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Simple molecular method to distinguish the identity of *Calanus* species (Copepoda: Calanoida) at any developmental stage

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Abstract Diagnostic morphological characteristics of copepods of the genus *Calanus* are restricted largely to minor variations in secondary sex characteristics. This presents a persistent problem in the identification of individuals to species level, especially for immature stages. We have developed a simple molecular technique to distinguish between the North Atlantic *Calanus* species (*C. helgolandicus*, *C. finmarchicus*, *C. glacialis* and *C. hyperboreus*) at any life stage. Using the polymerase chain-reaction (PCR), the mitochodrial large subunit (16S) ribosomal RNA (rRNA) gene was 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% agarose (Metaphor, FMC Ltd), produced a characteristic pattern for each species. The versatility of the method is demonstrated by the unambiguous identification to species of any life stage, from egg to adult, and of individual body parts.

Introduction

Copepods of the genus *Calanus* form a predominant proportion of the zooplankton biomass in the North Atlantic. They play an important role in marine food webs, both as consumers of primary production and as prey species for the larvae of commercial fish (Runge 1988). Indeed, there are believed to be more individuals of the genus *Calanus* alive at any one time than any other animal (Bucklin et al. 1996). Despite the relatively high abundance and ecological importance of the genus in both coastal and open oceanic planktonic assemblages, systematic relationships amongst the species are still unclear. Until the latter half of this century (Manwell et al. 1967; Frost 1974), it was not certain whether *C. helgolandicus* and *C. finmarchicus* were distinct species. *Calanus* species are reproductively isolated, but the manner in which this is attained results in extreme morphological similarity (Fleminger and Hulsemann 1977; Bucklin et al. 1995). Diagnostic morphological characteristics of *Calanus* species are restricted essentially to minor variations in their secondary sex characteristics, presenting a persistent problem in the identification of individuals to species level. Immature *Calanus* are the most problematic and, historically, the geographic location of collection has, at least partially, been relied upon as an indicator of specific identity.

When morphological characters are not practical for routine identification to species level, genetic characters can be used to provide unambiguous taxonomic discrimination. A reliable routine method to identify species is important for establishing their horizontal and vertical ranges (Fleminger and Hulsemann 1977). Such a method would also enable questions about the distribution and abundance of developmental stages to be answered.

Despite morphological similarity, *Calanus* species exhibit considerable base-sequence divergence in the mitochondrial large subunit (16S) ribosomal RNA (rRNA) gene (Bucklin et al. 1995). The 16S rRNA gene has been used to determine phylogenetic relationships in a wide range of organisms (Lane et al. 1985; DeLong et al. 1989; Rehnstam et al. 1989; Ward et al. 1990; Britschgi and Giovannoni 1991), and to examine the molecular systematics of *Calanus* (Bucklin et al. 1992).

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Although this mitochondrial gene varies sufficiently to discriminate closely related species, its intraspecific variation is low enough to warrant its use for species' identification. In this study, we build on the work of Bucklin et al. (1992, 1995), in which the DNA sequence of 16S rRNA genes was determined and used to compare phylogenetic relationships between *Calanus* species. We have further developed this system to produce a method whereby simple molecular techniques can be used to determine unambiguously the species of adults, juveniles and eggs of the dominant *Calanus* species found in the North Atlantic Ocean. Additionally, this technique is useful for the identification of small body parts of *Calanus* species which would enable their identification in gut-content analyses of predators.

To develop our molecular system of *Calanus* identification we used the four species *C. helgolandicus*, *C. finmarchicus*, *C. glacialis* and *C. hyperboreus*. Despite their distinct hydrographic affinities, these species are sympatric in many areas, with overlapping reproductive periods. *C. finmarchicus* is a characteristic northern boreal species that borders *C. glacialis,* primarily an arctic species (Frost 1974). In the eastern North Atlantic, *C. finmarchicus*, *C. glacialis*, and *C. hyperboreus* are sympatric in many areas including the region of the Polar Front. The former two species also co-occur in the region of the Gulf of St. Lawrence, Hudson and Davis Straits (Bucklin et al. 1995). In the eastern North Atlantic, the reproductive range of *C.helgolandicus* overlapped with that of *C. finmarchicus* and *C. glacialis* for appreciable periods in the history of the three species (Fleminger and Hulsemann 1977). *C. helgolandicus*, although a more temperate species, co-occurs extensively with *C. finmarchicus* in many areas of the North Atlantic and in the shelf seas around the United Kingdom (Williams and Conway 1980). The distribution, interactions and different life strategies of these two *Calanus* species are complex: they show converse vertical distributions in areas where they co-exist (Williams and Conway 1980), and their biogeographical boundaries are modified by the North Atlantic Oscillation (Fromentin and Planque 1996). These four species of *Calanus* were chosen for study not only because of their co-occurrence, but also because they constitute a major component of zooplankton in the North Atlantic and North Sea in terms of biomass and trophic role.

Materials and methods

Sample collection and preservation

Individuals of *Calanus helgolandicus*, *C. finmarchicus*, *C. glacialis* and *C. hyperboreus*, collected by net tows, were preserved in absolute ethanol for a minimum of 12 h, with ≃10 adults per 10 ml ethanol. *C. helgolandicus* were collected in April 1996 from a coastal station ≃10 km off Plymouth (western English Channel: 50°15′N; 4°13′W); *C. finmarchicus* were collected in April/May 1996 from Ganavan Bay (Oban, Western Scotland: 56°27′N; 5°27′W); and *C. glacialis* and *C. hyperboreus* were collected in June 1997 from the Norwegian Sea (77°01′N; 33°50′E).

DNA amplification

Amplifications of the 16S rRNA gene were performed on single, preserved individuals without prior purification of the DNA (Bucklin et al. 1995). Individual copepods were removed from ethanol, and rehydrated in 0.5 ml distilled water in a microcentrifuge tube for 6 to 12 h at room temperature. After rehydration, the water was removed and replaced with 34 µl ultrapure water and 5 µl 10x Dynazyme buffer (Flowgen Instruments Ltd.). The copepods were homogenized using a pellet pestle motor (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 m*M* dNTPs (Promega UK Ltd.), 2.5 µl each of primers 16SAR and 16SB2R (100 ng μl^{-1}), and 2 U of Dynazyme (Flowgen Instruments Ltd.)]. The amplification primers used were 16SAR (5'CGCCTGTTTAACAAAAACAT-3′; Palumbi and Benzie 1991) and 16SB2R (5′-ATTCAACATCGAGGT CACAAAC-3′; custom-designed from existing *Calanus* sequences). Amplification was carried out in an Autogene thermocycler (Grant). The cycling parameters included an initial denaturation step of 96 °C (5 min) followed by 40 cycles of 45 °C (2 min), 72 °C (1 min), and 96 °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. 10 µl aliquots of the amplification reactions were analysed by agarose gel electrophoresis (1.5%) to check amplification efficiency.

Sequencing

To determine the most suitable enzyme(s) for restriction-fragment length polymorphism (RFLP) analysis, the amplified 16S rDNA fragments were sequenced for each of the four *Calanus* species. Fragments were cloned into pBluescript SK^-, and sequencing was achieved following the chain-termination method (Sanger et al. 1977) using Sequenase II sequencing kit (Amersham).

Restriction mapping

The Genetics Computer Group (GCG) Sequence Analysis Software Package (Devereux et al. 1984) was used to restriction map the sequences of 16S rDNA for *Calanus helgolandicus*, *C. finmarchicus*, *C. glacialis* and *C. hyperboreus*. From these restriction maps, suitable enzymes were selected to differentially digest each of the four species.

Restriction digest

Restriction digests were performed on a 15 µl aliquot from each amplification by the addition of 0.5 µl of 5 *M* NaCl, 2 µl bovine serum albumin (1 mg ml^{-1}, Promega UK Ltd) and 5 U of each restriction enzyme (*Dde*I and *Vsp*I Promega UK Ltd.). Incubations were performed at 37 °C for 1 h. The digestion products were separated by electrophoresis through a 2% Metaphor agarose gel (FMC Ltd., Rockland, Maine, USA) and pre-chilled for 30 min at 4 °C to improve resolution. The gels were observed and photographed on a UV transilluminator.

Results

In this study, the amplification primers 16SAR and 16SB2R, have been used routinely to amplify 16S rDNA from single, preserved *Calanus* individuals. Amplification was successful on whole adults, animals, all copepodite stages, nauplii, eggs and body parts of copepods. Excessive material, resulting from the homogenisation of large adults, although successfully

amplified did in some cases produce less precise banding compared with reactions amplifying smaller amounts of template material.

Nucleotide-base sequences for this amplified region of mitochondrial 16S rDNA were determined for all four *Calanus* spp. (Fig. 1).

The sequenced rDNA regions were mapped for the presence of suitable RFLPs. The chosen restriction enzymes, *Dde*I and *Vsp*I, produced a unique restriction pattern for each *Calanus* species (Fig. 2).

The amplification of 16S rDNA and subsequent RFLP analysis produced a characteristic pattern for each species, providing an unambiguous statement of identity (Fig. 3). The versatility of this molecular method, allowing all life stages of a species including eggs to be identified, is demonstrated in Fig. 4. This method of species identification is also valid for small body parts, including the antennule, leg, thorax and urosome (Fig. 5).

Discussion

A simple molecular technique has been developed involving amplification of 16S rDNA and subsequent RFLP analysis, which can distinguish between *Calanus helgolandicus*, *C. finmarchicus*, *C. glacialis and C. hyperboreus* at any life stage. The method is preferable to the RAPD (randomly amplified polymorphic DNA) technique as it does not require prior purification of the DNA to obtain a reproducible result. Such a purification step would preclude the analysis of single eggs and nauplii by limiting amounts of starting material, and would also extend the overall time required for analysis.

Existing 16S rDNA-amplification systems (Palumbi and Benzie 1991; Bucklin et al. 1992) have been modified to provide a cost-effective PCR product suitable for subsequent RFLP analysis. The newly designed 16SB2R primer has a predicted melting temperature (Tm) closer to that of the forward 16SAR primer, allowing the annealing temperature used in the PCR to be increased to 45 °C. This increases the specificity of the amplification product which, in turn, produces a more distinct restriction profile. The reaction volume has been decreased by 50% to reduce the cost of reactants, and the extension phase of thermocycling reduced to save time.

The restriction enzymes were selected for their ability to produce a different restriction profile for each species whilst being optimally active in a single reaction buffer; in this way, simultaneous double-digestion could be performed in a single reaction. Additionally, there is no requirement for purification of the amplified product or buffer exchange prior to restriction digestion. A suitable buffer for activity of the two restriction enzymes is created simply by increasing the salt concentration of the PCR buffer by the addition of NaCl. This system, involving the amplification of rDNA sequence from single individuals and subsequent RFLP analysis, provides an accurate and reliable diagnostic technique for identifying *Calanus* species.

The sequence data obtained for each of the four 16S rDNA fragments amplified differed slightly from previously published sequence data for the same region of 16S rDNA for the same species (Bucklin et al. 1995). Variation was low for *Calanus helgolandicus* compared with *C. helgolandicus* collected previously from the English Channel (1%) and with *C. helgolandicus* collected

Fig. 1 *Calanus* spp. (*chel* = *C. helgolandicus*; *cfin* = *C. finmarchicus*; *cglac* = *C. glacialis*; *chyper* = *C. hyperboreus*). Sequence data for a region of the mitochondrial 16S rRNA gene (. = alignment gap; *n* = any base; **ct*n*ag** = *Dde*I restriction site; **attaat** = *Vsp*I restriction site; primers in *italics*)

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  cfin16S  cgcctgttta acaaaaacat cgtaaataat atttataata cctgctcagt ..aatattta aacagccgcg ttagtgttaa ggtagcatag
 cglac16S  cgcctgttta acaaaaacat cgtaaataat atttataata cctgctcagt g.aatattta aacagccgcg ttagtgttaa ggtagcatag
  chel16S  cgcctgttta acaaaaacat cgtaaaatag atttatattg cctgctcagt g.aatattta aacagccgca ttagtgttaa ggtagcatag
chyper16S  cgcctgttta acaaaaacat .gtaaattag atttataatg cctgctcaat gaaatattta aatagccgcg ttagtgttaa ggtagcatag

  cfin16S  taattagttt cttaattggg aaataggatg aatggtttca ctaaaatata gtttttatcc tcatttgcga aattttaatc taagtgaaaa
 cglac16S  taattagttt cttaattggg aaatggaatg aatggttt.a ctaaaatatg atatttattc taatttgcga aattttaatc tgagtgaaaa
  chel16S  taattagttt tttaattgga aaatggaatg aatggcccca ctaaagcata gtatttatac taaaaaatga aattttaatt taagtgaaaa
chyper16S  taattagttt tttaattgga aaatggaatg aatggtttca ctaagatatg gtatttatgc taataaatga aattttaatt taagtg.aaa

  cfin16S  tacttagcag ttgtactagg acgagaagac cctatgaagc tggcaaacta ttaat.acat attcctatta tttattagtt tatttttgg
 cglac16S  tactcagaag atatatttag acgagaaga. cctatgaagc tggtagactt ccaatgtaat tatacgatag ttcatgagtt tatttttgg
  chel16S  tacttaaatg atatatttag acgagaagac cctatgaagc tggtagacca taagtgtaat tatttcatag tag.caggtc tatttttgg
chyper16S  tacttaaaag atcttttaag acgagaagac cctatgaagc t.atagacta taaatataat tattataaat ttt.taagtt tatttttgg

  cfin16S  ggtaaaattt aataatacta ttaacacaat tgtactaaat tacatccttt aggaattatg aagaagctcc tctagggata ac.agcatta
 cglac16S  ggtaaaattt aataatagta ttaatattgg cttactaaat aatatcctct tggaattatg aaaaagctcc tctagggata acaagcatta
  chel16S  ggtaaaattt aataattata ttaatacaga tttgttcaaa cttatccttc tggaattatg aataagctcc tctagggata ac.agcatta
chyper16S  ggtaaaattt aataatttta tttaaataag cttattttaa ttgatccttt aggaattatg aaaaagctcc tctagggata ac.agcatta

  cfin16S  tgcttaaaag agttcttatc agaataagcg tttgtgacct cgatgttgaa t
 cglac16S  tgcttaaaag agttcttatc agaataagcg tttgtgacct cgatgttgaa t
  chel16S  tgcttaaaag agttcttatc agaataagcg tttgtgacct cgatgttgaa t
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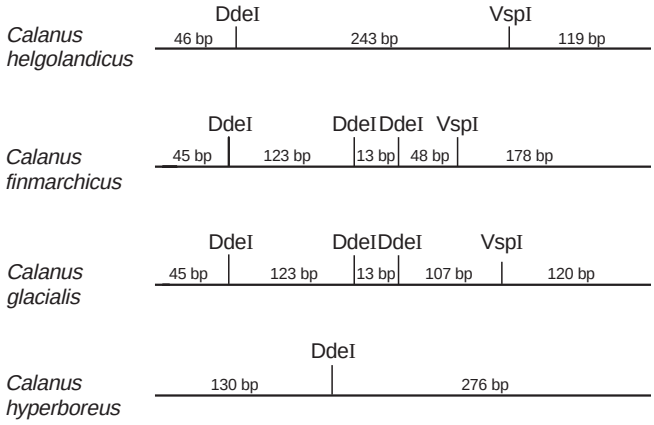

Fig. 2 *Calanus* spp. Restriction map of a region of mitochondrial 16S rDNA for *C. helgolandicus*, *C. finmarchicus*, *C. glacialis*, and *C. hyperboreus*. Both *Dde*I and *Vsp*I restriction sites are indicated. Resulting fragments are shown in base pairs (*bp*)

from the Azores (1.7%). Our sequence data for *C. finmarchicus* also varied only slightly from that for *C. finmarchicus* collected from the Gulf of Maine (0.03%) and from the Gulf of St. Lawrence (2.3%). *C. hyperboreus* showed a small variation of 2.3% from *C. hyperboreus* collected in the Gulf of St. Lawrence. *C. glacialis* displayed a slightly greater variation (9.9%) compared with the same species collected from the Gulf of St. Lawrence.

The differences are small in the case of *Calanus helgolandicus*, *C. finmarchicus* and *C. hyperboreus*, but significant in the case of *C. glacialis*. The latter difference may reflect the site of collection: the original sequence data were obtained for *C. glacialis* from the Gulf of St. Lawrence and our sequence data being obtained from *C. glacialis* collected from the Norwegian Sea. Although this difference is not as significant at that recorded between species (Bucklin and Lajeunesse 1994), it does pose

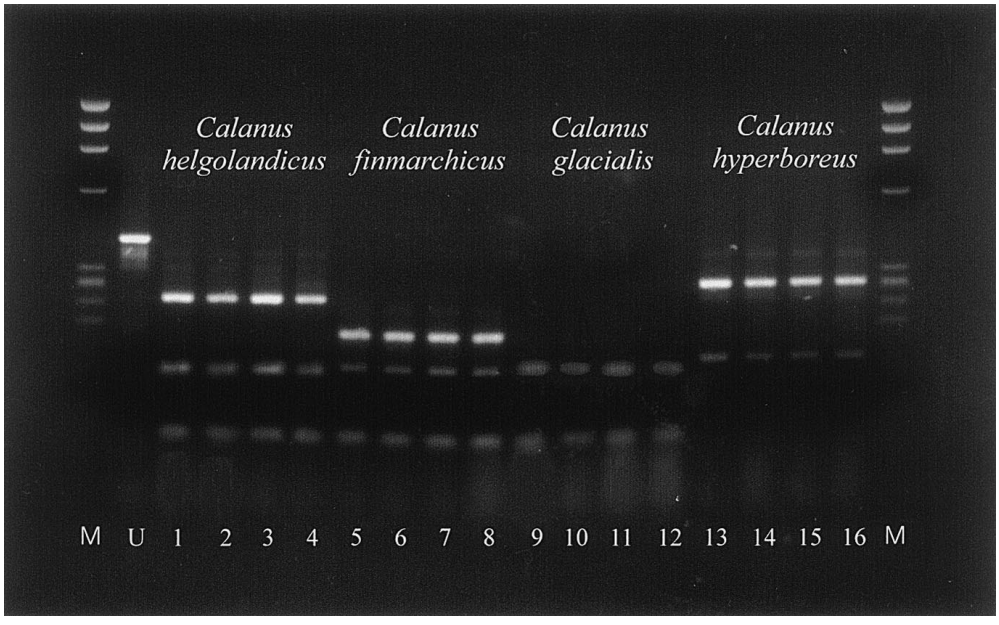

Fig. 3 *Calanus* spp. Amplification of 16S rDNA from whole adults and subsequent RFLP analysis with restriction endonucleases *Dde*I and *Vsp*I (*Lanes 1 to 4, C. helgolandicus*; *Lanes 5 to 8, C. finmarchicus*; *Lanes 9 to 12, C. glacialis*; *Lanes 13 to 16, C. hyperboreus*; *M*, øX174/*Hae*III DNA size-markers; *U*, amplified uncut *C. helgolandicus* DNA)

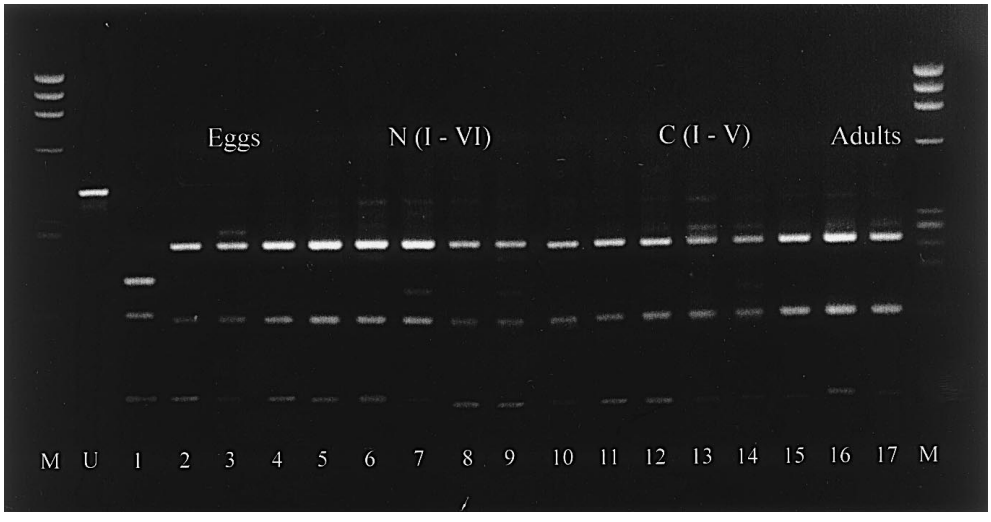

Fig. 4 *Calanus* spp. Amplification of 16S rDNA from major developmental stages of *C. helgolandicus* and subsequent RFLP analysis with *Dde*I and *Vsp*I. Restriction pattern produced by *C. finmarchicus* is shown for comparison (*Lane 1*) (*Lanes 2 to 4* individual eggs; *Lanes 5 to 10* Nauplius Stages I to VI, respectively; *Lanes 11 to 15* Copepodite Stages I to V respectively; *Lanes 16 to 17* Adult copepods; *M*, øX174/*Hae*III DNA size-markers; *U*, amplified uncut *C. helgolandicus* DNA)

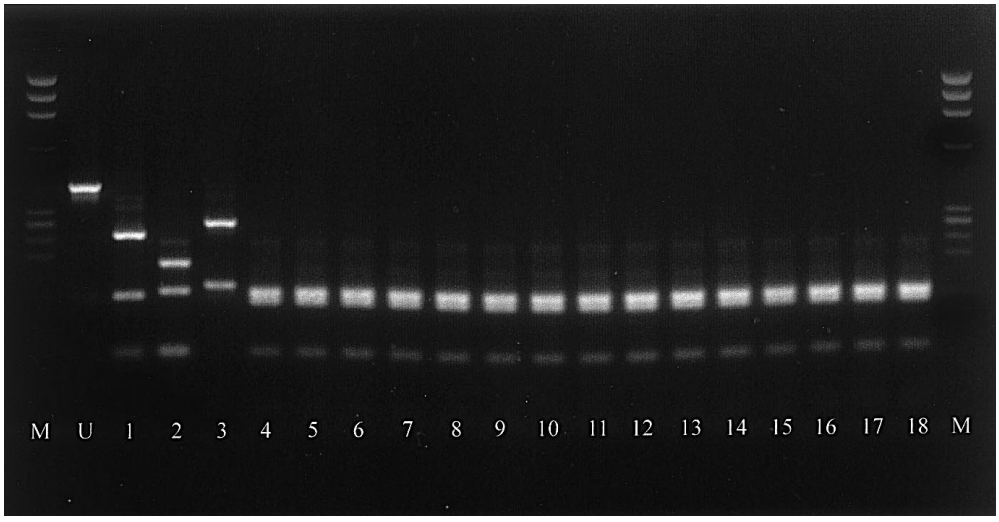

Fig. 5 *Calanus* spp. Amplification of 16 S rDNA from body parts of *C. glacialis* and subsequent RFLP analysis using *Dde*I and *Vsp*I. Restriction pattern produced by *C. helgolandicus* (*Lane 1*), *C. finmarchicus* (*Lane 2*) and *C. hyperboreus* (*Lane 3*) are shown for comparison (*Lanes 4 to 6* individual antennules; *Lanes 7 to 9* individual legs; *Lanes 10 to 12* individual urosomes; *Lanes 13 to 15* individual thoraxes; *Lanes 16 to 18* individual eggs; *M,* øX174/*Hae*III DNA size-markers; *U,* amplified uncut *C. helgolandicus* DNA)

some interesting questions about the phylogenetic relationships of geographically diverse *Calanus* species.

There are many practical applications for this simple molecular technique. It has been well demonstrated that environmental factors such as temperature, oceanic currents, turbulence, wind stress and nutrients can considerably influence the population composition of zooplankton (Colebrook 1982, 1985; Dickson et al. 1988; Fromentin and Planque 1996). Different combinations of *Calanus* species are known to co-occur (Frost 1974; Fleminger and Hulsemann 1977; Williams and Conway 1980). An unambiguous method to distinguish between *Calanus* species at any life stage will increase our understanding of the effect of these environmental factors on zooplankton assemblages, and thus enable a better understanding of their distribution, vertical migration and exploitation of habitat. This in turn will permit further investigations into how environmental changes affect individual zooplankton species with different physiological and biological properties.

Copepods, particularly eggs and nauplii, play an important role in marine food webs by providing an important food source for many animals including fish larvae, small species of fishes and euphausiids. Many experiments involve investigation of the gut content of such animals to determine their diet and role in the ecosystem. *Calanus* spp. eggs can remain undigested during the gut passage (Conway et al. 1994) and can, therefore, be identified easily using RFLP. In predators such as euphausiids, whose food is well masticated, only small body parts of *Calanus* spp. are found in the gut. In such cases, this molecular method, enabling identification of species from fragmentary material, will be particularly valuable.

In conclusion, our method for the identification of the North Atlantic *Calanus* species is reliable, reproducible and relatively simple and inexpensive. It has the benefit that analysis is performed on individuals preserved in ethanol, making it suitable for use on *Calanus* samples collected at sea.

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