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Molecular population structure of the marine benthic copepod *Microarthridion littorale* along the southeastern and Gulf coasts of the USA

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Abstract Relationships among populations (southeast Atlantic and northern Gulf coast, USA) of a ubiquitous, estuarine, harpacticoid copepod (*Microarthridion littorale* Poppe) were estimated using sequence data from two loci: one mitochondrial [cytochrome *b* (Cyt *b*)] and one nuclear [first internal transcribed spacer of ribosomal DNA (ITS-1)]. Copepods were collected from seven estuaries in 1997/98. Allelic phylogenies based on both genes were generally concordant, and suggested that *M. littorale* populations are structured over large geographic scales (hundreds of kilometers). Three well-supported groups were found in both gene trees comprising clades of alleles sampled from South Carolina, Florida, and Louisiana. Alleles from the Savannah, Georgia sample formed a monophyletic group using the Cyt *b* data, but this clade was not distinguishable with comparable ITS-1 data. A single specimen from Louisiana was classified in different clades depending on the locus assayed.

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Supplementary material Appendix 1: *Alignment of Cytb sequences in a NEXUS file format*, and appendix 2: *Alignment of ITS-1 sequences in a NEXUS file format* can be obtained at http://link.springer.de/link/service/journals/00227/index.htm

Introduction

Population subdivision can be attributed to geographic isolation and the subsequent cessation of gene flow (Endler 1977; Slatkin 1985). In the marine environment, population subdivision is relatively common in species with or without a life history conducive to high dispersal rates (Avise et al. 1987; Palumbi 1994). Non-conspicuous geographic features such as ocean circulation patterns, behavioral limits to dispersal, and natural selection can lead to population structures in marine animals that have comparatively larger effective population sizes and extensive geographic ranges (Avise 1994; Burton and Lee 1994; Palumbi 1994).

Harpacticoid copepods are ubiquitous, numerically abundant, and important components of benthic communities (Hicks and Coull 1983). Unlike many benthic invertebrates, most harpacticoid copepods do not possess planktonic larvae, suggesting no dispersal mechanism. Estuarine species can be passively dispersed within tidal creek systems through advection by tidal currents (Palmer and Gust 1985). Copepods, and more generally meiofauna, can occasionally be transported great distances by clinging to floating marine algal mats (Yeatman 1962) and by ballast of sailing vessels (Gerlach 1977). However, the contribution of these two means of transportation to dispersal and gene flow in harpacticoid copepods is unknown. Previous studies on population subdivision in harpacticoid copepods have reported various degrees of genetic differentiation on a scale between a kilometer and hundreds of kilometers. Distinct populations can be maintained over short distances (< 1000 m) among, for example, tide pools (Burton and Feldman 1981; Burton 1990; Burton and Lee 1994), salt marshes (Palmer 1980), or offshore habitats (Street and Montagna 1996). At larger scales, latitudinally separated populations of the harpacticoid *Coullana* (formerly *Scottolana*) *canadensis* (Willey) exhibit differences in growth (Lonsdale and Levinton 1985a), reproduction (Lonsdale and Levinton 1985b; Frey 1996), and energy

budgets (Lonsdale and Levinton 1989). These differences suggest that the geographically separated populations of *C. canadensis* could be genetically distinct.

In the present study we evaluate the likelihood that an estuarine harpacticoid copepod (*Microarthridion littorale*) might show population differentiation between estuaries separated by several hundred kilometers and across a well-known biogeographic boundary. Determining the spatial scale at which this differentiation occurs is essential to our understanding of population biology and microevolution of this species. *M. littorale* is a common inhabitant of subtidal sediments and is a useful organism for studies of sediment contamination (Marshall and Coull 1996; Chandler et al. 1997) and for intensive ecological studies (Morris and Coull 1992 and references therein). The estuarine habitats and lack of a planktonic dispersal stage of *M. littorale* could result in relatively low rates of dispersal.

We estimated large-scale patterns of genetic differentiation among populations of *Microarthridion littorale* from the southeastern Atlantic and northern Gulf of Mexico coasts of the USA. We determined sequence variation in two unlinked loci: the mitochondrial cytochrome *b* apoenzyme (Cyt *b*) gene and the first internal transcribed spacer region of the nuclear ribosomal cluster (ITS-1). Cyt *b* and ITS-1 are appropriate markers for population-level studies because they contain sufficient diversity to address intra- and interspecific phylogeny in invertebrates (Cyt *b*: Collins et al. 1996; Merritt et al. 1998; ITS-1: Schlötterer et al. 1994; Vogler and DeSalle 1994; Odorico and Miller 1997). Further, unlinked markers allow for independent assessment of phylogeographic pattern and an evaluation of concordant genetic patterns among loci. Concordant phylogeographic patterns among unlinked genes often result from historical factors limiting gene flow among populations (Avise 1994). Gene–gene concordance has strengthened assertions regarding phylogeographic patterns in many diverse systems, for example, among estuarine fish populations (Bernardi et al. 1993), harpacticoid copepods in rockpools (Burton and Lee 1994), beetles (Vogler and DeSalle 1993, 1994), and fungi (O'Donnell et al. 1998).

Materials and methods

Copepod samples

Microarthridion littorale Poppe were sampled in seven creeks along the southeastern Atlantic and Gulf of Mexico coasts of the USA from January 1997 to February 1998 (Fig. 1; specific location data available from the corresponding author). Salinity ranged from 20 to 27‰ at all locations except Cocodrie, Louisiana (5 to 7‰). Copepods were collected by scraping the upper centimeter of exposed sediment during low tide and passing it through 500 and 125 μm sieves. The 125 μm fraction was transported to the laboratory where copepods were separated using fiber optic light (Couch 1989). Individual *M. littorale* were identified under a dissecting microscope and were either used immediately for nucleic acid extraction or stored in 95% ethanol at −20 °C. *M. fallax* (Perkins) was collected from the Falmouth estuary, Plymouth, UK (50°12′N; 5°16′W) in May 1996.

DNA amplification and sequencing

DNA from individual copepods was extracted according to the "small metazoan" procedure of Schizas et al. (1997). Small aliquots of extracted nucleic acids (typically 2 to 3 μl) were used as template for polymerase chain reaction amplification (PCR; Saiki et al. 1988). Cyt *b* and ITS-1 amplifications used similar conditions: 50 m*M* KCl, 10 m*M* Tris-HCl, 2.5 m*M* $MgCl_2$, pH 8.3 (Perkin-Elmer Cetus), 200 μ*M* dNTP (Promega), 5 pmol forward and reverse primer, and 1 unit *Taq* DNA polymerase (Promega). Cyt *b* amplifications used primers 151F (5′-TGTGGRGCNACYGTWATYACTAA-3′) and 270R (5′-AANAGGAARTAYCAYTCNGGYTG-3′) (Merritt et al. 1998). ITS-1 amplifications used primers ITS-1F (5′-CACACCGCCCGTCGCTACTACCGATT-3′) and ITS-1R (5′-ATCGACCCATGAGCCGAGTGATC-3′). We constructed the ITS-1 primers by aligning the rDNA sequences of several arthropod taxa. Primers used for the amplification of ITS-1 correspond to starting positions 1825 and 2736 in the *Drosophila melanogaster* rDNA sequence, Accession Number M21017 (Tautz et al. 1988). Template DNA was denatured at 94 °C for 2 min followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C (52 °C for ITS-1) for 1 min, and extension at 72 °C for

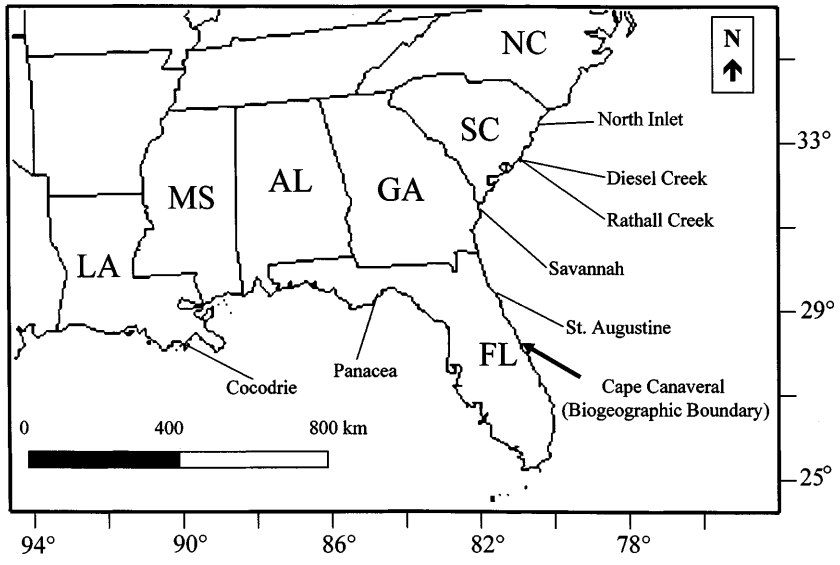

Fig. 1 Map of sites where *Microarthridion littorale* copepods were collected

1 min. A small aliquot (5 µl) of each amplification was loaded on a 1% agarose gel, stained with ethidium bromide, and visualized under UV light. Excess primer, dNTPs, and other impurities were removed from the remaining PCR solution by polyethylene glycol precipitation (Kusukawa et al. 1990). An aliquot (200 to 500 ng) of the precipitated PCR product was used as template for fluorescent sequencing using Thermo Sequenase (Amersham Life Sciences) and dye-labeled primers. Cycle sequencing amplifications used the following conditions: 94 °C for 45 s, 58 °C for 45 s and 72 °C for 90 s for 25 cycles (Cyt *b*); 94 °C for 30 s, 60 °C for 30 s and 72 °C for 90 s for 25 cycles (ITS-1). Electrophoresis was carried out on a LICOR 4000L sequencing system.

Phylogenetic analysis

Sequences were aligned using the profile alignment option in ClustalW (Version 1.7; Thompson et al. 1994). Sequences from North Inlet, Rathall, and Diesel copepods were aligned first, then aligned against other sequences in an incremental fashion. Since sequences from each sampling site had relatively few base pair differences for both genes, and practically no variation in the number and size of insertions/deletions for the ITS-1 region, this procedure resulted in a more meaningful alignment than a simultaneous alignment of all sequences. The resulting alignment of the ITS-1 region was adjusted manually, i.e. by eye. Alignments are available from http://link.springer.de in NEXUS format.

Relationships among haplotypes were explored in MacClade (Version 3.05; Maddison and Maddison 1992). After excluding the outgroup, a wide range in the number of variable sites among sequences was observed in the Cyt *b* (0 to 124) and the ITS-1 data (0 to 56). Templeton et al. (1992) suggested that phylogenetic methods do not perform well when sequence variation is both extensive and limited within a data set and proposed a method to identify closely related sequences that can be grouped with >95% confidence (P_j). The phylogenetic relationships among these closely related groups of sequences can be estimated by maximum parsimony. P_j was >95% when Cyt *b* and ITS-1 sequences differed by no more than seven and nine nucleotide sites, respectively (calculated by ParsProb, A. Templeton, Washington University, St. Louis). Individual sequences differing by fewer than seven or nine differences were constrained into groups, and this partially constrained topology was used as a starting point for exhaustive searches in PAUP (Version 3.1.1; Swofford 1993). For comparison, phylogenetic trees were also built with the maximum parsimony algorithm without ParsProb and the neighbor-joining algorithm (Saitou and Nei 1987) in MEGA (Kumar et al. 1993). Sequences from the congener *Microarthridion fallax* were used to root both phylogenies.

Results

Twenty-one *Microarthridion littorale* and two *M. fallax* specimens were sequenced for Cyt *b* and ITS-1. Sequences of both genes were obtained from the same individuals with four exceptions (Table 1). A total of 300 bp of Cyt *b* (131 parsimony informative sites) and up to 474 bp of rDNA (130 parsimony informative sites) were sequenced from each individual. The first 113 bp of each rDNA sequence were derived from the 3′ end of the 18S rRNA and contained relatively little variation. Cyt *b* and ITS-1 sequences from a single specimen (ML 5) have been deposited in GenBank (Accession Numbers AF102553 and AF102877, respectively).

Cyt *b*

A total of 14 haplotypes were observed for Cyt *b* (Fig. 2). Phylogenetic analyses under the parsimony criterion yielded a single most parsimonious tree (L = 226, CI = 0.95, RI = 0.96) containing four distinct clades comprising individuals from North Inlet, Rathall and Diesel Creek (South Carolina clade); Savannah (Georgia clade); St. Augustine and Panacea

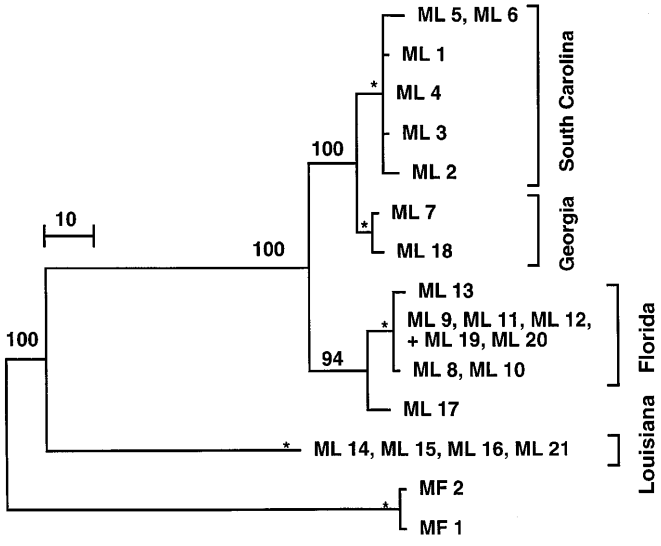

Fig. 2 *Microarthridion littorale*. The most parsimonious Cyt *b* tree based on the combination of maximum parsimony analysis and the Templeton et al. (1992) method. *M. littorale* (ML) and the outgroup *M. fallax* (MF). Branch lengths are proportional to the number of changes assigned to each branch. The scale bar represents the branch length of ten changes. Nodes with asterisks denote topological constraints supported at the $P \geq 0.95$ level using the method of Templeton et al. (1992). Strict-consensus bootstrap values are shown on bases of nodes supported by ≥50%. Numbers following taxon names indicate identification numbers of individual specimens

Table 1 *Microarthridion littorale* and *M. fallax*. Sampling locations, haplotypes, and number of specimens (*N*) sequenced for Cyt *b* and ITS-1. Haplotypes with the same number were derived from the same individual

Location	Cyt *b* (mtDNA) haplotypes	*N*	ITS-1 (rDNA) haplotypes	*N*
North Inlet	ML 1, ML 2	2	ML 1, ML 2	2
Diesel Creek	ML 3, ML 4	2	ML 3, ML 4	2
Rathall Creek	ML 5, ML 6	2	ML 5, ML 6	2
Savannah	ML 7, ML 18	2	ML 7, ML 22	2
St. Augustine	ML 8, ML 9, ML 10, ML 19	4	ML 8, ML 9, ML 10, ML 23	4
Panacea	ML 11, ML 12, ML 13, ML 20	4	ML 11, ML 12, ML 13, ML 24	4
Cocodrie	ML 14, ML 15, ML 16, ML 17, ML 21	5	ML 14, ML 15, ML 16, ML 17, ML 25	5
Plymouth UK	MF 1, MF 2	2	MF 1, MF 2	2

(Florida clade); and Cocodrie (Louisiana clade) (Fig. 2). Clade stability was assessed by 100 bootstrap replicates, and monophyly of each major clade was highly supported (Fig. 2). Partitioning of the haplotypes into four clades was verified with neighbor-joining analysis (Jukes–Cantor distances) and maximum parsimony without ParsProb constraints enforced (data not shown).

Within the South Carolina clade, sequences from Diesel Creek individuals were paraphyletic relative to North Inlet, whereas sequences from Rathall Creek formed a monophyletic group. The Georgia clade was more closely related to the South Carolina clade than to the Florida group and differed by three third-base transitions. Copepods from Florida differed from Georgia and South Carolina individuals by 2 first-base transitions, 2 second-base transitions, 16 third-base transitions, and 3 third-base transversions. With the exception of second-base transitions all the nucleotide changes were synonymous. Both Louisiana *Microarthridion littorale* and *M. fallax* copepods were separated by an unexpectedly large number of amino acid changes from the other clades, 38 and 36 respectively. Louisiana *M. littorale* and *M. fallax* were 36 amino acid changes apart.

ITS-1

A total of 19 haplotypes were observed for rDNA (Fig. 3). Topological constraints imposed by the method of Templeton et al. (1992) are illustrated in Fig. 3. Phylogenetic analyses of the ITS-1 data included alignment gaps as additional characters regardless of length. Parsimony analyses identified a single shortest tree (L = 160, CI = 0.963, RI = 0.967), which was concordant with the Cyt *b* tree with one notable exception: Georgia and South Carolina clades collapsed into a single well-supported group (Fig. 3). The South Carolina/Georgia clade is separated from the Florida clade by three synapomorphies: one C/T transition and two thymine insertion/deletion events. When we did not include the aligning gaps in the data analysis, the Florida clade collapsed into the South Carolina/Georgia clade (data not shown). Analyses using neighbor-joining and maximum parsimony without ParsProb constraints identified the Louisiana and Florida clades as monophyletic but failed to extract the South Carolina/Georgia clade as a monophyletic group in relation to the Florida clade (data not shown). The ITS-1 region sequences of the Louisiana copepods were approximately 20 bp shorter than the ITS-1 region of the other *Microarthridion littorale*, and 30 bp shorter than the ITS-1 region of the outgroup, *M. fallax*.

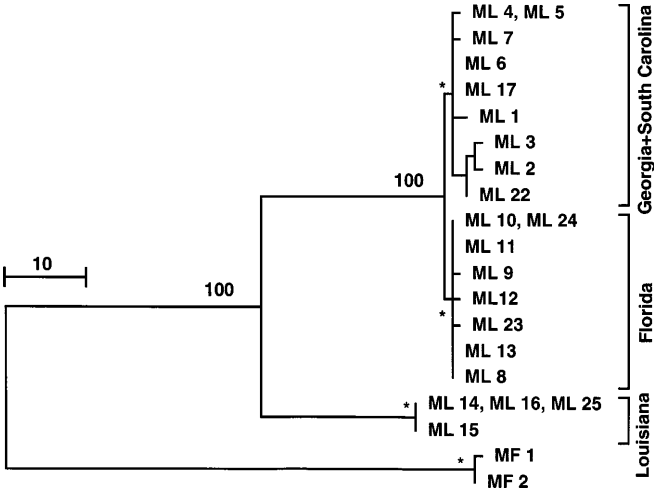

Fig. 3 *Microarthridion littorale*. The most parsimonious ITS-1 tree based on the combination of maximum parsimony analysis and the Templeton et al. (1992) method. *M. littorale* (ML) and the outgroup *M. fallax* (MF). Branch lengths are proportional to the number of changes assigned to each branch. The scale bar represents the branch length of ten changes. Nodes with asterisks denote topological constraints supported at the $P \geq 0.95$ level using the method of Templeton et al. (1992). Strict-consensus bootstrap values are shown on bases of nodes supported by ≥50%. Numbers following taxon names indicate identification numbers of individual specimens

Discussion

The genetic survey revealed an unexpected degree of genetic differentiation among populations of *Microarthridion littorale* along the southeastern and Gulf coasts of the United States. The two independently evolved molecular markers (Cyt *b* and ITS-1) yielded concordant patterns of population structure. The sample spacing was adequate to decipher inter-clade relationships but additional sampling will be required for all intra-clade comparisons. For the South Carolina clade, genetic relationships among creeks were inconsistent with the hydrography of the coast; Rathall and Diesel Creeks drain into the same estuary (Charleston Harbor) but Rathall Creek copepods had a unique haplotype not present in Diesel Creek and North Inlet. Cyt *b* sequences from the Charleston Harbor creeks (Rathall and Diesel) were more related to North Inlet sequences than to Savannah sequences, even though Charleston is only 50 km closer to North Inlet than it is to Savannah. We conclude the differences among these populations are not related to distance alone.

The polyphyly of the ITS-1 tree for the South Carolina and Georgia clades is not surprising given that the effective population size of a mitochondrial gene is four times less than that of a nuclear autosomal gene (Moore 1995). Recently delineated populations or species may be paraphyletic/polyphyletic in their mtDNA phylogeny if the time for lineage sorting of ancestral alleles is $< N_e$ (effective population size) generations (Neigel and Avise 1993). The same is true for nuclear genes although the time for thorough segregation of ancient polymorphisms is $> 4N_e$ generations (Moore 1995). The presence of ancestral polymorphisms within the mitochondrial (Avise et al. 1987) and nuclear (Pamilo and Nei 1988)

genes can lead to a population phylogeny that reflects random lineage extinctions rather than the "true" population tree. If the number of generations since separation of South Carolina and Georgia copepods is between N_e and $4N_e$, the ITS-1 phylogenetic signal may reflect the retention of ancestral polymorphisms resulting in a polyphyletic relationship between South Carolina and Georgia *Microarthridion littorale*, whereas the Cyt *b* data supports monophyly of the two clades. Alternatively, the monophyly of the South Carolina and Georgia clades supported by the Cyt *b* sequence data could be the result of sampling error (i.e. few copepods used to determine phylogenetic relationships of populations inhabiting adjacent sampling sites such as Charleston and Savannah).

Unequivocal phylogeographic patterns are established in studies where there is concordance in gene–gene and species–species phylogeny (Bernardi et al. 1993). In these cases, an overriding factor (e.g. physical barrier) influences the evolutionary history of the assayed fauna. The genetic discontinuities observed with both Cyt *b* and ITS-1 data between South Carolina/Georgia and Florida *Microarthridion littorale* imply the cessation of gene flow between copepods from these regions. These populations span the well-studied biogeographic barrier at Cape Canaveral on the Florida peninsula, separating temperate and tropical faunas into genetically distinct units (Avise 1992). Several marine and coastal taxa (horseshoe crabs, oysters, toadfish, diamondback terrapin, black sea bass, and seaside sparrow) have a trans-Floridian distribution and exhibit concordant phylogeographic patterns, with distinct Atlantic and Gulf of Mexico populations (Avise 1994). This phylogeographic pattern may reflect isolation of trans-Floridian distributed taxa caused by Pleistocene glacial advances (Avise 1992), and phylogeographically distinct units may be observed north and south of Cape Canaveral. Even though the presumed biogeographic barrier for the southeastern *M. littorale* seems to be located north of Cape Canaveral (between St. Augustine and Savannah), the present study is consistent with Avise's hypothesis since the phylogeographic break location in the Florida peninsula varies considerably among taxa (Avise 1992).

Current or recently ceased gene flow can result in the same degree of population homogenization. St. Augustine and Panacea *Microarthridion littorale* are not genetically differentiated, even though the discontinuous distribution of salt marshes around the Florida peninsula might suggest the absence of gene flow between the two sites (Figs. 2, 3). The most frequently encountered Cyt *b* haplotype in Florida *M. littorale* was present in both St. Augustine and Panacea individuals at 50% and 75% frequency, respectively (Fig. 2). The ITS-1 data provided a less structured phylogeny of the Florida copepods, compared to the Cyt *b* data, by supporting polyphyletic origins of the St. Augustine and Panacea specimens, further supporting the hypothesis that these *M. littorale* populations are panmictic (Fig. 3). The observed genetic patterns between St. Augustine and Panacea copepods are concordant with Avise's "Gulf haplotype leakage" hypothesis (Avise 1994), where the Gulf Stream may facilitate the transport of copepods suspended in the water column into the South Atlantic. Alternatively, the genetic relationship of the trans-Floridian populations of *M. littorale* may reflect the past distribution of salt marshes. The recession of salt marshes to their current range (Felder and Staton 1994) may have divided a once panmictic Floridian population and restricted gene flow among trans-Floridian populations of *M. littorale*, but recently enough that there has been no genetic differentiation.

Monophyly of the Cocodrie *Microarthridion littorale* was highly supported by sequences from both genes, notwithstanding specimen ML 17 (Figs. 2, 3). We sequenced the Cyt *b* gene from nine additional *M. littorale* from Cocodrie (data not shown) and all the resultant haplotypes unambiguously belonged to the monophyletic clade of Cocodrie. The inclusion of ML 17 in different clades depending on the gene is puzzling**.** The ITS-1 sequence of ML 17 could have resulted from the amplification of a non-homologous sequence (Hillis and Dixon 1991).

The extreme nucleotide divergence between the *Microarthridion littorale* copepods from Cocodrie and *M. fallax* copepods from England relative to copepods from the other clades was unexpected. When nucleotide differences are radically different than those expected, it is possible that nuclear mitochondrial-like sequences may be amplified instead of the target mitochondrial gene (Zhang and Hewitt 1996). It is unlikely that we amplified a pseudogene because (a) the inferred amino acid sequences contained no stop codons, and (b) the phylogenetic signal from both genes was concordant for the Cocodrie specimens. Additionally, the number of synonymous nucleotide substitutions per synonymous site (K_s) should equal the number of non-synonymous nucleotide substitutions per non-synonymous site (K_a). In our case, K_s significantly exceeded K_a implying that Cyt *b* may be under purifying selection. Pseudogenes should accumulate mutations in a neutral fashion (Kimura 1983) and by definition should be under no selection.

Alternatively, the Cocodrie specimens may constitute a related but undescribed *Microarthridion* species. The observed amino acid differences between *M. littorale* and Cocodrie copepods were comparable to those between *M. littorale* and *M. fallax*. Morphological similarity in the genus *Microarthridion* may be a misleading measurement of relatedness among specimens distributed along the southeastern and Gulf coasts of the United States. Harpacticoid assemblages in intertidal mudflats on all continents are characterized by a remarkable similarity in the predominant morphological forms (Hicks and Coull 1983). Specimens assigned to *M. littorale* throughout the USA are an integral part of these "isocommunities" (sensu Thorson 1957), but morphological similarities due to convergent evolution

from similar habitat constraints may mask genetic differences among these copepods.

The spacing of the sites and the number of individuals per site were adequate to identify population subdivision in *Microarthridion littorale* in the southeastern USA. The large genetic distances observed among the South Carolina, Florida, and Louisiana clades in both gene trees could be explained by the relatively large geographic distances between the successive sampling sites. Since we analyzed DNA sequences from *M. littorale* collected from only two sites in the Gulf of Mexico (Panacea and Cocodrie), it is possible that we missed "intermediate" populations. Finer-scale sampling in the southeastern USA might also have identified a transition zone between the South Carolina and Florida populations.

We feel confident that the gene trees reported here reflect the "true" population relationships of *Microarthridion littorale* for two reasons: (1) the major clades are separated from each other by large genetic distances and the monophyly of the major clades is supported $>95\%$ by bootstrap analysis and (2) two independently evolved loci (Cyt *b* and ITS-1) basically support the same phylogenetic pattern. Vogler and DeSalle (1993, 1994) inferred the phylogeographic patterns of the North American tiger beetle by utilizing DNA sequences from both mtDNA (16S, COIII and tRNA leu) and rDNA (ITS-1) and supported the presence of distinct US Atlantic and Gulf of Mexico populations.

The present study confirms previous evidence of marked subdivision in marine harpacticoid copepods (Palmer 1980; Burton and Lee 1994; Street and Montagna 1996). The observations that copepods in some habitats maintain distinct populations within meters of each other (Burton and Lee 1994), while in other habitats, reproductive isolation occurs between geographically separated (hundreds of kilometers) populations (Frey 1996), suggest that population subdivision may be pervasive throughout the Harpacticoida, regardless of their individual life history patterns. Physical and other environmental factors can be effective barriers to gene flow and structure copepod populations. This analysis of the population structure of *Microarthridion littorale* in southeastern USA estuaries makes it possible to ask specific questions about the scale on which contemporary gene flow occurs among adjacent creeks.

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