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Molecular diversity of the copepod, *Nannocalanus minor:* Genetic evidence of species and population structure in the North Atlantic Ocean

by Ann Bucklin[1,2], Todd C. LaJeunesse[1], Eva Curry[1], John Wallinga[1,3] and Karen Garrison[1]

ABSTRACT

The abundant calanoid copepod, *Nannocalanus minor,* is widespread from the Florida Straits (FS), throughout the Gulf Stream and the Sargasso Sea, to the eastern North Atlantic Ocean. Does the species represent a single, randomly-mating population across this extensive region, or does it comprise a number of genetically distinct populations or taxonomically distinct forms? What are patterns and pathways of dispersal of the copepod across the North Atlantic? These questions were addressed using population genetic analysis of DNA sequence variation of a 440 base-pair region of the mitochondrial 16S rRNA gene. This analysis separated *N. minor* into two genetically distinct types (distinguished by 10% sequence difference) that may represent the previously described *N. m.* forma major and *N.m.* forma minor. The two genetic types differed in size range and in geographic distribution: Type I individuals were larger and were most abundant in the western regions of the Gulf Stream; Type II individuals were smaller and became more abundant toward the eastern regions of the Gulf Stream. Significant differences in the size-frequency distributions of *N. minor* from different regions of the North Atlantic may result from mixtures of the two genetic types and environmental differences in food availability.

Within *N. minor* Type I, mtDNA sequence variation defined 68 haplotypes among 155 individuals. The haplotype frequency distribution was skewed: there were 40 individuals of one haplotype, 31 individuals of a second, and 60 unique individuals. Haplotype diversity, h, was very similar across the sampled range: $h = 0.886$ in samples from the FS and 0.874 for samples from the Gulf Stream Meander Region (GSMR). Nucleotide diversity, p_i, was significantly greater in the FS ($p_i = 0.00490$) than in the GSMR (0.00414), largely due to a number of genetically divergent individuals. Haplotype abundances did not differ significantly either within the regions (among FS samples, $P = 0.756$; among GSMR samples, $P = 0.336$) or between the regions ($P = 0.636$). Molecular genetic analysis can reveal cryptic species among marine taxa, and is particularly useful for taxa characterized by morphological similarity.

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1. Introduction

The calanoid copepod, *Nannocalanus minor,* is a cosmopolitan, surface-dwelling species that occurs from approximately 10N to 43N in the western North Atlantic, abundantly in the surface waters of the Florida Current (Owre and Foyo, 1967), in Gulf Stream Warm Core Rings (Copley *et al.,* 1989), and in the eastern North Atlantic (Grice and Hulsemann, 1965; Colebrook, 1986). Despite its cosmopolitan distribution, the species shows some evidence of morphological differentiation across its range. Size differences have been noted among populations (Sewell, 1947) that may be associated with temperature or food availability (Ashjian and Wishner, 1993a). In addition, differences in the shape of the pleiopod of females have been described (Sewell, 1929; Toda, 1986). The taxonomic significance of the morphological variation has not been determined.

This study uses molecular population genetic analysis to determine the systematic and ecological significance of morphological and genetic variation within *N. minor* in the North Atlantic Ocean. The questions addressed include: How genetically cohesive is the species across the North Atlantic? Are there genetically differentiated, geographically distinct populations within its range? What are the patterns and pathways of dispersal (gene flow) of *N. minor* in the Gulf Stream?

Samples of *N. minor* were compared from four oceanographic regions in the North Atlantic: the Florida Straits (FS), the North Atlantic Slope Water, the Gulf Stream Meander Region (GSMR), and the eastern North Atlantic. The Florida Current is the region of the Gulf Stream from south of the Dry Tortugas to Cape Hatteras. The intensification of flow through the FS makes this area ideal for characterization of the zooplankton entrained in the upstream portion of the Gulf Stream. Current velocities and transport volumes vary seasonally, with maximum flow during July (Niiler and Richardson, 1973). Since the Florida Current entrains shelf water with large populations of plankton, variation in current flow may affect plankton abundance both in the upstream regions of the Gulf Stream and in the sources of the entrained plankton. The GSMR flows eastward from Cape Hatteras to the Grand Banks; westward-flowing currents border it on each side (Robinson *et al.,* 1988). This region is characterized by a very complex hydrography and circuitous path (Richardson, 1981): meanders develop and intensify and cold- and warm-core rings spin off to the south and north, respectively, of the Stream. Mixing of water from different domains also occurs here. Transport in the Gulf Stream is thought to increase five-fold between the FS and Nova Scotia, due largely to entrainment of Sargasso Sea waters (Worthington, 1976) and shelf water.

The Southeast Newfoundland Rise is a region of Stream bifurcations. The first bifurcation results in a northward-flowing current along the eastern edge of the Grand Banks and an eastward-flowing current. The latter bifurcates again before

crossing the Mid-Atlantic Ridge, yielding a southern branch, called the Azores Current, which recirculates into the Sargasso Sea (Krauss *et al.,* 1990).

How distinct are these North Atlantic domains in terms of long-term, large-scale mixing processes? One measure of ocean structure is the population genetic structure of the plankton. The most compelling explanation for significant geographic variation in the genetic makeup of planktonic populations is that the persistent ocean structure prevents the mixing of populations from distinct sources and/or allows the genetic differentiation of populations by natural selection or possibly drift (see Bucklin *et al.,* 1989; Bucklin, 1991). In turn, population genetic characteristics may be used to define dispersal (gene flow) patterns and identify significant ecological boundaries. Previous work, using allozymes (genetic variants of enzymes), demonstrated significant genetic heterogeneity both within and among copepod species (Bucklin and Marcus, 1985; Sevigny and Odense, 1985; Sevigny and McLaren, 1988; Bucklin *et al.,* 1989; Sevigny *et al.,* 1989; Bucklin, 1991). Bucklin *et al.* (1989) and Bucklin (1991) concluded that genetic heterogeneity defined mesoscale structure in biological populations and was concordant with physical ocean structure. However, the forces maintaining genetic structure in a fluid regime are not well understood. Recent studies of the population genetics of zooplankton have used molecular, rather than biochemical, approaches (Bucklin, 1995; Bucklin and Kocher, 1995; Palumbi and Kessing, 1991; Palumbi and Brand, 1993).

Copepods collected in the GSMR may be the offspring of adult populations sampled in the FS, since *N. minor* can have several generations per year with nearly continuous reproduction (Ashjian and Wishner, 1993a). MtDNA (which is inherited without recombination) is an appropriate character to use to determine dispersal patterns, since descendants will be genetically identical to their maternal parent for mtDNA, while nuclear characters (which are recombined each generation) will lose this message during transport. The clonal inheritance pattern of mtDNA is also advantageous for studies of population structure, since mitochondrial genes frequently exhibit greater population differentiation than nuclear genes and will homogenize more slowly after differentiation (see Birky *et al.,* 1989).

Molecular characteristics can be informative of a species' population biology. In particular, the genetic distinctiveness of geographic populations and the frequencies of genotypes (or, for haploid genomes such as mtDNA, haplotypes) in different geographic locations may be statistically compared. Another useful characteristic is the molecular diversity of the population, measured either as the number of haplotypes or the haplotype diversity (h), or nucleotide diversity (p_i, the genetic divergence among haplotypes). These molecular characteristics are used here to determine whether there are genetically distinct populations of *N. minor* and to determine patterns of dispersal (gene flow) with respect to circulation patterns in the North Atlantic Ocean.

2. Materials and methods

a. Field collection of plankton samples. Zooplankton collections and physical measurements were made in the FS during February, 1993. Zooplankton for molecular analysis were collected by 10 integrated, oblique ring net tows (using a 333 μm mesh 1 m² net) along a transect across the FS (Figure 1; Table 1). In addition, vertically stratified samples were obtained by four casts of a 1 m² Multiple Opening and Closing Net and Environmental Sensing System (MOCNESS; Wiebe *et al.*, 1985). MOCNESS and ring net tows were taken during both day and night. This sampling plan, including numerous, closely-space tows, was designed to characterize the genetic diversity of the source populations by capturing the greatest range of variation possible in a short time.

Sampling in the GSMR was done during a fortuitous transit leg of the *RV Oceanus* from Woods Hole to the Azores in April, 1993 (Cruise OCE-258; Leg I). During the transit, the Gulf Stream was crossed six times, and 25 zooplankton samples were taken by integrated, oblique tows of a 1 m² ring net. Collections locations (shown in Figure 2 and Table 1) were in the North Atlantic Slope Water (OCE-1, -2, and -16), the Gulf Stream (OCE-3 through -15, -17 through -19, and -20 through -24), and the eastern North Atlantic (OCE-25).

b. Physical oceanographic measurements. Expendable bathythermograph (XBT) profiles were done in association with each meter net tow along the transect across the FS in February, 1993 (Fig. 1), to determine the relative position of the sample in the Stream cross-section. The MOCNESS tows provided additional hydrographic information at four stations across the Straits.

During the April, 1993, crossing of the Stream in the GSMR, hydrographic information was obtained from XBT profiles at 10 of the meter net tow sites (two in the Slope Water and eight in the Gulf Stream). In addition, current velocities were provided by continuous recording of the ship-mounted Acoustic Doppler Current Profiler (ADCP; RD Instruments, San Diego, CA). The ADCP transmitted an acoustic pulse at 1 per sec at 150 kHz. The current estimates were partitioned into discrete depth intervals of 8 m and averaged over 5 min (300 ensembles) to yield a relative current structure. To extract the absolute current, the ship's motion was removed using Global Positioning System (GPS) fixes by the ship's navigation system. The 5 min averaged ensembles were convolved with a 3 hr 10% cosine taper filter. This effectively removed any variability less than 3 hr and any outlier data from anomolous GPS fixes, without altering the overall structure of the measured currents. Analysis of the ADCP measurements yielded five cross-sections of the Stream in the meander region.

The delimitation of the Gulf Stream and associated features, the Slope Water, and the Sargasso Sea were estimated from facsimile copies of the NOAA/NOS oceano-
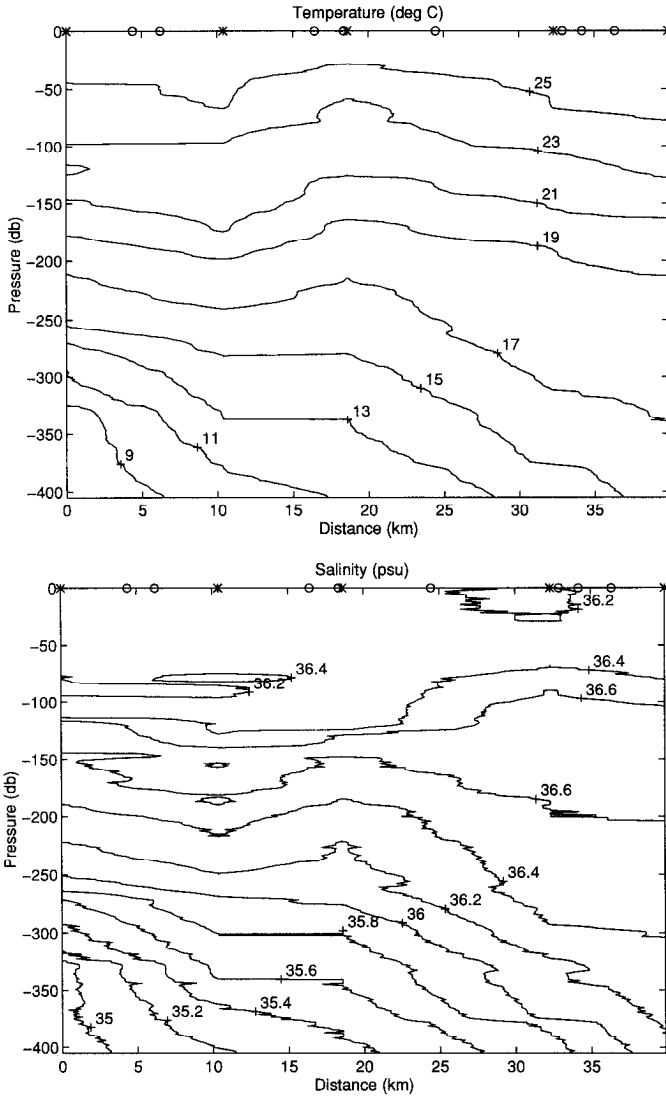
Figure 1. (a) Temperature and (b) salinity contours for the transect across the Florida Straits (22–24 February 1993) during which zooplankton collections were made. The positions of the MOCNESS casts, which yielded the CTD data, are given by asterisks at the top of the figure. Locations of the integrated meter net tows are indicated by circles. Collection information is given in Table 1.

graphic features analysis of sea surface temperatures obtained from satellite-based Advanced Very High Resolution Radiometry (AVHRR). The ship's position was plotted on charts obtained from NOAA daily during the cruise, and sampling strategies were determined in part from this information.

Table 1. Locations and sample collection information for *N. minor* in the North Atlantic.
Collection domains are indicated by the Gulf Stream Meander Region Transect number
(indicated by Roman numerals) or by water mass (SW = Slope Water, NEAtl = eastern
North Atlantic). See Figure 2 for placement of collection sites. All collection times are all
given in local time.

	Sta. #	Date	Time	Station coord.	Depth
RV Calanus (28 February–3 March, 1993)					
	MLF1	2/22/93	14:25	25°45.1'N; 79°40.0'W	25 m
	M2	2/22/93	21:30	25°45.0'N; 79°46.3'W	300 m
	M3	2/22/93	22:22	25°45.0'N; 79°47.4'W	300 m
	M5	2/23/93	18:49	25°45.5'N; 79°38.9'W	325 m
	M7	2/23/93	21:19	25°44.9'N; 79°35.3'W	125 m
	M8	2/23/93	22:40	25°45.0'N; 79°30.2'W	10 m
	M9	2/24/93	09:49	25°45.0'N; 79°28.1'W	125 m
	M10	2/24/93	10:19	25°45.0'N; 79°29.4'W	125 m
	M14	2/24/93	19:30	25°45.0'N; 79°52.8'W	125 m

Transect #	Sta. #	Date	Time	Station coord.	Depth
RV Oceanus (18–27 April, 1993)					
SW	OCE-1	4/20/93	16:27	40°45.8'N; 62°28.1'W	50 m
SW	OCE-2	4/20/93	21:40	40°45.0'N; 61°13.8'W	50 m
I	OCE-5	4/21/93	10:10	40°13.2'N; 58°40.9'W	100 m
I	OCE-7	4/21/93	12:11	40°11.8'N; 58°30.4'W	100 m
I	OCE-9	4/21/93	13:48	40°10.9'N; 58°18.8'W	100 m
I	OCE-10	4/21/93	14:30	40°10.0'N; 58°13.9'W	100 m
SW	OCE-16	4/22/93	06:52	39°46.3'N; 54°44.7'W	400 m
II	OCE-17	4/22/93	10:15	39°42.1'N; 54°05.4'W	200 m
II	OCE-18	4/22/93	11:38	39°41.5'N; 54°00.0'W	200 m
II	OCE-19	4/22/93	13:03	39°41.0'N; 53°54.0'W	200 m
III	OCE-20	4/23/93	03:10	39°25.9'N; 50°33.7'W	200 m
III	OCE-21	4/23/93	04:43	39°24.7'N; 50°27.9'W	200 m
IV	OCE-22	4/23/93	11:45	39°15.1'N; 48°43.6'W	100 m
IV	OCE-23	4/23/93	12:28	39°14.6'N; 48°38.8'W	100 m
IV	OCE-24	4/23/94	13:10	39°14.3'N; 48°33.5'W	100 m
NEAtl	OCE-25	4/24/93	02:05	38°43.3'N; 42°11.1'W	250 m

c. Molecular techniques. The DNA amplifications were done without purification of
the DNA. Individual copepods (only adult females were used) were sorted from the
samples in the laboratory, and allowed to sit in 0.5 ml distilled water for 6 to 12 hr
(until they sank to the bottom of the tube). The water was then pipetted from the
tube, and replaced with 88 μl of PCR amplification buffer (78 μl distilled water and
10 μl 10X Perkin Elmer PCR buffer). The copepods were homogenized using a
pipette tip. This homogenate was allowed to sit, under refrigeration, for 12 to 24 hr.
After this incubation (which presumably helped rupture cell and organelle mem-
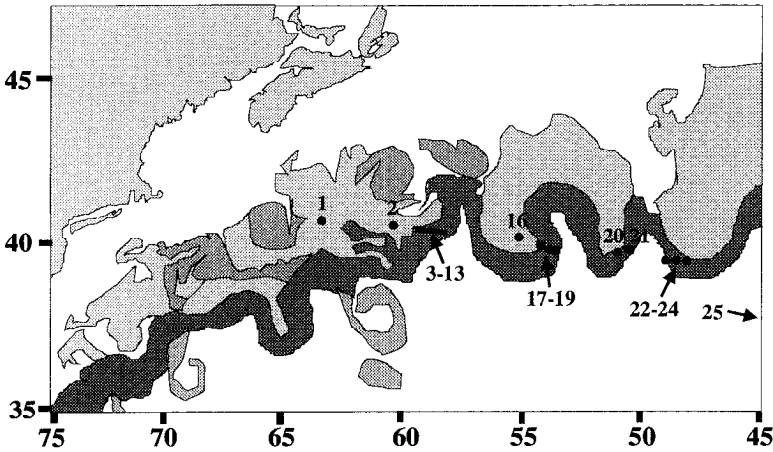branes), the remaining ingredients for the polymerase chain reaction (PCR) reaction

Figure 2. Oceanographic features in the western North Atlantic Ocean on 16 April 1993, as analyzed by the NOAA/NOS feature analysis of a sea surface temperature map based on Advanced Very High Resolution Radiometry (AVHRR) data. The positions are shown for the zooplankton samples collected for molecular and morphological studies. The outlines delimit different water masses, including the Gulf Stream, meanders, rings, and entrainment features, and the North Atlantic Slope Water. Three collections were made in the Slope Water (OCE-1, OCE-2, and OCE-16) and one collection was made in eastern North Atlantic (OCE-25). The other collections were made during four transects of the Gulf Stream: stations OCE-3 to OCE-13 were made during Transect I; OCE-17 to OCE-19 during Transect II; OCE-20 and OCE-21 during Transect III; and OCE-22 to OCE-24 during Transect IV. See Table 1 for collection information.

were added (10 μl 2 mM deoxynucleotide triphosphates, 1 μl of 10 μM solutions of each primer, and 0.5 unit Taq polymerase). The reaction mix was covered with 2 drops of mineral oil. The amplification reaction was done in a Perkin Elmer 480 ThermalCycler with the following program: 94°C (1 min); 37°C (2 min); 72°C (3 min) for 40 cycles.

The amplification primers used were 16SAR and 16SBR (Palumbi *et al.*, 1991) based on the *Drosophila yakuba* sequence (Clary and Wolstenholme, 1985). The sequences are:

<div style="text-align:center">

16SAR   5'-CGCCTGTTTAACAAAAACAT-3'

16SBR   5'-CCGGTTTGAACTCAGATCACGT-3'.

</div>

Amplification products to be sequenced were checked for size and purity by loading 10 μl on a 2% agarose gel. The remaining 90 μl of product was then loaded into wells of a 1% Nusieve gel with ethidium bromide and electrophoresed at 44 volts. Product bands were cut from the gel; the gel bands were digested overnight with 1 unit of agarase (Sigma Chemical Co., St. Louis, MO).

The sequencing reaction used 4 to 9 μl of template and was done in a Cetus-Perkin

Elmer 480 ThermalCycler using a Cycle-Sequencing Kit (Applied Biosystems, Inc., Beverly, MA). Fluorescently labeled dideoxynucleotides were incorporated during an asymmetrical amplification using one of the amplification primers. One cycle-sequencing reaction, using the 16SBR primer, was done for all templates. In addition, the complementary reaction using the 16SAR primer was done for 7 individuals in order to verify substitutions at particular sites. One variable site was removed from consideration on this basis.

Nucleotide sequencing was carried out in an Applied Biosystems, Inc., Model 373 Automated DNA Sequencer. The sequencer uses a 6% acrylamide gel, electrophoresed for 10 hrs. The sequences are produced as fluorescent emission spectra for each base, resulting in a four-color chromatogram. The sequence was further compiled using SeqEd (version 2.0) and checked thoroughly for accurate machine reading.

The Sequence Analysis Software Package was used for alignments and preliminary analyses. The programs, based on Smithies *et al.* (1981), have been published by Devereaux *et al.* (1984), and are commercially available (Genetics Computer Group, Madison, WI). The multiple sequence alignment program, PileUp, was used to align the sequences for each individual. PileUp is a simplification of the progressive alignment method of Feng and Doolittle (1987). Although the program has limitations, it is a safeguard against human subjectivity in the alignment process. Several parameters of PileUp can be altered; alignments were done using a range of values for the gap penalties and gap-length penalties. The definitive alignment used a gap penalty of 2.0 and a gap-length penalty of 0.3.

Molecular genetic diversity was measured in three ways for both regions (FS and GSMR). First, the number of haplotypes was determined. Then, haplotype diversity, h, was determined by:

$$h = 1 - \sum f_i^2 \tag{1}$$

where f_i is the frequency of the ith variant (Nei, 1975). Nucleotide diversity, p_i, was calculated as an estimate of genetic variation according to Nei (1987) by the formula:

$$p_i = \sum_{i<j} p_{ij}/n_c \tag{2}$$

where p_{ij} is the proportion of different nucleotides between the ith and jth haplotypes and n_c is the total number of sequence comparisons $[n(n - 1)/2]$.

A cluster diagram was generated by PileUp in order to identify distinct haplotypes. This analysis separated the individuals into two nonoverlapping groups, Type I and Type II, that differed by approximately 10% in base sequence. An alignment was done between the most common haplotype(s) of each of the two types. The distance between the two types was evaluated by reconstructing a phylogenetic tree, using

Neighbor Joining algorithms based on Tamura-Nei distances (Kumar *et al.,* 1993). The relative abundances of each type in each sample were determined for all samples used for molecular analysis. Since individual *N. minor* were picked from the samples without regard to size, this was a preliminary estimate of the geographic distributions of the two sequence types.

Further population genetic analysis was done for *N. minor* Type I, which was most abundant in the Gulf Stream from the FS to the GSMR. The unique haplotypes were pooled into a single haplotype, so that all analyses were done with eight haplotypes plus the pool. Differences in haplotype abundances among the samples, among the different transects of the Gulf Stream, and between regions were statistically evaluated by a chi-square test using a Monte Carlo simulation (Roff and Bentzen, 1989). This evaluation was done to determine the population genetic structure within *N. minor* Type I. Each comparison used 1000 replicates and generated a P value ± the standard deviation of results from the 1000 simulations.

An Analysis of Molecular Variation (AMOVA; Excoffier *et al.,* 1992) was performed for the *N. minor* Type I sequence data. AMOVA is a hierarchical evaluation of the partitioning of genetic variance within and among populations and groups, considering both haplotype frequency and nucleotide diversity. Samples of one transect were grouped to form "populations" to obtain sufficient sample sizes; the groups were FS and GSMR.

d. Size determinations and size frequency distributions. Samples for analysis of individual size were selected from four regions: the FS, the North Atlantic Slope Water, the GSMR, and the eastern North Atlantic. In some cases, different samples were used for the molecular and size frequency analyses, but several comparable samples from each oceanographic region were used for both analyses.

Samples from each of these regions (listed in Table 1) were subsampled using a wide-mouth pipette. The subsamples were placed in a petri dish and scanned at 25× magnification under a dissecting microscope. All adult female *N. minor* encountered in scans across the petri dish were removed to a vial, until sufficient individuals had been accumulated for sizing. The subsampling was repeated if necessary to obtain sufficient individuals. The copepods were then placed under a dissecting microscope attached to a computerized image analysis system in the University of New Hampshire's Image Analysis Laboratory. Images of the copepods were imported from the dissecting microscope to a digitizing image analysis computer program, *NIH Image,* Ver. 1.52 (free-ware available from the Soil Science Department, University of Minnesota). The cephalothorax lengths of individual copepods were determined and exported to a spread-sheet program on the computer.

The size-frequency distributions of the individuals in several samples from each of the four regions were determined. The distributional statistics (mean, median,

variance, standard deviation, standard error, and range) were calculated for each sample. Size frequency distribution, size range, and median size were plotted for each sample.

3. Results

Zooplankton samples were obtained from the upper 100 m of the water column in four regions of the North Atlantic: the Florida Straits (FS), the Gulf Stream Meander Region (GSMR), the North Atlantic Slope Water, and the eastern North Atlantic (Figures 1 and 2; Table 1). The collections were located in the physical structure of the ocean by ship-board physical measurements (including temperature, salinity, and doppler current velocities) and by satellite-based determination of sea surface temperature (Figures 1, 2, 3, and 4). Contouring of the temperature (Fig. 1A) and salinity (Fig. 1B) data for the FS provided little or no evidence of cross-Stream structure. The oceanographic feature analysis (Fig. 2) and the temperature profiles from XBT casts (Fig. 3) demonstrated that collections were made from several regions, including: the Slope Water (OCE-1, OCE-2, and OCE-16), the eastern North Atlantic (OCE-25), and the Gulf Stream itself. Several collections were made at the boundaries between these regions. The current path was evident in transects I, II, and IV in GSMR, based on ADCP analysis of along-Stream velocities (Fig. 4).

The calanoid copepod, *N. minor,* was abundant in nearly all samples. A subset of the samples was selected for molecular and morphological studies to allow characterization of small-scale patterns within the Gulf Stream and across the Stream axis, as well as description of large-scale patterns and differences among the four regions.

The sequences of 155 adult female *N. minor* from the four regions were determined for a 440 base-pair region of the mitochondrial 16S rRNA gene. The alignments revealed no large deletions or additions, but there was considerable variation in the base sequence among these individuals. The most divergent sequences differed by more than 10% of the bases (Fig. 5). A phylogenetic tree reconstructed by Neighbor Joining divided the individuals into two nonoverlapping groups on the basis of the mtDNA sequences. The distances between the two types were similar to those between congeneric species of other copepods (Fig. 6).

The abundances of the two genetic types of *N. minor* differed in samples across the sampling domain (Fig. 7). Type I individuals were abundant in the FS, Slope Water, and GSMR. Type II individuals were absent in the FS, and were relatively rare in the Slope Water and GSMR; they were the only type found in the eastern North Atlantic sample (OCE-25) (Fig. 7). Thus, there was some evidence of a trend of increasing abundance of Type II individuals in pooled samples for the four transects from west to east in the GSMR.

Individuals of the two genetic types differed in characteristic size and body shape (Fig. 8). Additional analysis of females in samples collected across the geographic range of the species revealed that average size was largest in the Slope Water
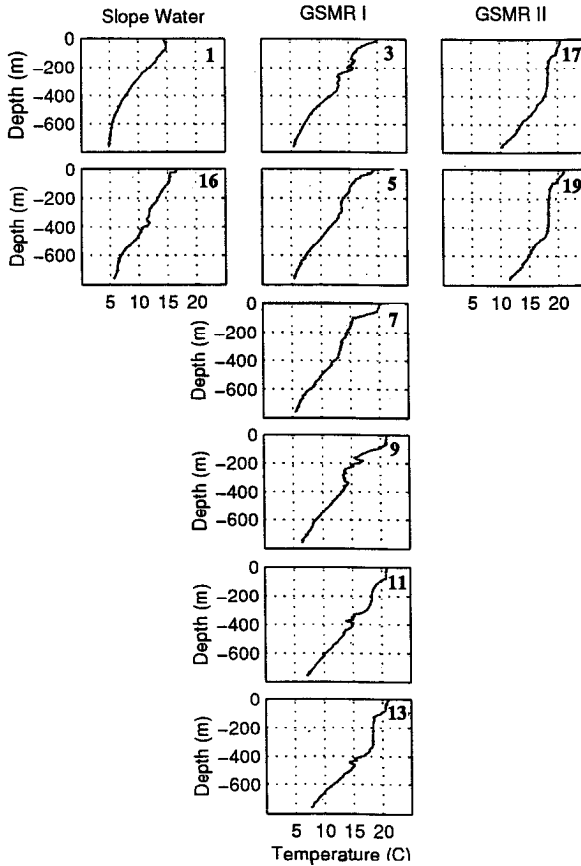
Figure 3. Temperature profiles for 10 stations where zooplankton collections were made by integrated meter net tow during cruise 258 (Leg I) of the *RV Oceanus,* which transected the Gulf Stream six times. The profiles provided additional confirmation of the placement of collections in different oceanographic regions. Relatively cool surface waters were indicative of the Slope Water (stations OCE-1 and OCE-16); surface water temperatures also helped place the collection in the axis of the Gulf Stream.

(OCE-1, OCE-2, and OCE-16), smaller in the FS (FLA-2, FLA-5, FLA-9, and FLA-14) and smaller still in the eastern North Atlantic (OCE-25; Fig. 9). The median sizes of female individuals collected from four samples in the FS were somewhat smaller (1.54 to 1.59 mm) than those of the GSMR (1.55 to 1.68 mm), with the exception of one sample at the southern boundary of the Stream, OCE-24 (1.40 mm). Females in the three Slope Water samples were significantly larger (1.72 to 1.81 mm). Several samples exhibited bimodal distributions, including one GSMR sample (OCE-24) and one Slope Water sample (OCE-1; Fig. 9).

Among 155 females of *N. minor* Type I, there were 68 haplotypes. The substitutions occurred throughout the length of the 440 base pair region (Fig. 10). Most
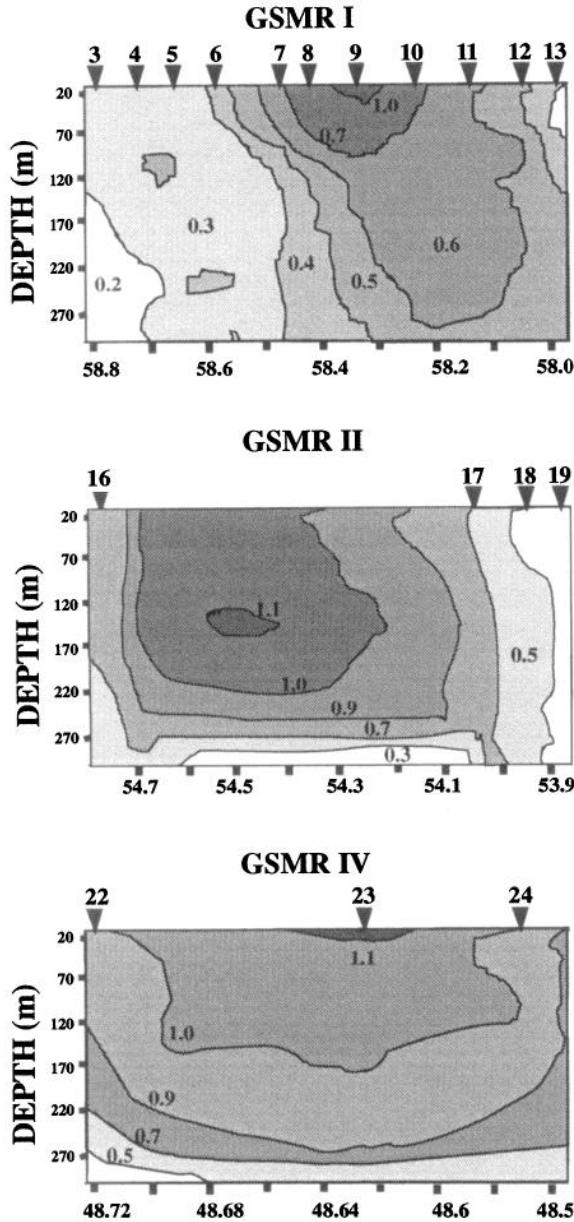
Figure 4. The positions of the GSMR zooplankton samples in the velocity structure of the Stream. The along-Stream current velocities were obtained by Acoustic Doppler Current Profiler for Transects I, II, and IV. Plankton collections were done by integrated oblique meter net tows at the points indicated along the top margins of the profiles. Numbers are station numbers corresponding to those given in Table 1, where the collection information is detailed. Contours indicate velocities in m^{-sec}.

```
          1                                                    50
TYPE I    AAATATTTTT AAGATACCTG CTCAATGAAT TTTTTAAATA CCCGCTTTAG
TYPE II   .......... .......... ....G..... A......... ..........

          51                                                   100
TYPE I    TGCTAAGGTA GCATAATAAT TAGTTTTTTA ATTGAAAAAT AGAATGAATG
TYPE II   .......... .......... .......... .......... ..........

          101                                                  150
TYPE I    GTTTTACTAA AATTGATATT TTAAAATAAT TAAACAAAAT TTTAATTTTA
TYPE II   .......... ....T..... ........CT. A....G..G. ....T.....

          151                                                  200
TYPE I    GTGAAAATAC TAAAATAATA TTTTTAGACG AGAAGACCCT ATGAAGCTAA
TYPE II   .......... .....A.... .......... .......... ..........

          201                                                  250
TYPE I    AAATCACTAT AAAAATTATA AATTTATCAG ATTTATTTTT TGGGGAAAAA
TYPE II   ......T..C ..G..C.... ..AAG.CTT. .......... ..........

          251                                                  300
TYPE I    ATTAATAAAT ATATTAATAA TCATTTATAA AAACTTATCC T---TCTAGG
TYPE II   T.....C... .......... .AT......T G..TA..... .TAA.AA...

          301                                                  350
TYPE I    AATGTTGAAA AAGCTCCTCT AGGGATAACA GCATAATATT TATTAGAGTT
TYPE II   ...A...... .......... .......... .......G.. ..........

          351                                                  400
TYPE I    CTTATCAGAA TAAATGTTTG TGACCTCGAT GTTGAATTAA ATA-TTTTTA
TYPE II   ......G... ....C..... .......... .......... ...T......

          401                                     440
TYPE I    TATGTGCAGG AGCTTATAAA AAAAGGTCTG TTCGACCTGA
TYPE II   .........A .......... .......... ........T.
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Figure 5. DNA sequence alignment for the most common haplotype of *Nannocalanus minor* Type I and the most common haplotype of *N. minor* Type II. The sequences of the two types differ by 10% of the bases in the 440 base-pair sequence. This degree of sequence difference is typical of that between congeneric species of the copepod, *Calanus* (Bucklin *et al.*, 1992, 1995), and suggests that the two types represent distinct species.

substitutions were rare, although two substitutions occurred in more than 25 individuals. There was a highly skewed frequency distribution: one haplotype was highly abundant and nearly ubiquitous, while nearly one-third the individuals assayed were genetically unique (Fig. 11).

Nannocalanus minor Type I exhibited similar genetic characteristics in populations in the FS and GSMR, based on the number of haplotypes (39 haplotypes among 74 individuals, and 35 haplotypes among 81 individuals, respectively). Haplotype abundances (Fig. 12; Table 2) did not differ significantly either within the regions (among FS samples, $P = 0.756$; among GSMR samples, $P = 0.336$) or between the regions ($P = 0.636$). Haplotype diversity measures were also similar: $h = 0.886$ in the FS
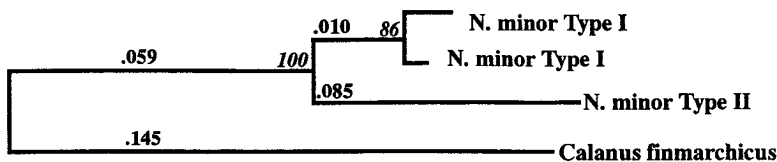

Figure 6. Neighbor joining tree showing the genetic divergence between *N. minor* Types I and II. Only the two most abundant haplotypes of *N. minor* Type I and the most abundant haplotype of *N. minor* Type II are shown. Despite some genetic variation within each type, there was highly significant separation of all individuals into one of the two types (100% of 1000 bootstrapped subreplicates exhibited the same tree topology for the separation of the types).
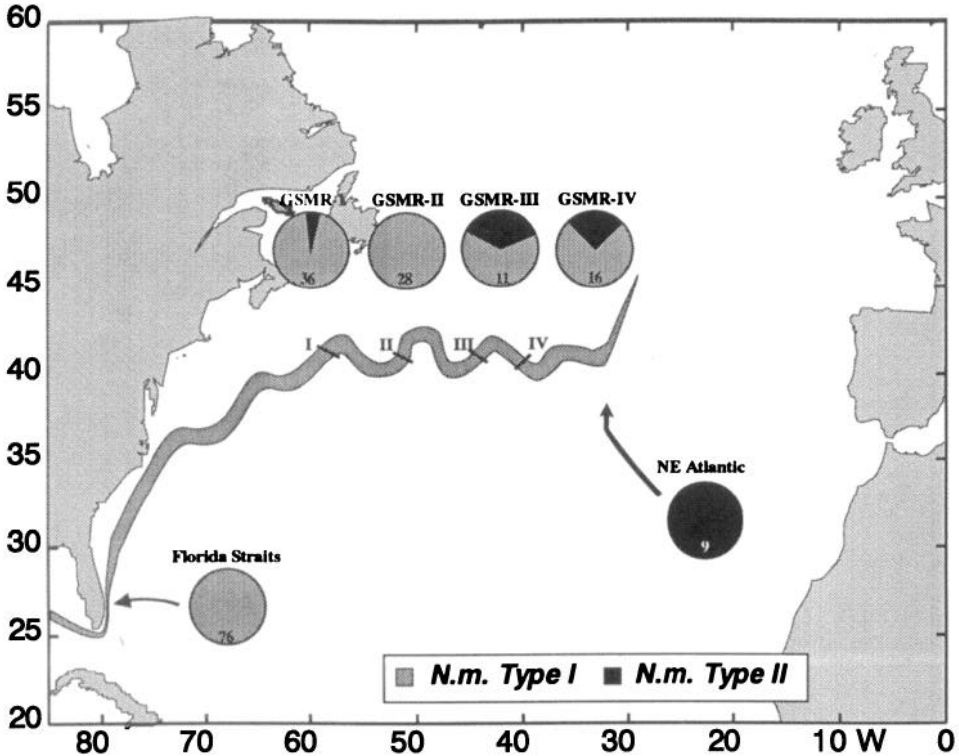
Figure 7. Relative abundance of *N. minor* Type I and Type II in pooled samples from the Florida Straits transect, the four Gulf Stream Meander Region (GSMR) transects, and the eastern North Atlantic sample, as determined by DNA sequence data. Individuals were drawn without regard to size from the samples, so these preliminary data may approximate the relative abundances of the types in the regions shown. Type I is abundant in the western portions of the North Atlantic, including the Florida Current and the Slope Water. Both types are abundant in the GSMR, although the relative abundance of Type II appears to increase from west to east, as shown in the pooled samples of the four GSMR transects. Only Type II occurred among the individuals assayed in the eastern North Atlantic sample.

samples and 0.874 in GSMR samples. However, nucleotide diversity (Fig. 13) differed significantly between FS ($p_i = 0.00490$) and GSMR samples ($p_i = 0.00414$).

The AMOVA (Excoffier *et al.,* 1992) demonstrated that nearly all of the total variance resulted from variance within populations (97.81%), with only a small fraction among populations within groups (1.37%) and among groups (0.81%).

4. Discussion

a. Nannocalanus minor consists of two genetically distinctive forms. The most obvious finding from the molecular population genetic analysis of the calanoid copepod, *N. minor,* is the discovery that what might be expected to be a homogeneous and
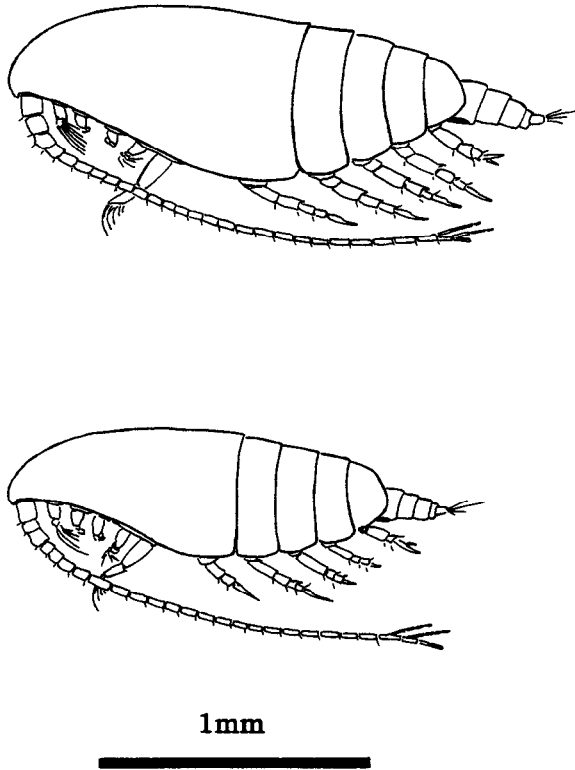
1mm

Figure 8. Diagrams of *N. minor* Type I and Type II indicating the relative size and body shape of individuals of the two types. Type I individuals are larger and have a more rebust body shape than Type II individuals.

well-mixed species population is composed of two forms that differ markedly. The two forms differ by 10% of the bases in a 440 base-pair sequence of the mitochondrial 16S rRNA gene (Fig. 5) with no overlap in the identity of the haplotypes. The level of difference for this gene portion is typical of that between congeneric species of copepods (Bucklin *et al.*, 1992; 1995), and indicates that the forms are actually distinct species (Fig. 6).

Type I individuals are larger than Type II (Fig. 8), suggesting that the genetically-defined forms may correspond to the previously-described varieties, *N. m.* forma major and *N. m.* forma minor (Sewell, 1929, 1947). Morphological examination of the forms will be required to confirm or establish diagnostic taxonomic characters; these studies are currently underway by an expert in copepod taxonomy.

The two genetic types of *N. minor* may also differ in geographic distribution (Fig. 7). Analysis of samples from four regions in the North Atlantic revealed that Type I individuals were abundant in the FS, GSMR, and in the North Atlantic Slope Water. Type II individuals were not found (at least in this preliminary survey) in the

Florida Straits   **Slope Water**
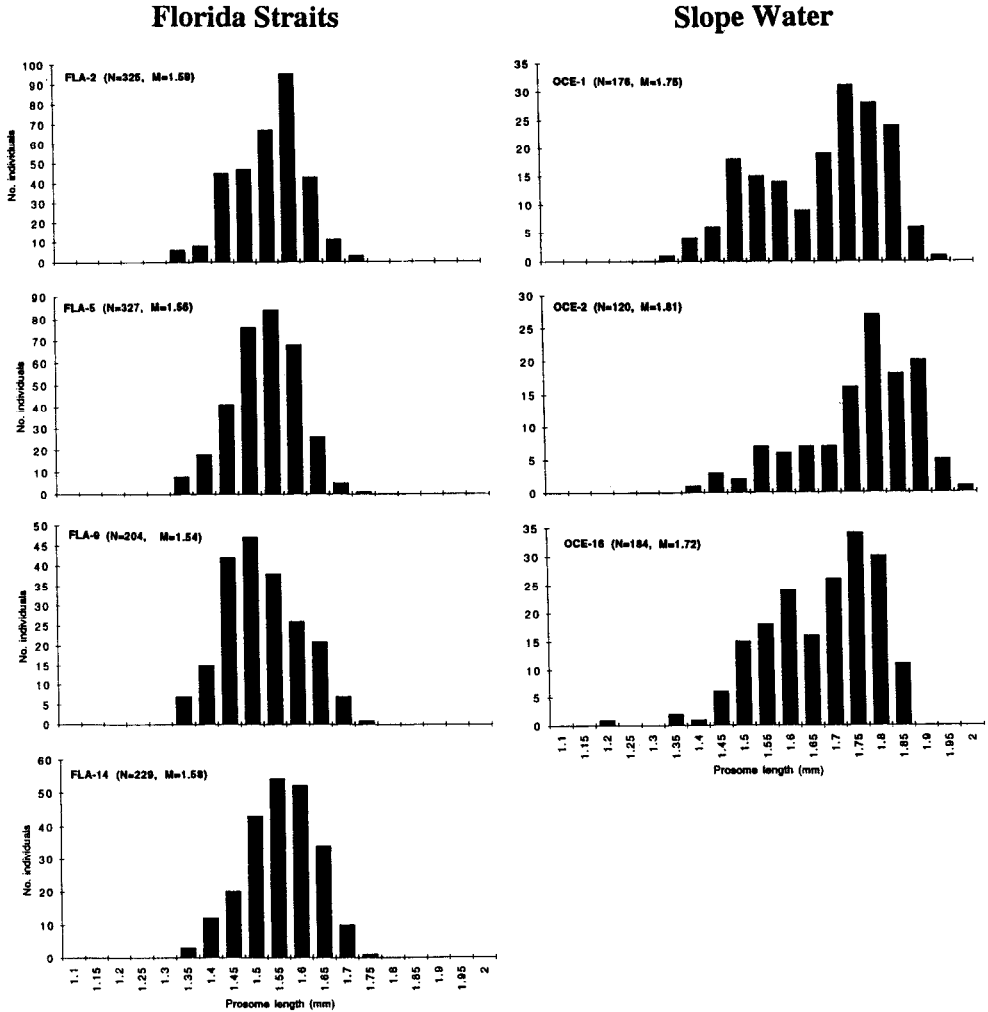

Figure 9. Size frequency distributions for *N. minor* females in samples from the Florida Straits (FLA-2, FLA-5, FLA-9, and FLA-14), Slope Water (OCE-1, OCE-2, and OCE-16), Gulf Stream Meander Region Transect I (OCE-5, OCE-9, OCE-13), Transect II (OCE-18), and Transect IV (OCE-22, OCE-24), and Northeast Atlantic (OCE-25). Prosome length was measured by image analysis. Sample sizes (N) and median sizes (M) are given for each sample. The apparently bimodal distributions of several of the samples may result from an intermixture of the two genetic types: e.g., OCE-24 was collected at the southern boundary of the Gulf Stream, where water masses intermix.

FS and were less abundant than Type I individuals in the GSMR and in the Slope Water. The relative abundance of Type II individuals increased from west to east in the GSMR (Fig. 7). Only Type II individuals were found in the eastern North Atlantic sample.
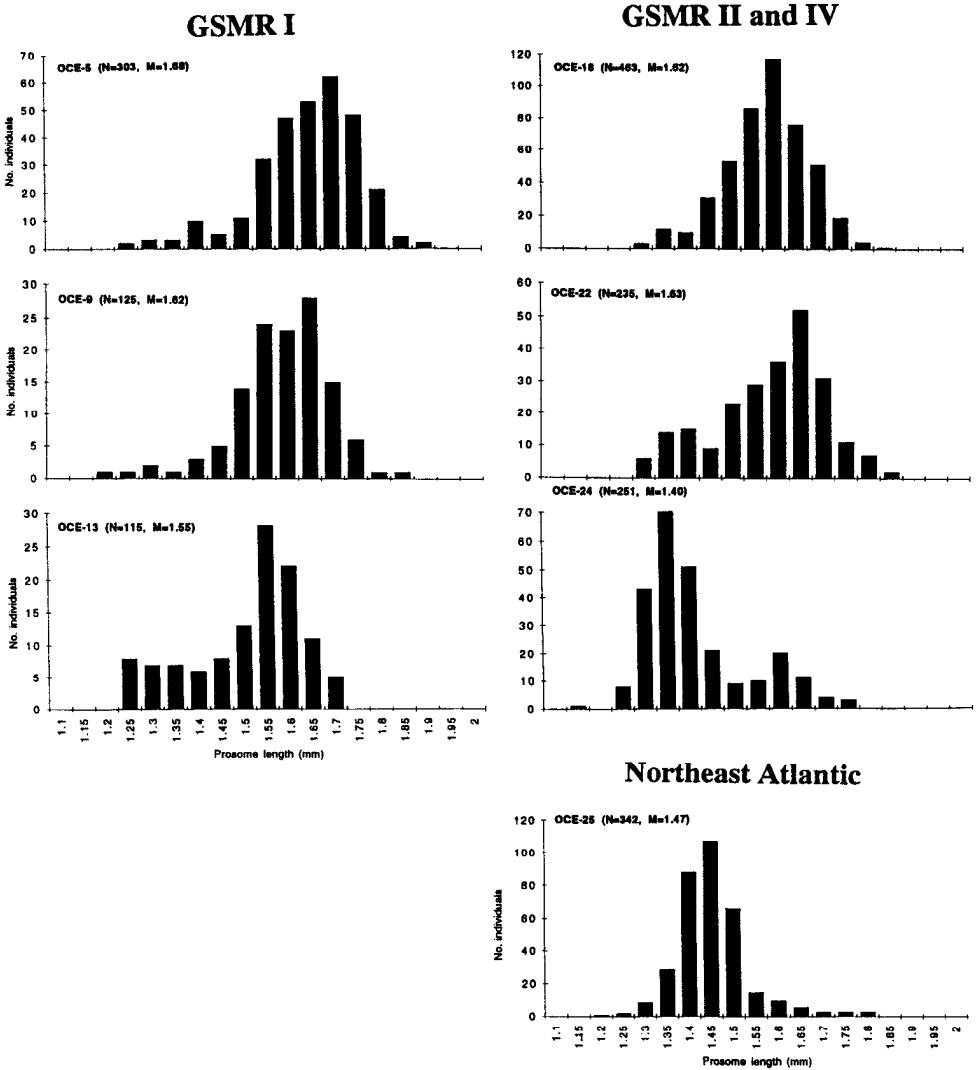
GSMR I

GSMR II and IV

Figure 9. (Continued)

We hypothesize that Type I is characteristic of the Continental Shelf and Slope Waters, as well as the Florida Current, although some individuals may be advected off the shelf in the Gulf Stream. Mixing into the waters adjacent to the Stream by the physical action at the edges of the Stream may explain their occurrence in both the waters immediately to the south of the Stream and also the Slope Water to the north. *Nannocalanus minor* Type II are characteristic of the eastern North Atlantic; these individuals may be swept into the GSMR in the same mixing action that results in the presence of Type I individuals outside the Stream. In addition to physical processes

```
Type I    AAATATTTTTAAGATACCTGCTCAATGAATTTTTTAAATACCCGCTTTAGTGCTAAGGTA 60
Subst.    ...............................A.......................C........

Type I    GCATAATAATTAGTTTTTTAATTGAAAAATAGAATGAATGGTTTTACTAAAATTGATATT 120
Subst.    C....G...............A.....G......................G....A.....

Type I    TTAAAATAATTAGACAAAATTTTAATTTTAGTGAAAATACTAAGATAATATTTTTAGACG 180
Subst.    ......T..T....AT........................AA................A
                 *                                     *

Type I    AGAAGACCCTATGAAGCTAAAAATCACTAT-TAAAATTATAAATTTATCAGATTTATTTT 239
Subst.    ....................T.TC.CTA.......C.......CAG..........
                                  G

Type I    TTGGGGAAAAAATTAATAAATATATTAATAATCATTTATAAAAACTTATCCTTCTAGGAA 299
Subst.    ......G....T.....................T.........G...........T.T....

Type I    TGTTGAAAAAGCTCCTCTAGGGATAACAGCATAATATTTATTAGAGTTCTTATCAGAATA 359
Subst.    .A..A................................................C............

Type I    AATGTTTGTGACCTCGATGTTGAATTAAATATTTTTATATGTGCAGGAGCTTATAAAAAA 419
Subst.    .........................G...............A..........G..

Type I    A-GGTCTGTTCGACCTGAT                                       437
Subst.    GA.............A...
```

Figure 10. DNA sequence alignment and sites of substitutions for a 440 base pair region of the mitochondrial 16S rRNA for *Nannocalanus minor* Type I. Substitutions were observed throughout the sequence and were usually unique, with the exception of the substitions occurring at sites 133 and 164 (indicated with an asterisk), which occurred 25 and 26 times, respectively. Haplotype variation between Type I individuals was less than 1%, compared to the 10% variation observed between Type I and II individuals.

governing water mass mixing along and across the axis of the Stream (see e.g., Wishner and Allison, 1986) the geographic distributions and relative abundances of each type may depend upon differences in the environmental conditions preferred by each type of *N. minor.* The two types may be adapted to different food availabilities, temperature regimes, and seasonal patterns.

The geographic pattern of variation in size frequency distributions (Fig. 9) may result from both genetically-determined differences in size and environmental
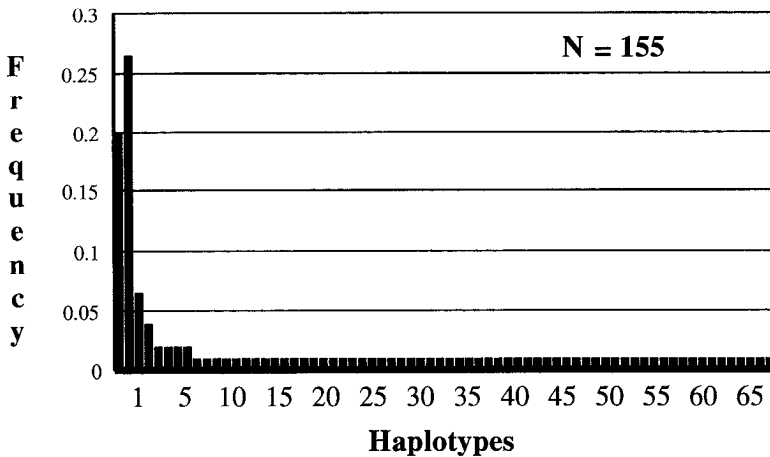

Figure 11. Distribution of haplotype frequencies for *N. minor* Type I. There are numerous unique haplotypes, and two haplotypes that are abundant and nearly ubiquitous. This skewed haplotype frequency distribution is typical of mtDNA haplotype frequency distributions previously observed in marine fish and invertebrates.
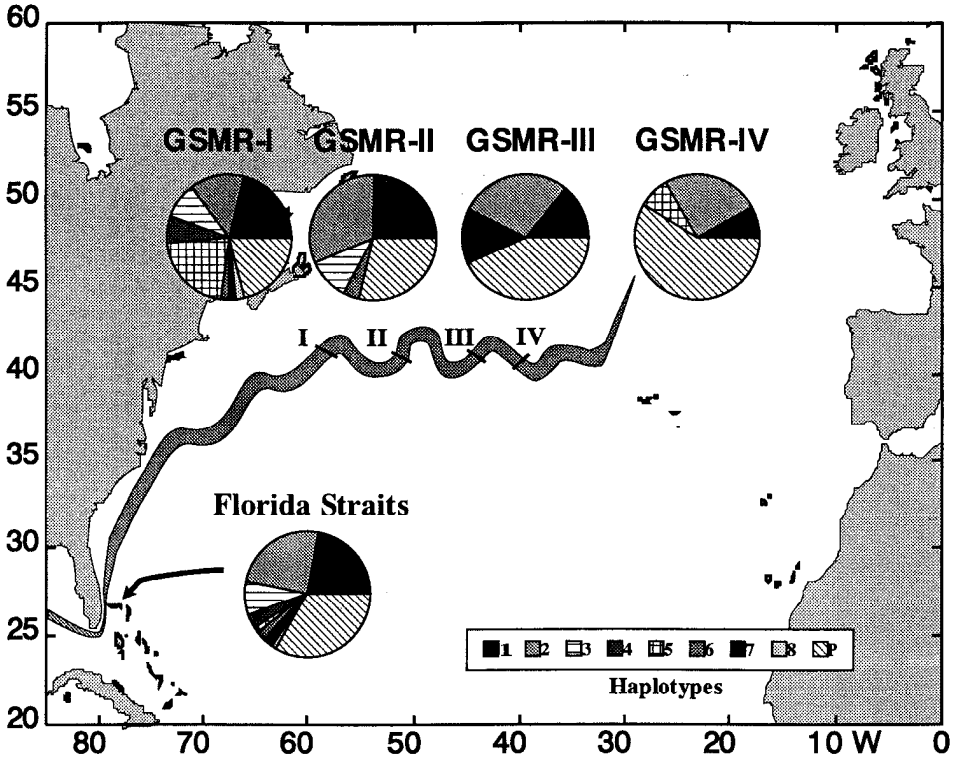
Figure 12. Haplotype frequencies of *N. minor* Type I samples pooled by transect (see Table 1 for collection information). Haplotype abundances did not differ either among samples within a region, among pooled samples of the four transects of the Gulf Stream Meander Region, or between the pooled samples of each region.

causes. In regions where one of the two types is nearly exclusive, such as the FS and the eastern North Atlantic, the size frequency distribution is unimodal (Fig. 9). In boundary regions where it might be expected that the two types are intermixed, such as at boundaries between the Slope Water and Gulf Stream (sample OCE-1) and at the southern edge of the GSMR (OCE-24), the size frequency distributions appear to be bimodal, as might result from a mixture of two distinct populations.

Food availability and temperature have both been suggested as determinants of individual size of *N. minor* (Ashjian and Wishner, 1993a). Our studies indicate similar trends as observed in this previous study: individuals are larger in the cooler, food-rich waters of the Slope Water than in the warmer, more oligotrophic waters of the FS—although Type I is predominant in both regions. A more extensive study of the geographic distribution and physiological condition of *N. minor* in the four regions will be needed to distinguish between genetic and environmental determinants of size in *N. minor*.

Table 2. Haplotype abundances for *Nannocalanus minor* Type I from all samples collected in the Florida Straits and the Gulf Stream Meander Region, with regional totals from the pooled samples in each region. Data summarized are limited to those samples used for molecular analysis. Geographic differences in haplotype abundances were analyzed using a chi-square test by Monte Carlo simulation (Roff and Bentzen, 1989). Simulation results are given at the bottom of the table. Since $P > 0.05$ for all comparisons, there were no significant differences either between samples within a region, between the pooled samples of the GSMR transects, or between the two regions. Ranges given for the P values result from variation among the results of the 1000 simulations.

Samples	Haplotypes								
Florida Straits									
M2	4	4	0	0	0	0	0	0	4
M3	1	0	0	0	0	0	0	0	1
M5	3	4	0	1	0	0	0	0	8
M7	2	2	1	2	0	0	0	0	3
M8	0	1	0	0	0	0	0	0	2
M9	2	6	2	0	1	0	0	1	10
M10	1	2	0	0	0	0	0	0	2
MLF1	0	1	0	0	0	0	0	0	3
TOTAL	13	20	3	3	1	0	0	1	33
Gulf Stream Meander Region									
OCE5	3	3	0	0	0	0	1	0	3
OCE7	3	1	1	0	0	1	0	0	3
OCE9	2	1	1	3	0	0	0	1	2
OCE10	1	1	2	0	0	0	0	0	1
OCE17	1	5	2	0	0	1	0	0	0
OCE18	2	2	1	0	0	0	0	0	5
OCE19	4	2	0	0	0	0	0	0	3
OCE20	1	1	0	0	0	0	1	0	1
OCE21	0	1	0	0	0	0	1	0	1
OCE22	1	1	0	0	1	0	0	0	4
OCE23	0	1	0	0	0	0	0	0	0
OCE24	0	1	0	0	0	0	0	0	3
TOTAL	18	20	7	3	1	2	2	1	27

Results of Chi-square tests by Monte Carlo simulation

Between regions	$P = 0.636 \pm 0.015$
Among FS samples	$P = 0.756 \pm 0.014$
Among GSMR samples	$P = 0.336 \pm 0.015$
Among GSMR Transects	$P = 0.392 \pm 0.015$
Among all samples	$P = 0.550 \pm 0.016$

b. Nannocalanus minor Type II exhibits characteristic molecular genetic variation. Within the large, western North Atlantic *N. minor* Type I, mtDNA sequence variation defined 68 haplotypes among 155 individuals. The highly skewed haplotype frequency that was observed (Fig. 11) is characteristic of many zooplankton species
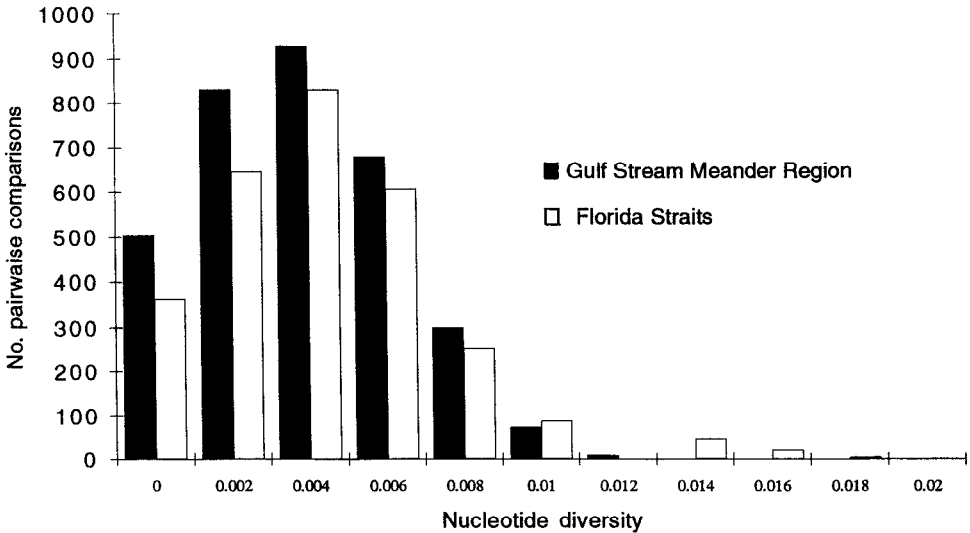
Figure 13. Distributions of haplotype diversities, p_i, for pooled samples collected from the Florida Straits and the Gulf Stream Meander Region. The two distributions are significantly different (by a t-test, $P < 0.001$). The most evidence difference between the distributions is the presence of a number of particularly genetically distinctive individuals in the Florida Straits samples.

(Bucklin *et al.*, 1995; Bucklin and Kocher, 1996). Sixty individuals were unique, while 40 individuals shared one haplotype and 31 individuals shared a second haplotype. This characteristic haplotype frequency distribution is unfortunate for studies for population structure, since unique haplotypes are not geographically informative.

The haplotype abundances did not differ significantly either within the regions (among FS samples, $P = 0.756$; among GSMR samples, $P = 0.336$), between pooled samples of the four GSMR transects ($P = 0.392$), or between the two regions ($P = 0.636$) (Fig. 12; Table 2). Since haplotype abundances are a usual measure of population genetic structure, the lack of geographic variation in this character suggests that *N. minor* Type I experiences significant dispersal along the Gulf Stream from the FS to the GSMR.

One other molecular characteristic indicated that populations of *N. minor* Type I were genetically similar across the range: haplotype diversities (a weighted index of the number of haplotypes) were similar in the two regions: $h = 0.886$ in FS samples and $h = 0.874$ in GSMR samples. Interestingly, another characteristic revealed a significant difference between populations in the two regions. Nucleotide diversity (a weighted index of the degree of difference between haplotypes) was higher in samples collected from the FS than in those from the GSMR ($p_i = 0.00490$ and 0.00414, respectively) (Fig. 13). The difference may be due to a number of genetically divergent individuals in the FS samples. It is possible that the divergent haplotypes in FS samples originated from distinctive source populations. The many distinctive

source regions for copepods—including the North Atlantic and Caribbean—may ensure high genetic diversity of the zooplankton entrained in the upstream portion of the Gulf Stream System. Despite significant differences in nucleotide diversity among FS and GSMR, nearly all of the molecular variance was accounted for by variance within populations (97.81%), with small amounts among populations within groups (1.37%) and among groups (0.81%). Since "populations" for this analysis were an artificial grouping (resulting from pooling samples along each transect) the within population variance may be spuriously high. Further examination of sub-mesoscale genetic variation will be required to assess the significance of mesoscale and larger patterns.

c. Transport pathways in the Gulf Stream System and North Atlantic Ocean. Zooplankton transport in the Gulf Stream is problematical in part because of the long and circuitous pathway of the current. The results of seeding isopycnal floats in the Stream indicate that particles may require months to travel any distance along the Stream path (Rossby *et al.,* 1983). Analysis of zooplankton transport is further complicated by the physical processes of upwelling and downwelling in meanders, which act to detrain and entrain transported particles, including zooplankton (Ashjian, 1993). One of the goals of this work was to determine whether molecular genetic characteristics can be used as "tags" of transport pathways of planktonic copepods. The "tag" used (the mtDNA 16S rRNA gene) did not reveal significant genetic structure within *N. minor* Type I, indicating high levels of dispersal along the Gulf Stream. This gene may not be sufficiently variable to reveal smaller-scale structure and dispersal pathways, and we are currently working to identify additional molecular tags.

The results presented here indicate that there is at least one significant change in the genetic makeup of the entrained copepods during their transport from the upstream waters of the FS, through several meanders of the GSMR. That change is the apparent loss of a number of genetically divergent individuals during transport (Fig. 13). Because entrained *N. minor* may pass through a number of generations between the FS and the GSMR (Ashjian and Wishner, 1993a), there are many forces that might drive genetic differentiation of downstream populations. The stability of the other molecular genetic characteristics analyzed (haplotype frequencies and haplotype diversity) suggests that *N. minor* Type I in the Gulf Stream System represent a single, genetically cohesive population.

Downstream of the GSMR, the North Atlantic Current flows eastward at about 50N latitude; a significant portion of the Current flows into the Norwegian Sea (Krauss, 1986). The eastern North Atlantic is a region of complex hydrography, due in part to intrusions of Mediterranean water and to variation in the position of the southern edge of the Polar Front (Gould, 1983). The mesoscale eddy field has not been adequately resolved; eddies may result from pinched-off meanders of the North

Atlantic Current (Pollard, 1982 in Krauss, 1986). The sources and transport pathways of water from the Gulf Stream into the eastern North Atlantic may be amenable to study using molecular characteristics of the entrained zooplankton as markers of their origin. In particular, since *N. minor* Type I is a western North Atlantic form, its presence in the eastern North Atlantic may be useful as a tracer of water transported from the western North Atlantic.

The occurrence of two, congeneric species in the North Atlantic basin—one distributed on the western side of the North Atlantic ridge and the other on the eastern side—is known for other zooplankton groups. Certainly the hypothesized distributions of *N. minor* Type I and II are known for other zooplankton species. Van der Spoel and Heyman (1983) describe a *Euphausia pseudogibboides* type distribution: the species distribution is centered between 20S and 20N but is carried northward as an expatriot in the eastern boundary current (p. 62). This description resembles that of *N. minor* Type I based on this study, except that *N. minor* actively reproduces in the Gulf Stream (Ashjian and Wishner, 1993a) and does not appear to be an expatriot in these waters. A number of warm-water species exhibit similar distributions, and may be circumglobal despite restriction to mid-latitudes. The distribution of *N. minor* Type II may be largely restricted to the eastern North Atlantic, similar to several pteropod species, such as *Cavolinia tridentata* forma atlantica (Van der Spoel and Heyman, 1983, p. 75). *Nannocalanus minor* is currently known from the North Pacific Ocean: whether this is either or both of the *N. minor* types or another type or species is currently unknown.

How these distinctive geographic patterns are maintained in the face of considerable mixing across oceanographic domains is an open question. Adaptation to different environmental conditions must be part of the answer. Western North Atlantic species will experience cool temperatures and high food availability, while the warmer, more oligotrophic waters of the eastern North Atlantic may constitute a very different bio-physical domain. Different species are associated with each of these domains; the physical boundaries constitute faunal boundaries for planktonic species. Within the domains, however, there is no evidence of genetic divergence of geographic populations—at least not of *N. minor* Type I. The physical gradients and heterogeneous biotic conditions may not be sufficiently persistent or marked to drive and maintain differentiation of conspecific populations.

There are unanswered questions at several spatial scales regarding dispersal of both types of *N. minor* throughout the North Atlantic Ocean. At small scales, there are questions about how position in the Stream may affect transport. At large scales, there are questions about the causes of the difference in the genetic makeup (nucleotide diversity) of populations at the source *vs* farther downstream. Additional questions include: are there biological and/or physical retention mechanisms that maintain population abundances and geographic distributions of both types of *N. minor* in the North Atlantic Ocean? How common among copepods and other

zooplankton are the patterns observed for *N. minor* (see Ashjian and Wishner, 1993b)?

Molecular analysis of marine zooplankton is likely to continue to reveal taxonomically-significant genetic partitioning of species populations, including cryptic species. This may be especially true for calanoid copepods, which as a group comprise numerous sibling species assemblages that may be discriminated by a very few, subtle morphological characters. The presence of sibling species among marine groups is quite common (Knowlton, 1993). Despite their morphological similarity, congeneric and sibling species of several copepod genera have been shown to differ significantly in genetic character (see e.g., Bucklin *et al.*, 1992, 1995).

Molecular technologies will also help to disentangle the biological-physical coupling in the ocean, especially as it determines plankton transport and dispersal processes. Application of these new technologies may help to understand the ways in which physical properties of the ocean control the distributions and abundances of zooplankton populations in time and space. The long-term goal of this study is to work toward a predictive understanding of plankton distributions on meso- to large scales.

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