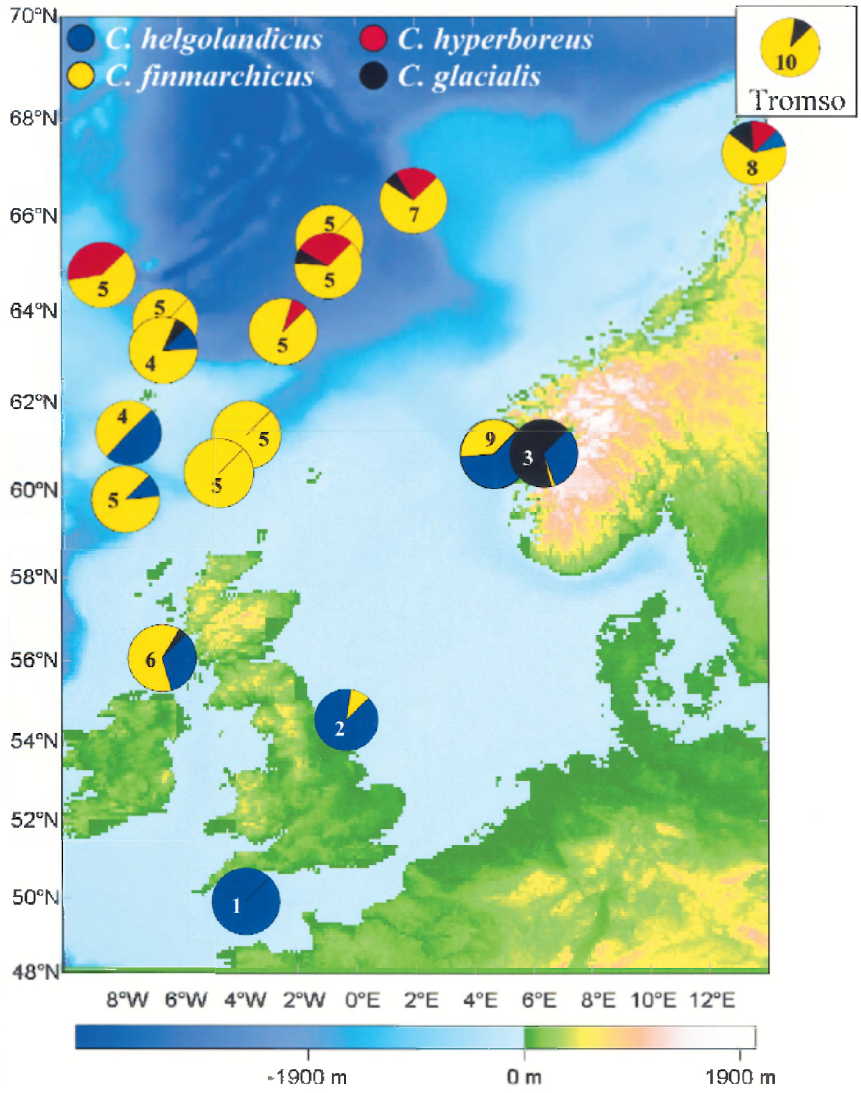


FIG. 2. – Distribution of *Calanus helgolandicus*, *C. finmarchicus*, *C. glacialis* and *C. hyperboreus*, identified using 16S rDNA amplification and RFLP analysis.

of the locations sampled, and co-occurred with *C. helgolandicus, C. finmarchicus and C. glacialis.* Only at one location did all four *Calanus* species occur together (Saltenfjord, 67°N; Fig. 2).

## DISCUSSION

The simple molecular identification technique, involving amplification of 16S rDNA and subsequent RFLP analysis (modified from Lindeque *et al.*, 1999), has been applied successfully to a number of samples of *Calanus* collected from a wide geographical area. The method was able to distinguish unambiguously between *Calanus helgolandicus*, *C. finmarchicus*, *C. glacialis* and *C. hyperboreus* with an amplification success rate of >99%.

Animals stored in absolute ethanol, even at a high density of approximately 100 individuals per 1 mL of ethanol, were suitable for DNA amplification. This system of preservation is simple to use at sea, and appears suitable for transportation and long-term storage prior to molecular analysis.

The success rate for amplification of the 16S rDNA fragment from preserved animals was over 99%. The small proportion of individuals, less than 1%, that failed to produce an amplification product, can probably be attributed to insufficient homogenisation of the animal. In all cases of successful amplification, the DNA product was digested successfully with *Vsp* I and *Dde* I to give a restriction profile characteristic of one of the four *Calanus* species. There were no aberrant restriction profiles from any geographical location. The application of the molecular technique has confirmed that the method can consistently discriminate unambiguously between the four *Calanus* species common to the North Atlantic. Over the geographical region studied the nucleotide sequences at the *Dde* I and *Vsp* I restriction sites are suitably conserved, proving that geographically distant conspecific populations do not affect the method. Hill *et al.* (2001) have also confirmed that sequence variation in *Calanus* mitochondrial DNA can be a diagnostic and accurate indicator of species identity. Using mitochondrial Cytochrome Oxidase I sequence variation a molecular technique for *Calanus* species identification, based on a multiplexed species specific PCR-protocol has been designed (Hill *et al.*, 2001). Both protocols are suitable for the molecular diagnosis of *Calanus* species in the North Atlantic by untrained biologists and oceanographers.

An interesting result, revealed by the application of this molecular diagnostic technique, is the species composition of *Calanus* identified in Lurefjorden. Lurefjorden is virtually landlocked and has an invertebrate-dominated predator system. Compared to other fjords, Lurefjorden has a higher standing stock of *Calanus* that exhibit a high mortality rate early in life but a lower mortality rate in the early copepodite stages (Eiane *et al.*, 1999). As with neighbouring fjords, it was thought that the Lurefjorden *Calanus* species composition predominantly included *C. finmarchicus* and *C. helgolandicus.* In contrast, the Lurefjorden species exhibited different egg production rates and predator/prey relationships (Eiane *et al.*, 1999). The application of a molecular technique has revealed the presence of high numbers of *C. glacialis* in the fjord. Fortuitously the presence of three *Calanus* species in Lurefjorden has also been revealed by Bucklin *et al.*, (2000) based on the DNA sequence of mt16S rDNA. The molecular-based systematic approach to *Calanus* has proved invaluable, exceeding the capabilities of traditional morphological taxonomy for the *Calanus* species in Lurefjorden. This molecular technique has alerted ecologists to the presence of three *Calanus* species in the fjord and has provided a tool for further research of this unusual ecosystem.

Comparison of the morphological and molecular identification of *Calanus* species in Lurefjorden highlighted the weaknesses of traditional morphological methods. The morphological criteria used for identification were unable to discriminate between male *Calanus*, and did not allow separation of *C. helgolandicus* and *C. glacialis.* Although comparison between morphological and molecular characterisation techniques has obviously been limited, it is clear that a simple method of molecular identification is effectual, being more extensive and less ambiguous than morphological methods.

Molecular identification of *Calanus* species from a wide geographical area within the North Atlantic has not only proved the accuracy and reproducibility of the technique, but has also stimulated some new interpretations of dispersal. The distribution of *Calanus* species determined by molecular analysis is mostly reflective of traditional results. However, the molecular analysis has clearly shown extended areas of distribution and co-occurrence. These results demonstrate the limitations of identification of *Calanus* species based solely on morphology and have highlighted the potential errors associated with relying on geo-

graphical location for species determination. *Calanus helgolandicus* is a warm-temperate water species found in southern areas of the North Atlantic, the Celtic Sea, North Sea and in coastal waters south of England (Fleminger and Hulsemann, 1977; Planque and Fromentin, 1996). Recently the use of Generalized Additive Models in the analysis of Continuous Plankton Recorder (CPR) data have shown that *C. helgolandicus* are advected around the north of Scotland and into the North Sea through the Fair Isle Channel (Beare and McKenzie, 1999). These results indicate that the population of *C. helgolandicus* is increasing and colonising further north within the North Sea overtime. The occurrence of *C. helgolandicus,* determined by this molecular study, is generally supportive of the traditional distribution described above. It is however, surprising to find *C. helgolandicus* north of the Faroes, especially considering that the sample was collected in April when the water temperature was still low. Possibly, *C. helgolandicus* found in this area result from advective transport in the Atlantic stream.

*Calanus finmarchicus*, a more cold-temperate water species than *C. helgolandicus*, is abundant in coastal and Atlantic water around Iceland (Gislasson and Astthorsson, 1995), and is found in the East Icelandic current water and the Arctic intermediate water around the Faroes. In this study, *C. finmarchicus* was observed as far south as 54°N in the North Sea and as far north as Grotsund, Tromsø. Traditionally, *C. finmarchicus* is thought to overlap extensively with *C. helgolandicus* in many areas of the North Atlantic and in the shelf seas around the United Kingdom (Williams and Conway, 1980). The two *Calanus* species co-occur extensively in surface waters of the Northeast Atlantic during spring and in the surface waters of the North Sea during summer and autumn (Planque and Fromentin, 1996). The molecular analysis of samples clearly reflects this distribution, with a co-occurrence of *C. finmarchicus* and *C. helgolandicus* seen in the Faroe and west of Scotland samples collected in April and May, and in the North sea sample obtained in July. Planque and Fromentin (1996), from an analysis of CV and CVI animals collected in the subsurface layer, estimated that the region of co-occurrence of *C. finmarchicus* and *C. helgolandicus* extended from 52°N to 57°N in the North Sea and Northeast Atlantic. It is obvious from this molecular study that the sympatric area of these two species extends well above 57°N.

*Calanus glacialis*, predominantly an Arctic water species (Frost, 1974), has a reproductive range overlapping with both *C. helgolandicus* and *C. finmarchicus* (Fleminger and Hulsemann, 1977). From molecular analysis there are four regions where these three species of *Calanus* co-occur: north of the Faroe isles; Saltenfjord and Lurefjorden, western Norway; and Oban, western Scotland. It was perhaps not anticipated to observe *C. glacialis* as far south as Oban compared to traditional, morphologically determined distributions.

*Calanus hyperboreus* is a northern Arctic cold water species that has a population centred in the Greenland Sea (Conover, 1988; Hirche, 1991). *Calanus hyperboreus* shows overlapping distributions with *C. finmarchicus* and *C. glacialis* at the front between the Atlantic and Arctic water masses in the Northeast Atlantic (Hirche *et al.*, 1994). Molecular identification of *Calanus* has shown three sympatric areas of *C. hyperboreus*, *C. glacialis* and *C. finmarchicus.*

This molecular study has demonstrated a greater co-occurrence of *Calanus* species than traditionally thought. The extent of the sympatric areas may not have been previously realised, because geographical location has played a significant role in species determination, with obvious consequences. Taking into account the difficulties associated with morphologically distinguishing between *Calanus* species, especially considering the extent of overlap in the length distributions of *C. finmarchicus* and *C. glacialis* (Jaschnov, 1972; Frost, 1974), it is probable that *Calanus* species have been misidentified previously, affecting knowledge of geographical distributions. Traditionally, *Calanus* distributions have been based on CV and adults, because of the difficulties in identifying earlier stages. Williams and Conway (1980) gave evidence that the vertical temperature gradient was responsible for differences in the vertical distributions of co-occurring *C. finmarchicus* and *C. helgolandicus*, and between different stages within a species. However, because of the difficulty in identifying early developmental stages, assumptions on the vertical distribution of different stages from a site of sympatric distribution had to be drawn from analysis of isolated species. The molecular tool, allowing the unambiguous identification of *Calanus* species at any developmental stage, has obvious implications in describing the distributions of *Calanus* juveniles. In areas of extreme distribution, or unexpected co-occurrence, it is not known whether adult populations are coin-

cident with younger populations. Such a question is of ecological importance because the presence of young stages would be indicative of a growing population. If no eggs or nauplii were present, it may be assumed that the older stages have been transported to their site of collection. Answering such questions is only possible with the developed molecular technique.

The four *Calanus* species, for which this molecular identification technique has been designed, are the predominant species in the North Atlantic. However, *Calanus marshallae* has been identified recently in Arctic waters in Isfjorden, Spitsbergen (Sundt and Melle, 1998) by sequence data analysis of an amplified region of 16S rDNA. This appears to be an isolated case and, therefore, does not warrant extension of the developed technique to include such species. However, were future samples to produce aberrant restriction profiles, sequence data would be obtained for the 16S amplification product to obtain species identity, and the profile noted and used in future diagnosis.

The relatively simple and inexpensive molecular method, developed for the identification of the North Atlantic *Calanus* species, is reliable and reproducible. The technique has shown a huge potential for better describing and understanding the distribution of this important copepod genus. Correct species identification is the keystone of ecological studies, and accurate descriptions of the spatial and temporal patterns of *Calanus* species are invaluable for understanding and predicting the response of the different species to environmental conditions.

## ACKNOWLEDGEMENTS

This work was funded by the NERC special topic PRIME (Plankton Reactivity in the Marine Environment) and was partially supported by funding from the European Commission through the TASC project (MAS3-CT95_0039). We are grateful to E. Bagoien, K. Eiane, E. Gaard, S. R. Gonzalez, B. Hansen, X. Irigoien, K. Olsen, and S. S. Oosterhuis for supplying samples of *Calanus* species. We would also like to thank Joanne Shepherd for all her help with the molecular identification of *Calanus* species during a work-experience placement.

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