Marine Biology (2002) 140: 585–594
DOI 10.1007/s00227-001-0728-y

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Sympatry of distinct mitochondrial DNA lineages in a copepod inhabiting estuarine creeks in the southeastern USA

Received: 1 August 2000 / Accepted: 12 September 2001 / Published online: 20 November 2001
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Abstract The population genetic structure of the meiobenthic harpacticoid copepod *Microarthridion littorale* (Poppe) was examined with a geographic survey of a 348 bp fragment of the mitochondrial cytochrome *b* gene. Copepods were collected from ten locations on the coast from North Carolina to Georgia, USA, from January 1997 to November 1998. Sequence divergence among 198 individuals was as much as 4.3%, and three divergent mitochondrial clades were uncovered that differed by six to nine nucleotide changes. A rapid assay was developed to distinguish among mitochondrial clades, and an additional 333 specimens were surveyed. The three lineages co-occurred in seven of ten sampling locations. Data analyses were carried out separately for individuals assayed by DNA sequencing as well as for a combined data set that included individuals typed by restriction endonuclease digestion. An analysis of molecular variance indicated that a significant proportion of the total genetic variance could be partitioned among populations, although no significant correlation between geographical and genetic distance was detected.

Communicated by J.P. Grassle, New Brunswick

Supplementary materials: Additional documentary material has been deposited in electronic form at and can be obtained from http://dx.doi.org/10.1007/s00227-001-0728-y.

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Introduction

Increased interest in the biodiversity of marine ecosystems has revealed an unexpectedly large number of species in almost all habitats examined. The notion that the marine environment is a heterogeneous medium is well established and is consistent with the presence of many species. With an increased application of molecular tools in biodiversity studies, higher than expected intra- and interspecific genetic variation from seemingly uninterrupted population or species distributions has frequently been observed. Distinct genetic populations or lineages may have different evolutionary trajectories, and the sum of all evolutionary trajectories within a species determines its long-term fate. Knowledge of evolutionary trajectories (i.e. genetic composition) of individual species is becoming increasingly necessary for conservation in the marine environment, which traditionally depends on comprehensive surveys of species composition.

The study of spatial genetic variation is useful for elucidating the processes causing population differentiation (Gould and Johnston 1972), and often reveals unexpected patterns of population relatedness. Geophysical, climatic, and biological barriers produce distinct "breaks" in the geographic distribution of populations and species (Knowlton et al. 1993; Avise 1994, 2000). The same processes causing divergence among species contribute to increased polymorphism within species. The present study focuses on a marine copepod species with distinct mitochondrial lineages,

and an unexpectedly large amount of genetic variation within locations separated by short geographic distances (tens of kilometers) in the southeastern USA.

Seemingly large census population sizes in relatively small areas, the ease with which individuals can be collected, and a relatively short life cycle for most temperate species are demographic attributes that make marine benthic copepods an attractive model system for population-level questions (Hicks and Coull 1983). Because of their seemingly limited vagility and their obligatory benthic life style, harpacticoid copepods are often found as genetically isolated populations occupying short geographic distances (Burton and Lee 1994; Burton 1998). The population structure of only a few harpacticoid copepod species has been studied (Lonsdale and Levinton 1989; Burton and Lee 1994; Frey 1996; Burton 1998; Schizas et al. 1999; Rocha-Olivares et al. 2001), and, in all cases, differentiation has been inferred to occur in allopatry, which is frequently correlated with obvious biogeographic barriers (Burton and Lee 1994; Schizas et al. 1999; but see Burton 1998). In sympatry, divergent lineages have been reported only from the harpacticoid copepod *Cletocamptus deitersi*, but the lineages most likely represent cryptic species (Rocha-Olivares et al. 2001).

One of the most studied harpacticoid species, *Microarthridion littorale*, is distributed worldwide and is the most abundant, sediment-dwelling copepod in estuaries along the southeastern US coast (Fleeger 1979; Coull and Dudley 1985). *M. littorale* enhances the biomineralization of detritus (Morris and Coull 1992), is considered an essential food for demersal juvenile fish, and is sensitive to estuarine pollutants (Chandler et al. 1997; Kovatch et al. 2000). *M. littorale* has a relatively short life cycle, i.e. egg to egg in 30 days at 25°C (Palmer and Coull 1980). Although it is restricted to benthic habitats within estuaries, *M. littorale* can be passively transported for short distances (10s to 100s of meters) in the water column (Palmer and Gust 1985). The dispersal capacity of *M. littorale* beyond individual creeks is unknown.

A previous molecular study, based on a fragment of the cytochrome apoenzyme *b* (cytochrome *b*) and ITS-1 sequences, revealed an unexpectedly high degree of differentiation within *M. littorale* collected along the southeast USA and Gulf of Mexico (Schizas et al. 1999). Copepods collected from estuaries of South Carolina and Georgia, east and west Florida, and Louisiana comprised distinct populations and were phylogenetically distributed in three clades. All clades were reciprocally monophyletic. Copepods from Louisiana were found to be as genetically distinct from the South Carolina and Georgia copepods as those were from the designated outgroup *M. fallax*, which was collected from Plymouth, England (Schizas et al. 1999). We were interested in estimating patterns of gene flow among populations on a smaller geographic scale (by order of magnitude) than the previous regional study (Schizas et al. 1999). We sequenced a fragment of the cytochrome *b* gene of the mitochondrial genome (mtDNA) from populations within the geographic region encompassing the South Carolina clade (sensu Schizas et al. 1999). We hypothesized that short-distance dispersal through suspension and advection by tidal currents (Palmer and Gust 1985), and consequent gene flow would lead to populations with minimal or no genetic differentiation. Contrary to our expectations, we identified three distinct mtDNA lineages occurring in sympatry at most sampling locations. We tested the isolation-by-distance model (Slatkin 1993a) by correlating pairwise genetic distances to geographic distances among populations. Our sampling regime included both contaminated and clean creeks. To further understand the role of environmental variables in the maintenance of haplotype diversity within populations, we attempted to interpret the frequency distribution of the three mtDNA lineages in terms of a crude assessment of contaminant history at each location.

Materials and methods

Sample collection

Microarthridion littorale (Poppe) were collected from ten estuarine creeks along the coast of North Carolina, South Carolina, and Georgia, USA (Fig. 1; Table 1), from the upper 1 cm of sediment over an area of approximately 5 m^2 from January 1997 to September 1998. *M. littorale* from the east and west coasts of Florida were used as outgroups (Schizas et al. 1999). Fiber optic lights on darkened aquaria were used to collect individuals (Couch 1989).

PCR amplification and sequencing of cytochrome *b*

DNA from individual copepods was extracted as previously described (Schizas et al. 1997). A small aliquot of extracted nucleic acids (2 µl) was used as template for polymerase chain reaction amplification (PCR, Saiki et al. 1988). Initial conditions for the PCR amplification were: 50 mM KCl, 10 mM Tris-HCl, 2.5 mM $MgCl_2$, pH 8.3 (Perkin-Elmer Cetus), 200 µM dNTP (Promega), 5 pmol forward and reverse primer, and 1 U *Taq* DNA polymerase. Template DNA was initially denatured at 94°C for 3 min, followed by 35 cycles at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min on an MJ thermocycler. For PCR amplification of cytochrome *b*, we used the forward 151F (5′-TGTGGRGCNACYGTWATYACTAA-3′) and reverse 270R (5′-AANAGGAARTAYCAYTCNGGYTG-3′) primers of Merritt et al. (1998). Five microliters were removed from the 50 µl PCR amplification and loaded on a 1% gel, stained with ethidium bromide, and observed under UV light. The remaining PCR solution (45 µl) was purified by the polyethylene glycol precipitation procedure (Kusukawa et al. 1990). We used the following conditions for cycle sequencing amplifications: 94°C for 45 s, 58°C for 45 s, and 72°C for 90 s for 25 cycles. Electrophoresis was carried out on an ABI 377 sequencing system.

PCR-RFLP of cytochrome *b*

For the enzymatic digestion of the cytochrome *b* amplicon, 13 µl of a 50 µl PCR amplification were digested using 2 U of the restriction enzyme *Mbo*I in 1.6 µl of the buffer C 10× (Promega) at 37°C overnight. The endonuclease *Mbo*I specifically recognizes and cuts at the 5′-GATC-3′ sequence. The resulting DNA fragments were separated in 2% agarose gels, stained with ethidium bromide, visualized and photographed under UV illumination. DNA frag-

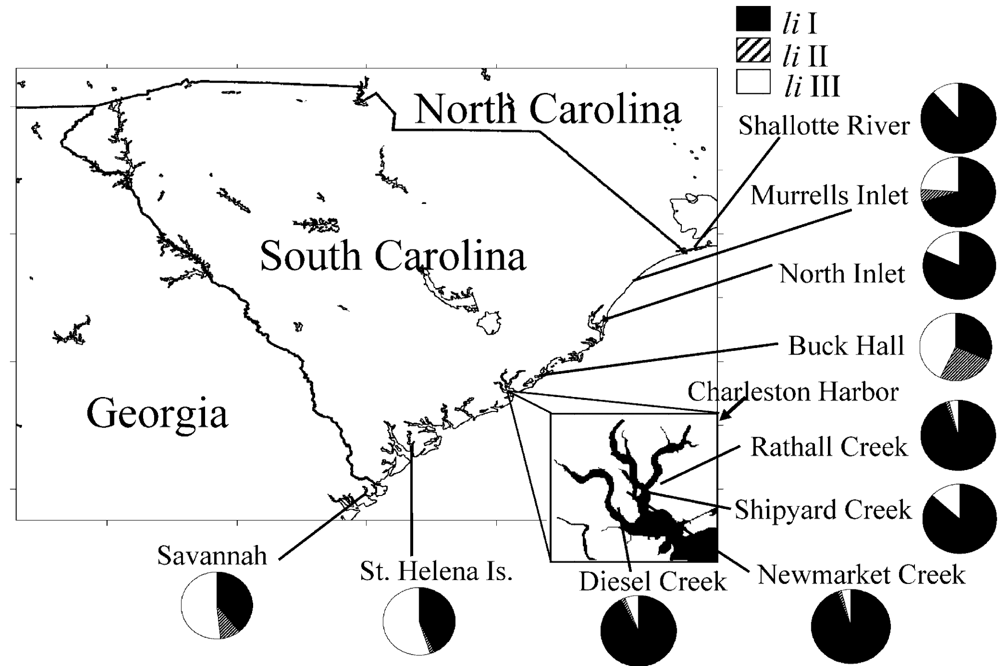

Fig. 1 *Microarthridion littorale*. Map of sample locations in North Carolina, South Carolina, and Georgia and relative frequencies of three mitochondrial clades (*li*I, *li*II, *li*III) in the ten samples

ments were electrophoresed against a 100 bp DNA step ladder (Promega) for comparison.

Statistical analysis

Cytochrome *b* sequences were aligned manually in a text editor. Identical haplotypes were filtered and then merged using MacClade (v.3.1; Maddison and Maddison 1992). We constructed a haplotype network based on the resulting number of unique haplotypes using NTSYSpc v.2.02h (Rohlf 1998). For the haplotype network, a distance matrix of the among-haplotype number of base substitutions was first generated in MEGA v.2.1 (Kumar et al. 2001) and then imported in NTSYSpc. Phylogenetic relationships among the cytochrome *b* sequences were assessed by the neighbor-joining method implemented in PAUP* 4.0b7 (Swofford 1999). Three sequences, representing *M. littorale* from Florida, were used as outgroups. Clade support (Efron 1979; Felsenstein 1985) was estimated with 1,000 bootstrap pseudosamples. Standard measurements of DNA polymorphism in populations, such as nucleotide diversity (π) (Nei 1987), gene diversity (h) (Tajima 1983; Nei 1987), and Tajima's D (Tajima 1989), were calculated using subroutines of the software Arlequin v.1.1 (Schneider et al. 1996). π is the number of nucleotide differences per site between two randomly chosen sequences, and h is the probability that two sequences randomly chosen from the population are different. Tajima's D is a DNA neutrality test that takes into consideration a measurement of the difference between the number of segregating sites and the average number of nucleotide differences among a sample of DNA sequences (Tajima 1989).

The partition of molecular variance was calculated with the analysis of molecular variance (AMOVA) program (Excoffier et al. 1992) as implemented in Arlequin. The AMOVA procedure calculates variance components and Φ-statistics (analogues to Wright's F-statistics, Wright 1951). The variance components and Φ-statistics were partitioned among and within sampling locations. The significance of the variance components was tested by comparing observed values with values generated by non-parametric methods (10,000 random permutations), since molecular data are not normally distributed (Excoffier et al. 1992). Φ-statistics were calculated separately for the 198 sequenced individuals and then for all the processed copepods (sequenced specimens and PCR-RFLP specimens) assigned to the three mitochondrial clades.

To test the isolation-by-distance model (Slatkin 1993a), we examined the relationship between F_{ST} and geographic distance among populations (Appendix 1, electronic supplementary material). The significance of relationships between Slatkin's linearized F_{ST} (Slatkin 1993a) and geographic distance was assessed with the Mantel test of matrix association (Mantel 1967; Smouse et al. 1986). Geographic distances were measured as the shortest, continuous water-surface distance between sampling locations. Matrix comparisons were performed using the matrix correlation subroutine in NTSYSpc (Rohlf 1998). The confidence of the resulting t-statistic was assessed with 5,000 random permutations.

Table 1 *Microarthridion littorale*. Collection locations. ΣERM is the sum of ratios of toxicant concentrations and their associated effect range-medians (*ERM*); ERM is the toxicant concentration at which 50% of toxicity studies show biological effect; ΣERM > 1 is considered contaminated. Sediment chemistry data compiled from several studies (toxicants and their concentrations for each location available from the corresponding author)

Collection location	Latitude	Longitude	ΣERM
Shallotte R., N.C.	33°55′11″N	78°22′31″W	0.1542
Murrells Inlet, S.C.	33°33′19″N	79°1′28″W	1.3847
North Inlet, S.C.	33°17′55″N	79°13′14″W	0.1672
Buck Hall, S.C.	33°1′15″N	79°34′56″W	0.0177
Rathall Ck., S.C.	32°51′22″N	79°52′48″W	1.0277
Shipyard Ck., S.C.	32°50′23″N	79°56′46″W	13.2645
Diesel Ck., S.C.	32°48′56″N	79°57′45″W	5.6947
Newmarket Ck., S.C.	32°48′25″N	79°56′4″W	4.5771
St. Helena Is., S.C.	32°18′24″N	80°32′54″W	0.1815
Savannah, Ga.	31°59′41″N	80°55′38″W	0.4574

Contamination history of the ten locations

Sediment chemistry data from the sampling locations were available from the Tidal Creek Project (Hyland et al. 1996, 1998; Sanger et al. 1999a,b). Chemical contaminant data included levels of pesticides, trace metals, and polycyclic aromatic hydrocarbons (PAHs). A useful way to summarize toxicity data in a biologically relevant context is the effect range-median (ERM), which is defined

as the toxicant concentration at which 50% of the toxicity studies testing or measuring this toxicant have shown a biological effect (Long et al. 1995, 1998). To obtain the ΣERM value for each creek, the ratio of each in situ toxicant concentration and its associated ERM value were calculated and subsequently all the ratios were summed (Table 1). For a ΣERM > 1, the creek was considered "contaminated"; otherwise, the creek was considered "clean". It was our intent to use all available data on chemical contaminants. However, since the chemical contaminant data were pooled from independent surveys, listings of measured chemicals from each location varied. The ΣERM method of summarizing all available chemical contaminant data from each location is an attempt to minimize subjectivity in classifying locations as "contaminated" or "clean" but the method is relatively crude.

Results

We sequenced 348 bp of the cytochrome *b* gene from 198 individuals of the copepod *Microarthridion littorale*. Eight of the 198 sequences were previously published (Schizas et al. 1999) and are included for comparison (Appendix 2, electronic supplementary material). Fifty-four unique haplotypes were recorded (Fig. 2). In addition, 57 variable nucleotide sites (16.4%) and 23 parsimony informative sites were found within the 348 bp fragment of the gene. Forty-six parsimony informative sites were observed when three sequences of *M. littorale* from Florida were included. Pairwise DNA differences between haplotypes ranged from 0.3% (a single substitution) to 4.3% (15 base substitutions), the majority being third base transitions. Haplotypes M32, M33, and M34 shared a first-base transition (i.e. A to G), resulting in the only consistent amino acid difference (Val to Ile) shared by more than one individual (Appendix 2, electronic supplementary material). The geographic distribution of the 54 haplotypes is shown in Table 2. The most common haplotype (M1, $n = 86$) was present at all locations (Table 2). The second most-frequently observed haplotype (M39, $n = 15$) was absent (except for one specimen) from the four sampling locations in Charleston Harbor, North Inlet, and Shallotte River. Haplotypes M1 and M39 differed by nine base substitutions (Appendix 2, electronic supplementary ma-

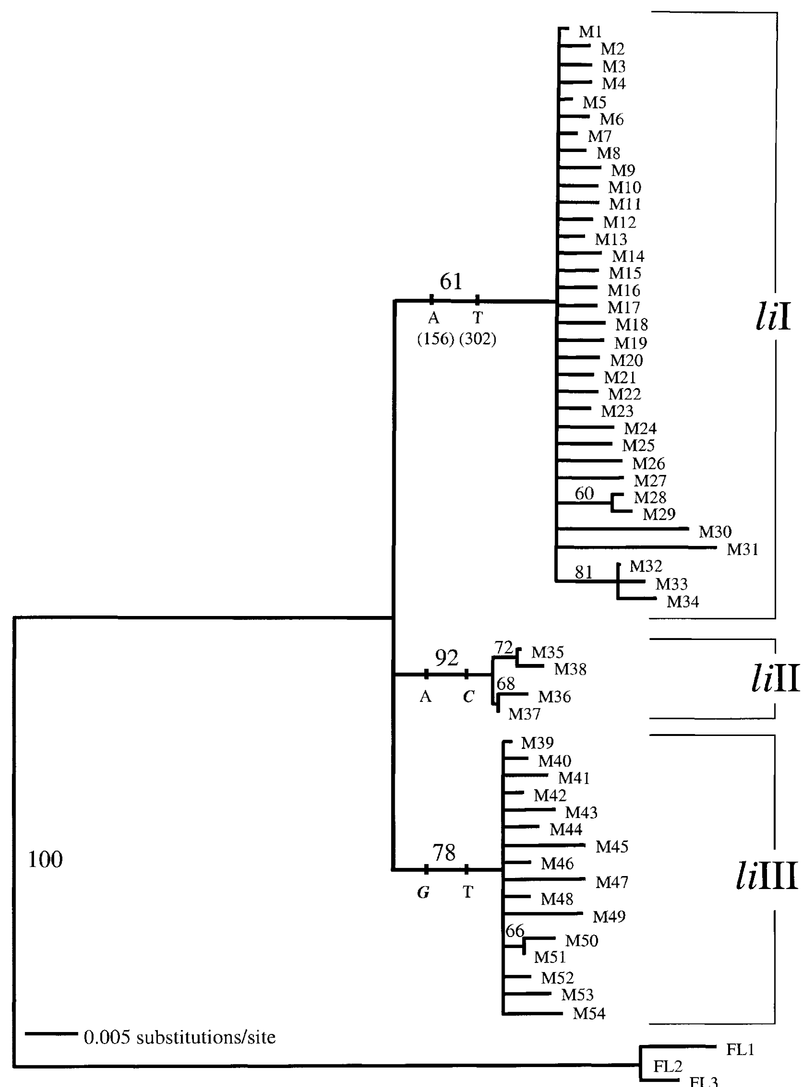

Fig. 2 *Microarthridion littorale*. Neighbor-joining phylogram showing relationships among haplotypes. Sequences from Florida (*FL*) were used to root the tree. Distances between haplotypes calculated with the HKY (Hasegawa et al. 1985) model of nucleotide substitution. Branch lengths represent estimated number of substitutions per site. Numbers above branches indicate bootstrap support obtained by 1,000 pseudoreplicates. Only bootstrap values > 60 are included. Starting trees were obtained via stepwise addition with random addition sequence; bootstrap estimates were calculated with the fast-heuristic search option in PAUP* 4.0b7. Diagnostic nucleotides used to distinguish the *li*I, *li*II, and *li*III clades with *Mbo*I presented on each branch with small perpendicular hatches. Position of nucleotides relative to submitted sequences in *parentheses*. All sequences deposited in GenBank (AF296559–AF296615)

Table 2 *Microarthridion littorale*. Distribution of 54 haplotypes among sampling locations. *Numbers* represent number of individuals sharing each haplotype

Location	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	M15	M16	M17	M18	M19	M20	M21	M22	M23	M24	M25	M26	M27
Shallotte R.	12	0	1	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Murrells Inlet	7	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	1	0	1	3	0	0	1	0
North Inlet	8	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Buck Hall	4	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Rathall Ck.	17	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Shipyard Ck.	7	0	0	0	2	0	0	2	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	1	0	0	0
Diesel Ck.	16	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0	0
Newmarket Ck.	4	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0
St. Helena Is.	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	1	0	0
Savannah	3	0	0	0	2	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	86	1	1	1	5	2	1	2	1	1	2	1	1	3	1	1	1	1	1	1	1	2	5	1	1	1	1

Location	M28	M29	M30	M31	M32	M33	M34	M35	M36	M37	M38	M39	M40	M53	M42	M43	M44	M45	M46	M47	M49	M50	M51	M52	M53	M54	Total
Shallotte R.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	17
Murrells Inlet	0	0	0	0	0	3	0	0	0	0	0	2	0	0	0	0	0	0	0	1	0	0	0	2	0	0	23
North Inlet	0	0	1	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	1	0	0	0	0	0	0	17
Buck Hall	0	0	0	0	0	0	0	2	2	1	1	8	0	0	0	0	0	0	0	0	0	0	0	2	0	0	21
Rathall Ck.	1	1	0	2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	26
Shipyard Ck.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	14
Diesel Ck.	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	23
Newmarket Ck.	0	0	0	0	2	2	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	2	16
St. Helena Is.	0	0	0	0	0	0	0	0	0	0	0	2	4	1	0	2	0	0	0	0	1	0	1	0	0	0	22
Savannah	0	0	0	0	0	0	1	0	0	0	0	2	1	0	0	2	5	1	1	0	0	0	0	0	0	0	19
Total	1	1	1	2	2	7	1	3	2	1	1	15	9	1	1	4	5	1	1	2	1	1	1	4	1	2	198

terial). Of the 54 haplotypes identified, 34 were represented by only one individual (singletons).

Evaluation of the phylogenetic relationships of the 54 haplotypes and the three outgroup sequences with the neighbor-joining method supported three clades: *li*I, *li*II, and *li*III (*li* derives from *littorale*) (Fig. 2). The three clades are also outlined in the haplotype network (Appendix 3, electronic supplementary material), which visualizes the sequence divergence within and among the three mitochondrial clades, but does not depict accurate phylogenetic relationships among the 54 haplotypes. The monophyly of the *li*I, *li*II, and *li*III clades was supported by 61%, 92%, and 78% bootstrap values, respectively (Fig. 2). We regard the bootstrap support of clade *li*I as weak. In population level studies, high bootstrap support is not readily attained when there is a large amount of polymorphism within populations and limited divergence among populations. We applied the ParsProb method to the cytochrome *b* data (Templeton et al. 1992) as implemented in PARSPROB v.1.1 (Posada 1998). This method identifies closely related sequences that can be grouped together with >95% confidence. For a 348 bp DNA segment, such as the cytochrome *b* data set, there is a 0.97 probability of a parsimonious linkage between two haplotypes differing at six nucleotide sites and a 0.96 probability of a parsimonious linkage between two haplotypes differing at seven nucleotide sites. For more than seven sites the connections are non-parsimonious at the 0.95 level. The PARSPROB results indicate that the haplotypes making up each of the three mitochondrial clades can be grouped together with >95% confidence, at least from the parsimony point of view. The most common haplotype of *li*I differed by nine nucleotide substitutions (2.6%) from the most common haplotype of *li*II and *li*III, whereas those of *li*II and *li*III differed by six substitutions (1.7%). We do not know whether the three clades are biologically meaningful (i.e. represent lineages with distinct evolutionary trajectories).

The largest number of haplotypes was found in the Newmarket Creek sample, while the highest nucleotide diversity was observed in the St. Helena Is. sample (Table 3). Shallotte River, Shipyard Creek, Diesel Creek, and Rathall Creek samples showed the greatest reduction in nucleotide diversities. With the exception of the Shallotte River, the other three creeks are considered contaminated. Clade *li*III exhibited significantly higher gene diversity than clade *li*I (data not shown). Tajima's *D*-values in all contaminated creeks, except Newmarket Creek, were negative (Table 3). Two out of the four contaminated locations and the Shallotte River displayed significantly negative Tajima's *D*-values.

Restriction digestion of the amplicon with the endonuclease *Mbo*I resulted in three unambiguous banding patterns diagnostic of the three clades. Digested DNA samples of clade *li*I resulted in three DNA fragments (155, 145, and 50 bp), digested DNA samples of clade *li*II resulted in two fragments (195 and 155 bp), and digestion of samples of clade *li*III produced two

Table 3 *Microarthridion littorale*. Gene diversity, h (Nei 1987), nucleotide diversity, π (Tajima 1983; Nei 1987), and Tajima's D (Tajima 1989) estimates for each sampling location based on sequenced specimens. Data represent means ± SD (*$P<0.05$)

Location	h	π	D
Shallotte R.	0.515 ± 0.145	0.005 ± 0.003	–2.201*
Murrells Inlet	0.885 ± 0.048	0.015 ± 0.008	–0.035
North Inlet	0.772 ± 0.097	0.016 ± 0.009	–0.022
Buck Hall	0.824 ± 0.065	0.017 ± 0.010	0.960
Rathall Ck.	0.579 ± 0.114	0.007 ± 0.004	–2.098*
Shipyard Ck.	0.747 ± 0.111	0.003 ± 0.002	–1.499
Diesel Ck.	0.526 ± 0.126	0.005 ± 0.003	–2.281*
Newmarket Ck.	0.925 ± 0.047	0.018 ± 0.010	0.314
St. Helena Is.	0.840 ± 0.061	0.019 ± 0.011	1.462
Savannah	0.906 ± 0.045	0.017 ± 0.010	0.371
All locations	0.790 ± 0.031	0.016 ± 0.009	

fragments (300 and 50 bp). Results from the PCR-RFLP assay are presented in Table 4. Haplotypes of clade *li*I were the predominant haplotypes at seven of ten sampling locations. Haplotypes of clade *li*III were the predominant haplotypes in St. Helena Is. and Savannah samples. Total frequencies of haplotypes of clades *li*I and *li*III were approximately the same as those derived from the sequence data, and the total frequency of clade *li*II was twice as much compared to the sequence data.

Different sampling locations shared haplotypes, but the presumed migration rates between these locations were not sufficiently large to mask population structure in *M. littorale* (Table 5). When all sequences were combined, AMOVA analysis indicated that there was significant population subdivision ($P<0.01$), and 22% of the molecular variance was allocated among sampling locations (Table 5). Further analysis of the sequences allocated to their respective clades revealed that in clade *li*III, the significant population structure was attributed to among-sampling-location variance (34.72%, Table 5). Haplotypes of clade *li*I were also significantly structured ($P<0.01$), even though the among-sampling-location variation contributed only 6% to the total genetic variance.

Table 4 *Microarthridion littorale*. Distribution of three mitochondrial clades (*li*I, *li*II, *li*III) at ten sampling locations. Mitochondrial clade assignment based on RFLP assay

Location	*li*I	*li*II	*li*III	Total
Shallotte R.	30	0	5	35
Murrells Inlet	19	3	7	29
North Inlet	32	0	5	37
Buck Hall	12	6	12	30
Rathall Ck.	24	4	1	29
Shipyard Ck.	31	0	5	36
Diesel Ck.	27	2	0	29
Newmarket Ck.	35	1	4	40
St. Helena Is.	11	1	17	29
Savannah	15	5	19	39
Total	236	22	75	333

Table 5 *Microarthridion littorale*. Hierarchical analysis of variance across ten populations. Φ-statistics provided separately for DNA sequence data and for frequencies of three clades (including 198 DNA sequences). Data analyzed as combined or partitioned into three clades (*li*I, *li*II, *li*III). For each analysis, the number of specimens (n), the percentage variance explained (%), probability of more extreme values from 10,000 permutation tests (P), and Φ-statistics for among-location variation are presented (*$P<0.05$)

Variance component	n	%	P	Φ
DNA sequence data				
All groups	198			
Among locations		21.99	*	0.220
Within locations		78.01	–	–
*li*I	141			
Among locations		5.95	*	0.060
Within locations		94.05	–	–
*li*II[a]	7			
Among locations				
Within locations				
*li*III	50			
Among locations		34.72	*	0.347
Within locations		65.28	–	–
Haplotype frequency data				
All groups	531			
Among locations		20.33	*	0.203
Within locations		79.67	–	–

[a]No AMOVA analysis for individuals in *li*II because number was too low

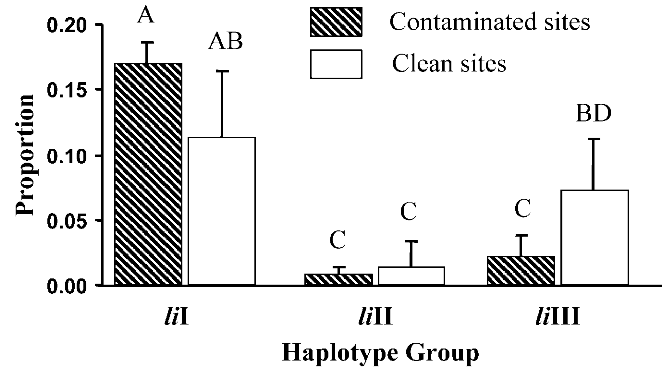

Fig. 3 *Microarthridion littorale*. Comparison of total frequencies of three mitochondrial clades (*li*I, *li*II, *li*III) collected from five contaminated and five clean sampling locations. *Bars with the same letters* are not statistically different from each other. *Error bars*: one standard deviation from the mean

Genetic distance was not correlated with geographic distance (Mantel test, $r=0.0023$, $t=0.011$, $P=0.5$). Therefore, haplotypes of clades *li*I, *li*II, and *li*III were distributed among the sampling locations in a random fashion and did not conform to the "isolation-by-distance" model, which would predict that genetic distance between populations increases as geographic distance increases (Slatkin 1993a).

When all processed specimens were pooled according to the condition status of the sampling location (five clean vs. five contaminated creeks), we observed differences in the frequency distribution of the three clades (Fig. 3), but they were not significant at $\alpha=0.5$

($P = 0.087$). Haplotypes of clade *li*III were more likely to be found in clean locations than in contaminated locations (Fig. 3). Haplotype frequencies of clade *li*II in clean and contaminated locations were not statistically different (Student's *t*-test), although insufficient numbers of *li*II haplotypes were collected to make meaningful comparisons. Overall, the contamination condition of the sampling location was not statistically significant (at the $\alpha = 0.05$ level) in determining the frequency of the three haplotype groups in each creek and in all clean versus all contaminated creeks.

Discussion

The most intriguing observations in this study are the elevated levels of differentiation among the clades *li*I, *li*II, and *li*III and the existence of the three clades or mitochondrial lineages in sympatry. While divergence levels between samples from South Carolina and Florida were high (10%, 10%, and 9% between *li*I, *li*II, and *li*III vs. Florida copepods, respectively; in the present study and Schizas et al. 1999), the divergence observed among sympatric haplotypes in South Carolina was even more unexpected. High divergence rates have been reported in populations of the well-studied harpacticoid copepod *Tigriopus californicus* across Point Conception (Burton and Lee 1994) and across inconspicuous barriers on the Californian coast (Burton 1998). Population divergence ranged from 15% to 22% in cytochrome oxidase subunit I (COX-1) from *T. californicus* collected in the Southern California Bight. Rocha-Olivares et al. (2001) examined multi-allelic molecular and morphological data in the harpacticoid copepod *Cletocamptus deitersi* and observed an even higher degree of divergence (25% and 36% in COX-1 and 16S rDNA, respectively). Three mitochondrial types (I, II, III) were discovered from copepod samples collected from Alabama, Louisiana, California, and the Pacific coast of Mexico. Types I and II were sympatric in Alabama and Louisiana and were distinguished both morphologically and genetically. The three mitochondrial types of *C. deitersi* reported by Rocha-Olivares et al. (2001) and the three mitochondrial lineages of *Microarthridion littorale* reported in this study probably represent opposite ends of a spectrum in the speciation process. Most likely, the three mitochondrial types of *C. deitersi* represent different cryptic species (Rocha-Olivares et al. 2001), whereas the mitochondrial lineages of *M. littorale* may be the initial phases of species differentiation.

The three mtDNA lineages of *M. littorale* may represent ancient lineages that once maintained large population sizes. In large populations, such as those of *M. littorale*, extinction of major lineages is prevented and gradual divergence among the major lineages may occur. The levels of intraspecific diversity in *M. littorale* could also be explained by unusually fast rates of mtDNA evolution. So far, molecular studies of three harpacticoid species, *T. californicus*, *C. deitersi*, and *M. littorale*, have discovered a surprising level of genetic diversity (Burton and Lee 1994; Schizas et al. 1999; Rocha-Olivares et al. 2001). As more harpacticoid copepod species are used for population genetics and phylogenetic studies, it remains to be seen if high levels of intra- and interspecific divergence are widespread phenomena among members of the order Harpacticoida and beyond. Within the order Calanoida, molecular data and reproductive isolation experiments support the hypothesis that the cosmopolitan calanoid copepod *Eurytemora affinisis* represents an assemblage of cryptic species (Lee 2000).

The observed genetic divergence among the three mitochondrial clades might be attributed to the presence of cryptic sibling species. The presence of cryptic species in the marine environment is not unusual (Knowlton 1993), and re-examination of what were previously regarded as widely distributed single species has resulted in the discovery of presumably cryptic species (Knowlton 1993; Garcia-Rodriguez et al. 1998; Lee 2000; Colborn et al. 2001). In the superficially homogenous realms of benthic habitats, sibling species have been encountered in both the deep sea (Hessler and Sanders 1967; Chase et al. 1998) and shallow water habitats (Grassle and Grassle 1976; Hoare et al. 2001; Rocha-Olivares et al. 2001). We did not expect to identify three distinct mitochondrial clades from our sampling locations; therefore, this study was not designed to decipher the systematic identity of the three clades. The extent to which clades *li*I, *li*II, and *li*III represent different biological species could probably be answered by classical genetic approaches (i.e. inter-clade breeding experiments) and use of molecular markers from independently evolved loci, e.g., nuclear genome. If the three clades were reciprocally monophyletic, the assertion that each clade represents a species would be strengthened. Analysis of sequences of *M. littorale* ITS-1 (Schizas et al. 1999) suggests that there is no differentiation among the three mitochondrial lineages in that portion of the *M. littorale* nuclear genome. The discordance between the cytochrome *b* and the ITS-1 data could be explained by the ITS-1 gene not being variable enough to resolve intraspecific questions, at least for *M. littorale*. Rather, ITS-1 is a suitable marker for deciphering phylogenetic relationships at the species and higher levels (Schizas et al. 1999). However, we do not discount the possibility that significantly different evolutionary forces are influencing the evolution of *M. littorale* mtDNA and nDNA.

Whatever the taxonomic status of the three mitochondrial lineages, the observed genetic differentiation is either due to secondary contact of once isolated populations or to divergence in sympatry. *M. littorale* typically inhabits estuarine mudflats, which are separated by long stretches of sandy beaches in the southeast USA. Since representatives of clade *li*II were rarely found in the four sampling locations in Charleston Harbor, it is possible that coastal currents have hindered their

dispersal. Southward coastal currents have most likely shaped the population structure observed in several macrofaunal species with planktonic larvae across Point Conception, California (Wares et al. 2001). Secondary contacts, resulting from changes of oceanographic features, or changes in the South Carolina coastline, could potentially explain the present distribution of the three lineages.

Alternatively, divergence of the three mitochondrial lineages might have occurred in sympatry. Meiobenthic copepods are approximately 0.5 mm in length, and different species can be vertically segregated in both sandy (Fleeger and Gee 1986; Foy and Thistle 1991) and muddy habitats (Coull et al. 1989; Fleeger et al. 1995). Copepods that occupy different depths in the sediment or are at different intertidal heights might encounter gradients of food quality and quantity, oxygen, predation, and currents (Palmer 1980; Fleeger et al. 1995). Therefore, the number of possible microhabitats and niche specializations within the sediment for *M. littorale* is large. Currently, it is not possible to evaluate whether mitochondrial differentiation has occurred in historically separated populations with subsequent secondary contact or in sympatry.

The observed population structure of *M. littorale* does not fit the isolation-by-distance model. Matrix correlations indicated no relationship between pairwise estimates of genetic and geographic distance. Interestingly, copepods from Buck Hall were somewhat isolated genetically from geographically adjacent sampling locations, and their inclusion in the analysis disproportionally disrupts any relationship between geographic distance and estimates of gene flow. Copepods from Buck Hall were more closely related to isolates of St. Helena Is. and Savannah than to those from the geographically adjacent North Inlet and the creeks in Charleston Harbor (Appendix 1, electronic supplementary material). This was unexpected since Buck Hall is in the southeastern US intercoastal waterway, where boat traffic could increase the potential for dispersal of copepods north and south from this location. We know little about dispersal in estuarine benthic copepods, since studies provide estimates of dispersal rates within individual creeks (Bell and Sherman 1980; Palmer and Gust 1985), but not for greater distances (e.g. ocean waters).

Failure to detect any association between F_{ST} and geographic distance could also be a reflection of historical relationships among populations of *M. littorale*. Genetic drift and migration may not be in equilibrium for recently isolated populations or for populations recently established (Slatkin 1993b, 1994). Clade *li*I harbors many more haplotypes with "new" mutations (i.e. haplotypes that differ by one mutation from the most frequently encountered haplotype) than clade *li*III. Relationships among haplotypes of clade *li*I could be characterized as those of a star phylogeny, indicative of an expanding population, a selective sweep or background selection. Haplotypes of clade *li*III display a larger degree of genetic differentiation, indicative of a more ancient lineage.

Our motivation for including the contamination condition of each sampling location originated from previous observations on benthic copepods, where the presence of sediment-bound contaminants was linked to decreased genetic diversity in six species sampled at varying distances from offshore oil platforms. Population bottlenecks caused by the introduction of contaminants from the construction and operation of the oil platforms was one of the proposed explanations for the reduced genetic variability near these facilities (Street and Montagna 1996). In laboratory experiments, the harpacticoid copepod *Nitocra lacustris* exposed to sublethal levels of PAHs experienced a severe population bottleneck during the first generation, but remained stable over the next three generations (Street et al. 1998).

Population bottlenecks are usually observed in species with small population sizes (i.e. small N_e). Empirical studies of natural population bottlenecks suggest that even after a severe bottleneck (95% mortality) all measures of genetic diversity regained pre-bottleneck levels within 2–3 years of the crash (Keller et al. 2001). This rapid recovery was due to immigration, even though the observed immigration rates were low (Keller et al. 2001). Small-scale meiofauna recolonization experiments indicate that recolonization can be detected as early as 12 h after an initial sediment disturbance (Sherman and Coull 1980; Chandler and Fleeger 1983). *M. littorale* is one of the most abundant benthic invertebrates in southeastern US estuaries (thousands of specimens can be collected from a 5 m^2 area throughout the year at all sampling locations; Schizas, personal observations). Impacted sediments can potentially be re-populated to their previous levels through immigration of copepods from adjacent unimpacted sediments. It is unlikely that *M. littorale* has experienced population bottlenecks caused by exposure to toxicants, as suggested by the observed patterns of haplotype and genetic diversity, unless a particular genotype(s) is more fit than others among exposed individuals in the impacted sediments.

Some sampling locations yielded low levels of haplotype diversity and small values of π, irrespective of their classification as clean or contaminated. We observed negative Tajima's D-values for samples from clean and contaminated creeks, where most sampled haplotypes belonged to only one clade, suggesting recent directional selection, background selection, or population expansion. Both selective forces and demography of the species (e.g. population growth) are known to produce nucleotide patterns inconsistent with DNA neutrality, but indistinguishable from each other. Therefore, we cannot differentiate the demographic effects from any possible selective forces acting on the mitochondrial genome of *M. littorale*. Nevertheless, our rough classification of sampling locations as contaminated or clean suggests a possible weak relationship between contamination load and unequal lineage frequencies in each

location (Fig. 3). Copepods of clade *li*I were abundant throughout the sampled region, whereas copepods of clades *li*II and *li*III were more likely to be found in clean locations. Copepods may also respond to other environmental variables that we have not considered (e.g. sediment grain size, salinity, organic C content, N content) that happen to co-vary with the toxicant concentrations. In future studies we will examine the susceptibility of the three mtDNA lineages to specific contaminants found in the contaminated creeks (see Schizas et al. 2001).

It is clear that the observed genetic patterns in the mitochondrial genome of the copepod *M. littorale* can be explained in many ways which are not necessarily mutually exclusive. Genetic divergence in *M. littorale* is probably caused by interaction among evolutionary forces, ecological characteristics of the estuarine habitats, and physical forces acting on the coastal zone. If selection pressures differ from creek to creek, genetic differentiation may increase among populations of *M. littorale*. In contrast, migration of *M. littorale* from creek to creek mediated by coastal currents, marine birds, and boats may homogenize or limit population differentiation resulting from selection or other stochastic processes.

Acknowledgements The following individuals contributed in the collection of copepods: G. Street, J. Staton, M. Gordos, L. Snow, C. Kovatch, and S. Klosterhaus. G. Street and J. Staton contributed to this project further by providing valuable comments and ideas. J. Staton also assisted in drawing Fig. 1. We are grateful to G. Scott (NOAA-NMFS, Charleston, SC) for providing the sediment chemistry from Buck Hall. This project was supported by grants from the EPA (assistance ID no. R-825439 to B.C.C., G.T.C., and J.M.Q.), the SC SeaGrant consortium award R/ER-15 to G.T.C. and B.C.C., the NSF (OCE-9814172 to J.M.Q.), and the Slocum-Lunz Foundation (to N.V.S.). N.V.S. was the recipient of a SETAC and Taylor & Francis advanced training fellowship and a Lerner Gray Fellowship for marine studies during the initial phases of the research. A portion of this manuscript was written while N.V.S. was financially supported by the DOE/SLOAN postdoctoral fellowship for Computational Molecular Biology. Although the research described in this article has been funded wholly or in part by the United States Environmental Protection Agency through EPA assistance ID no. R-825439 to B.C.C., G.T.C., and J.M.Q., it has not been subjected to the Agency's required peer and policy review and therefore does not necessarily reflect the views of the Agency, and no official endorsement should be inferred. This is contribution no. 1294 from the Belle W. Baruch Institute for Marine Biology and Coastal Research, University of South Carolina.

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