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Molecular phylogeography and evolutionary history of the estuarine copepod, *Acartia tonsa*, on the Northwest Atlantic coast

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Abstract

Coastal estuaries are useful model systems to study the ecological and evolutionary responses of organisms to highly variable, discontinuous habitats. For this study, the molecular population genetic diversity of the planktonic calanoid copepod *Acartia tonsa* (Dana, 1849) was described based on DNA sequence variation for a 183 base-pair region of the mitochondrial 16S rRNA gene. Samples of *A. tonsa* were collected from four estuaries on the Atlantic coast of the USA during 1993 and 1994, one estuary on the Gulf of Mexico coast in 1994, and one site on the Pacific coast of the USA in 1994. Dispersal of *A. tonsa* was shown to be restricted, with significant population genetic structuring between different estuaries. For all but the closely-adjacent MA and RI samples, frequencies of haplotypes and/or length polymorphisms within one haplotype (caused by insertion/deletion mutations) revealed highly significant genetic differentiation and geographic isolation. Mt16S haplotypes of *A. tonsa* from Atlantic and Gulf of Mexico estuaries were assorted among four deeply-diverged clades. Haplotypes within each clade differed by <2%, while differences among clades of 10% to 14% approached those between described *Acartia* species (e.g., 19% to 28% among *A. clausi*, *A. hudsonica*, and *A. longiremis*). Atlantic and Pacific coast samples identified as *A. tonsa* had no haplotypes in common and genetic differences between haplotypes ranged from 18% to 29%; phylogenetic analysis supported the separation of Pacific coast *A. tonsa* as a distinct species. We hypothesize that the observed patterns of molecular genetic diversity and structure of *A. tonsa* resulted from responses to historical climatic variation, including episodic range compression and displacement, and alteration of NW Atlantic coastal and estuarine environments.

Introduction

The spatially discontinuous nature of estuarine habitats suggests that obligately-estuarine species may be composed of geographically distinct, genetically differentiated populations. However, there should be ample opportunity for planktonic dispersal among estuaries, particularly during periods of high freshwater input when organisms may be flushed out of estuaries (Miller, 1983; Boicourt et al., 1987; Gaines & Bertness, 1992). The fate of estuarine holoplankton outside estuaries is unclear and is likely influenced by species-specific responses to changes in temperature, salinity, food environment, physiological condition, and other factors (Paffenhöfer & Stearns, 1988; Gaines & Bertness, 1992). For many planktonic species, at least short-term survival (days) outside of estuaries is possible and patterns of advective transport in coastal waters suggest the potential for dispersal among estuaries.

The highly-abundant, widely-distributed, and characteristically estuarine holoplanktonic calanoid copepod, *Acartia tonsa* (Dana, 1849), is an excellent study system for population genetic and phylogeographic investigations to examine present-day dispersal among estuaries and responses to historical climatic variation.

The genus dominates coastal estuaries world-wide (e.g., McKinnon et al., 1992) and *A. tonsa* has been a frequent subject of ecological and oceanographic studies (Uye & Fleminger, 1976; Durbin et al., 1983; Paffenhöfer & Stearns, 1988; Roman et al., 1988; White & Dagg, 1989; Saiz et al., 1993; Kleppel, 1992; White & Roman, 1992; Purcell et al., 1994; Clarke & Walsh, 1993).

Acartia tonsa has been described from estuaries of the Atlantic coast of North and South America from the Miramichi estuary, Canada (47.5° N, Bousfield, 1955) to Argentina (45° S, Sabatini, 1990); it has also been reported from the Pacific coast of North America (Uye & Fleminger, 1976; Durbin et al., 1983). In estuaries within this range, *A. tonsa* is frequently the dominant zooplankton species during the summer, in terms of both numbers and biomass (Durbin & Durbin, 1981). South of Cape May, New Jersey, U.S.A., *A. tonsa* is present year-round; farther north, the species occurs seasonally and overwinters as benthic diapause eggs (McAlice, 1981). The seasonal cycle has been described by McAlice (1981): during the fall, copepod abundance declines as water temperature decreases; females switch from producing subitaneous (i.e., immediately hatching) eggs to benthic diapause eggs, which overwinter in the sediments. In the spring, diapause eggs hatch when water temperature exceeds 10° C. Adult populations increase rapidly above temperatures of 15–18 °C, with several generations of females producing subitaneous eggs during the summer.

Molecular population genetic analysis can provide new insights into the ecological dynamics and evolutionary history of estuarine species, including patterns of dispersal and exchange among populations; estimation of the timing of population differentiation and speciation; impacts of historical geological and climatic events; and occurrence of genetic bottlenecks (see Avise, 2000, for review). Such studies have previously examined the evolutionary histories of marine organisms in Northwest Atlantic coastal environments, which have been particularly affected by climatic changes over the past several million years. In particular, the southward expansion of an ice sheet over the North American continent beginning 2.5 to 3.1 Ma (Zachos et al., 2001) led to dramatic compression and latitudinal displacement of isotherms (Cronin, 1988). Local sea level was lowered, coastal estuaries emptied, and sea temperatures cooled (Lambeck & Chappell, 2001). For obligately estuarine species, glaciation profoundly altered the nature and availability of suitable habitat, resulting in range compression, genetic bottlenecks, and loss of genetic diversity (see, e.g., Wares & Cunningham, 2001). It is possible that such species experienced local extinction during range compression, with subsequent range expansion that may have subsampled the genetic diversity of source populations through founder events. Bucklin & Wiebe (1998) speculated that historical range compression resulting from climatic variation may explain the low genetic diversity of highly abundant, pelagic copepods. Such scenarios may serve as useful hypotheses for understanding the molecular evolutionary consequences of historical climatic variation in Northwestern Atlantic coastal regions.

In recent decades, the biogeographical distribution of coastal and estuarine planktonic species – including *A. tonsa* – have almost certainly been significantly impacted by man's activities (see Carlton, 1985, for review). Recently categorized as a cosmopolitan species by Razouls & de Bouvee (1998; but see Bradford, 1976), *A. tonsa* may be exchanged among active harbors in the ballast tanks of commercial ships (Brylinski, 1981; Ohtsuka & Ueda, 1999; Gollasch et al., 2000; Guvanova, 2000; Wonham et al., 2001). The possible role of anthropogenic exchange of organisms at local to circumglobal scales must be considered in discussions of the determinants of present-day patterns of population genetic diversity and structure.

For this study, molecular genetic diversity of *A. tonsa* was examined for samples from four estuaries along the Atlantic coast of the U.S.A. Comparative analysis was done for one Gulf of Mexico and one Pacific coast collection, and for collections of *A. hudsonica* and *A. longiremis*. Individual copepods collected during 1993 and 1994 were examined for DNA sequence variation of a portion of the mitochondrial 16S rRNA gene. Geographic patterns of molecular genetic variation were used to estimate present-day dispersal among estuarine populations. Population genetic and phylogeographic analyses provided a basis for hypotheses about the evolutionary consequences of historical climatic variation in the Northwest Atlantic coast for this copepod.

Materials and methods

Sample collection

Zooplankton samples containing species of *Acartia* were collected in coastal estuaries using oblique tows

Table 1. Locations, coordinates, and dates of collection for the *Acartia* spp. used in this study. See Caudill (1995) for details.

Species	Sampling region	Location	Sampling dates	Sample size
Acartia tonsa	Great Bay, NH, U.S.A.	43° 03′ N, 70° 50′ W	6 November, 1993	29
			11 August, 1994	14
			7 November, 1994	15
	New Bedford Harbor, MA, U.S.A.	41° 38′ N, 70° 55′ W	10 October, 1993	28
			15 November, 1994	30
	Narragansett Bay, RI, U.S.A.	41° 34′ N, 71° 27′ W	13 October, 1993	30
			29 November, 1994	30
	Savannah River Estuary, GA, U.S.A.	31° 55′ N, 81° 02′ W	3 October, 1993	33
			21 November, 1994	30
	Nueces Bay, TX, U.S.A.	27° 55′ N, 97° 10′ W	15 September, 1994	31
	LaJolla, CA, U.S.A.	32° 50′ N, 117° 16′ W	8 September, 1994	6
A. longiremis	Håkoybotn Fjord, Norway	69° 42′ N, 19° 00′ E	1 December, 1994	4
A. hudsonica	Narragansett Bay, RI, USA	41° 34′ N, 71° 27′ W	29 November, 1994	2

of either 0.25 m or 1 m zooplankton nets with 333 μm mesh. Collected samples were immediately preserved and stored in 95% ethanol until analysis. Several samples were examined by taxonomic experts N. J. Copley (Woods Hole Oceanographic Institute) and B. J. McAlice (University of Maine) to confirm species identifications.

Samples of *A. tonsa* were collected from coastal regions of the U.S.A., including four estuaries along the Atlantic coast (Great Bay, NH; Buzzards Bay, MA; Narragansett Bay, RI; Savannah River Estuary, GA) during 1993 and 1994; one estuary on the Gulf of Mexico (Nueces Bay, TX) in 1994 and one site on the Pacific coast (La Jolla, CA) in 1994 (Table 1). Samples were designated by state abbreviation and year of collection. Samples of two additional *Acartia* species were obtained for molecular analysis: *A. longiremis* was collected from Håkoybotn, Norway and *A. hudsonica* from Narragansett Bay, RI (Table 1).

DNA amplification and sequencing

Individual fifth stage copepodites or adult females were rehydrated in 300–400 μl distilled, autoclaved water for 1–72 h. Longer soaking (>24 h) often improved results. Copepods were homogenized in 1× PCR buffer and the remaining PCR reagents were added; PCR reaction volumes ranged from 50 μl to 100 μl. A 340 base-pair (bp) region of the mitochondrial 16S rRNA gene was amplified using the primers 16S-167 (Bucklin et al. 1998; sequence 5′-GACGAGAAGACCCTATGAAG-3′) and 16sbr-H (Palumbi 1996; sequence 5′-CCGGTTTGAACTCAGATCATGT-3′). The PCR protocol was 94 °C (1 min), 37 °C (2 min), and 72 °C (3 min) for 38 or 40 cycles. DNA sequencing was carried out in an Applied Biosystems Inc., Automated DNA Sequencer (Model 373) using protocols described in Bucklin et al. (1992, 1995). All base assignments were confirmed visually using SeqEd v.1.0.3 (Applied Biosystems Inc., Foster City, CA). DNA sequences were submitted to the molecular database, GenBank (http://www.nlm.nih.ncbi.org) and were assigned a GenBank Accession Number.

Molecular phylogenetic, population genetic, and phylogeographic analyses

Nucleotide sequences obtained for mt16S rRNA from individual copepods of *A. hudsonica*, *A. longiremis*, and *A. tonsa* were aligned using the CLUSTAR algorithm in MegAlign DNA* (DNASTAR, Inc., Madison, WI). A published sequence for the same gene portion for *A. clausi* (GenBank Acc. No. AF295332, Bucklin et al. 2000) was included in the analysis. Gap penalty was set to 10 and the gap length penalty was 5, resulting in a multiple alignment of 189 base-pairs (including six alignment gaps in the *A. tonsa* sequences). Pairwise percentage nucleotide differences, as well as Tamura/Nei gamma distances (Tamura & Nei, 1993) were calculated using Molecular Evolutionary Genetic Analysis (MEGA), Ver. 2.1 (Kumar et al., 2001). Phylogenetic examination of *A. clausi*, *A. hudsonica*, *A. longiremis*, and *A. tonsa* using several tree-building algorithms did not

resolve evolutionary relationships among the species with statistical significance.

A phylogeographic tree for *A. tonsa* based on mt16S rRNA sequence variation was reconstructed using MEGA, Ver. 2.1 (Kumar et al., 2001). The outgroup used was *A. longiremis*, which eliminated all gaps in the multiple sequence alignment. Maximum parsimony, distance-based, and maximum likelihood algorithms were used to reconstruct relationships among the haplotypes and clades of *A. tonsa* based on mt16S rRNA. For the parsimony and distance-based analyses, all substitutions were included and equally weighted; tree topologies were evaluated by bootstrap replicates (1000). A distance-based tree reconstructed by Neighbor Joining (Saitou & Nei, 1987) with Tamura-Nei gamma distances, $\alpha = 2$ (Tamura & Nei, 1993) yielded the highest overall bootstrap values and most nodes with bootstrap values $\geq$50%.

Analysis of Molecular Variance (AMOVA, Excoffier et al., 1992) was done to calculate fixation indices for each level of an hierarchical analysis of covariance and to partition molecular covariance components among estuaries (Φ_{ST}), among collection years for a given estuary (Φ_{SC}), and within samples (Φ_{CT}). Temporal and spatial variation of haplotype frequencies of *A. tonsa* were evaluated using Arlequin Ver. 2.000 (Schneider et al., 2000); a non-parametric approach with 1000 permutations was used to evaluate significance of the Φ-statistics (Excoffier et al., 1992). Pairwise Φ_{ST} values were calculated for all samples and statistically evaluated with a non-parametric permutations test.

An initial analysis was based on all haplotypes and included all Atlantic and Gulf of Mexico samples of *A. tonsa*; sequence differences between pairs of haplotypes were used as Euclidean distance measures. Subsequently, separate analyses were done for Clades A and B (the only clades with sufficient sample sizes) and excluding the TX sample, in order to examine geographic structure at smaller scales. AMOVA analyses were done for all samples collected during 1993 and 1994 from the four Atlantic estuaries, including: (1) Euclidean analysis for Clade A, including three haplotypes defined by substitutions; (2) equidistant analysis for Clade A (see Shoemaker & Jaenike, 1997), including three substitution haplotypes plus an additional 29 haplotypes defined by insertion/deletion events causing a length polymorphism (which were grouped within haplotype A1 in the first analysis); and (3) Euclidean analysis for Clade B, including three substitution haplotypes.

Results

DNA sequence variation of mt16S rRNA

DNA sequence variation for a 183 base-pair region of mt16S rRNA resolved seven different haplotypes among 239 individuals collected from Atlantic estuaries in 1993 and 1994 (based on analysis of 28 to 33 individuals for each estuary each year; Table 1). Among 31 individuals analyzed from a sample collected from Gulf of Mexico in 1994, six haplotypes were resolved, four of which were unique to that sample (A3, B4, C1, and C2). Haplotypes were designated by a clade reference (A, B, C, or D; see below) and a number, with numbers assigned in order of decreasing frequency in pooled samples from both years. GenBank Accession Numbers were obtained for 11 haplotypes of *A. tonsa*: A1 (AF502368); A2 (AF502369); A3 (AF502370); B1 (AF502376); B2 (AF502378); B3 (AF502379); B4 (AF502375); C1 (AF502383); C2 (AF502382); D1 (AF502373); and D2 (AF502372). In addition to the haplotypes defined by nucleotide base substitutions, one Atlantic coast haplotype, A1, had an insertion/deletion length polymorphism that included a variable number of T's (ranging from three to nine), followed by a variable number of A's (seven to 14); this variation defined another 29 'indel' haplotypes (see Caudill, 1995).

Six individuals identified as *A. tonsa* were analyzed from the Pacific coast sample; two additional haplotypes, P1 (AF502384) and P2 (AF502386), were resolved which were unique to that sample. Haplotype P1 differed from Atlantic coast *A. tonsa* haplotypes by 18% to 29% (Table 2). Pairwise nucleotide differences among the four described species of *Acartia*, *A. tonsa*, *A. hudsonica* (AF5023890), *A. longiremis* (AF502387), and *A. clausi* (Bucklin et al., 2000), ranged from 19% to 28% (Table 2).

Phylogenetic and phylogeographic analysis of Acartia

All phylogenetic analyses supported the separation of the recognized species of *Acartia*, although tree topologies and species relationships varied widely with different algorithms. It seems likely that the selected region was 16S rRNA was too short to reliably resolve phylogenetic relationships among described species of *Acartia*. The two haplotypes occurring in the Pacific sample of *A. tonsa* were paraphyletic to the Atlantic and Gulf of Mexico haplotypes in most analyses, although they were associated with different described species in different trees.

Table 2. Proportion of nucleotide differences for a 189 base-pair region of mt16S rRNA for pairwise comparisons between *Acartia* species, including representative haplotypes of four *A. tonsa* clades (A1, B1, C1, and D1), a putative species from the Pacific coast of the USA, *Acartia* sp. (*A.* sp. P1), *A. clausii* (data from Bucklin et al. 2000), *A. longiremis*, and *A. hudsonica*.

	A. tonsa						
	A1	B1	C1	D1	*A.* sp P1	*A. clausii*	*A. longiremis*
*A.tonsa*_B1	0.112						
*A.tonsa*_C1	0.134	0.106					
*A.tonsa*_D1	0.128	0.123	0.140				
A. sp. P1	0.213	0.184	0.212	0.230			
A. clausii	0.236	0.246	0.257	0.208	0.287		
A. longiremis	0.218	0.207	0.223	0.218	0.225	0.279	
A. hudsonica	0.220	0.206	0.191	0.199	0.227	0.248	0.227

Phylogeographic analysis of mt16S rRNA variation within *A. tonsa* resolved nine haplotypes assorted among three deeply-divided clades, with pairwise differences between haplotypes of different clades ranging from 11% to 13% (Table 2). Two haplotypes restricted to the Gulf of Mexico sample formed a fourth clade differing from the others by 12% to 14% (Table 2, Fig. 1). The majority of individuals fell within clade A and most of these were identical in sequence except for the length polymorphism resulting from insertion/deletion mutations. Clade B haplotypes were less common, although at least two individuals within this clade were sampled at every location. Clade C was only present in the TX sample; sample sizes were insufficient to test geographic patterns within clade D (Figs 2 and 3). Among Atlantic samples, three of the four clades were widespread (A, B, and D; Fig. 2). Several of the indel haplotypes were restricted to a single location (e.g. AG, AJ, AL, Fig. 3).

The phylogenetic analysis using any algorithm confirmed the monophyly of the four clades of *A. tonsa*. Evolutionary relationships among the *A. tonsa* clades were resolved with highest bootstrap values in the distance-based analysis (Fig. 1). Pairwise differences among haplotypes within a given clade were small (<2%); differences among haplotypes of different clades were much larger, ranging from 10% to 14% .

Population genetic analysis of A. tonsa

A single hierarchical AMOVA analysis, considering haplotypes of all clades occuring in Atlantic and Gulf of Mexico samples, provided evidence of significant spatial structure accounting for 50% of the variation ($\Phi_{ST} = 0.582$, $p < 0.0001$). Interannual differences between samples from the same estuary (excluding TX, which was sampled only in 1994) accounted for 9% of the variation ($\Phi_{SC} = 0.172$, $p < 0.0001$). There were significant differences between 1993 and 1994 samples from RI and GA ($p < 0.0001$), but not NH ($p = 0.480$) or MA ($p = 0.502$). In some cases, significant interannual variation resulted from sampling haplotypes of a given clade in only one of the two years (Fig. 2).

Separate analyses by hierarchical AMOVA for only Atlantic estuary samples (NH, MA, RI, and GA) revealed significantly different temporal and spatial patterns when based on just Clade A substitution haplotypes, Clade A indel haplotypes, or Clade B substitution haplotypes. The Euclidean analysis of Clade A substitution haplotypes revealed significant geographic structure ($\Phi_{ST} = 0.229$, $p < 0.0001$) that explained 71% of the variation, while interannual differences accounted for only 6% of the variation ($\Phi_{SC} = 0.079$, $p = 0.0020$; Table 3). The Clade A Equidistant analysis, including indel haplotypes, revealed significant geographic structure ($\Phi_{ST} = 0.097$, $p < 0.0001$) that accounted for 90% of the variation, while interannual differences were not significant ($\Phi_{SC} = 0$, $p = 0.4565$; Table 3). All pairwise comparisons among samples based on Clade A indel haplotypes were significant, with the exception of MA94 vs RI93 ($p = 0.207$). Ninety-five percent of all Clade B individuals shared a single haplotype (B1). Nonetheless, the Clade B analysis provided some evidence of geographic structure ($\Phi_{ST} = 0.228$, $p = 0.0323$) and strong evidence of interannual variation (Φ_{SC} = 0.497, $p < 0.0001$; Table 3). No

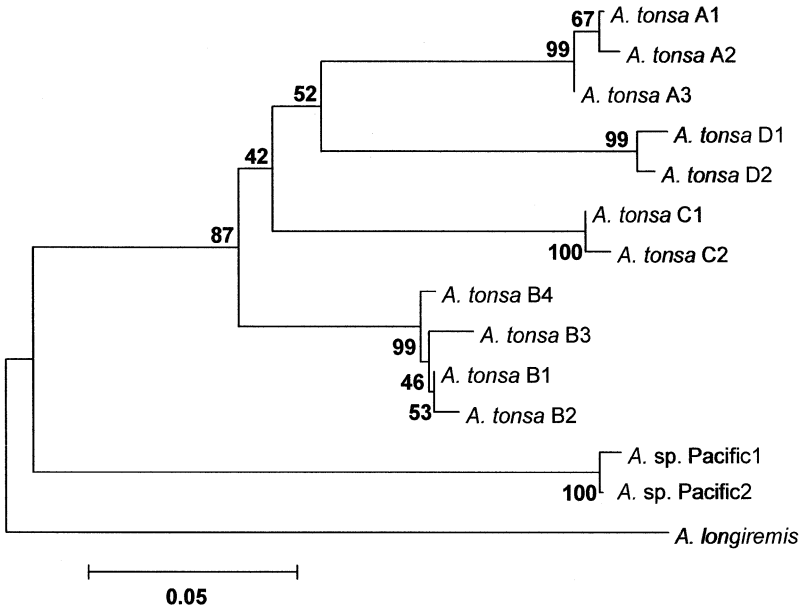

Figure 1. Mt16S rRNA gene tree for *Acartia tonsa* reconstructed using the Neighbor Joining algorithm and Tamura Nei gamma distances, with $\alpha = 2$. *Acartia longiremis* was used as an outgroup; the tree was bootstrapped 1000×. Taxon names indicate the clade designation for *A. tonsa* (A, B, C, or D) and haplotype number (as submitted to GenBank). Numbers at nodes are bootstrap values (i.e., percentage of times that node appears among 1000 replicates). Scale bar shows Tamura Nei distances.

pairwise comparisons among Atlantic samples based on Clade B haplotypes were significant.

Discussion

Phylogeography of A. tonsa *in Atlantic estuaries*

Patterns of molecular genetic diversity and structure of *A. tonsa* reflected an exceptional degree of intraspecific variation of mt16S rRNA compared to other copepods. For Atlantic coast samples, four deeply-diverged clades had closely-related terminal taxa; haplotypes differed by <2% within a given clade, while among clades, haplotypes differed by 10% to 14%. In comparison, this same region of mt16S rRNA differed by 10% to 20% among congeneric species of other calanoid copepod genera (Bucklin et al., 1998), although among described species of *Acartia* differences ranged from 19% to 29% (Table 2). Although observed levels of mtDNA divergence among *A. tonsa* clades approached that typical of distinct species, we cannot assume that the taxon comprises a suite of cryptic species. For the purposes of this discussion, we treat the clades as conspecific and focus on the ecological and evolutionary processes that may have created this phylogeographic phenomenon. Clearly, the exceptional degree of divergence among *A. tonsa* clades requires further morphological and molecular systematic examination.

Geographic patterns of population genetic structure among *A. tonsa* populations of Northwest Atlantic coast estuaries were also notable. Mt16S rRNA haplotype frequencies were significantly geographically heterogeneous for all analyses ($p < 0.0001$ for all except Clade B Euclidean, $p = 0.0323$; Table 3). The frequencies of 'indel' haplotypes (i.e., length polymorphisms within haplotype A1) in each estuary were stable between years (Caudill, 1995), and proved to be particularly useful characters for detecting geographic patterns of population genetic structure (Fig. 3; Table 3). The fact that many of the indel haplotypes were restricted to single estuaries suggests that populations of *A. tonsa* in different estuaries are currently isolated. Of the Atlantic coast samples, only one pairwise comparison among samples (MA94 vs RI93) did not differ significantly for indel haplotype frequencies. Notably, these samples were collected from closely adjacent estuaries – albeit in different years – between which exchange may be relatively more frequent.

In addition to geographic structure, population genetic analysis of *A. tonsa* in Atlantic estuaries revealed some evidence of interannual variation. Fre-

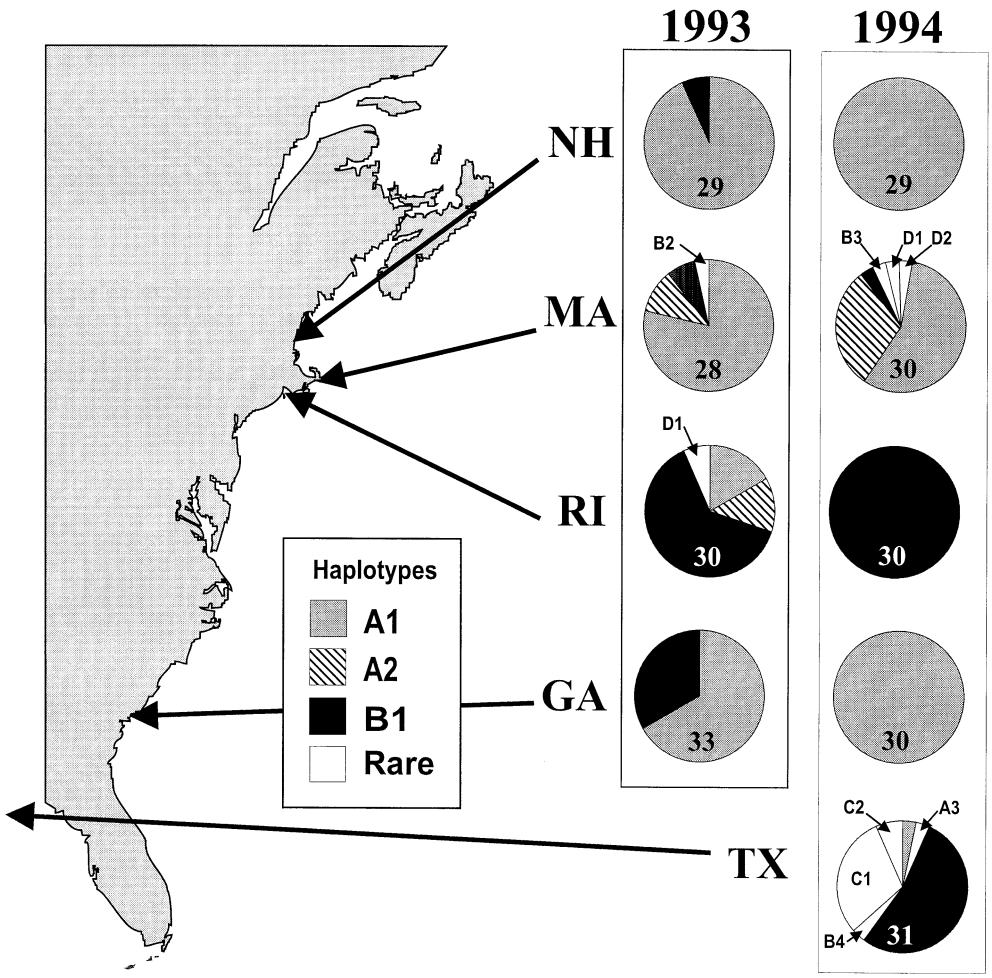

Figure 2. Frequencies of substitution haplotypes defined by a 183 base-pair region of mt16S rRNA, observed in samples of *A. tonsa* collected during 1993 and 1994 from estuaries in New Hampshire (NH), Massachusetts (MA), Rhode Island (RI), and Georgia (GA) along the Atlantic coast of the USA (shown in figure). Haplotype names are as given in the text: A, B, C, and D represent clades; numbers are haplotypes within each clade. Rare haplotypes are indicated by arrows to unshaded pie sections; numbers on pies are sample sizes. This analysis excluded 29 'indel' haplotypes defined only by a length polymorphism within haplotype A1 (see Fig. 3 and text for explanation).

quencies of all mt16S rRNA haplotypes considered together differed significantly between 1993 and 1994 in samples from two of the four Atlantic estuaries (RI and GA). Significant interannual variation in mtDNA haplotype frequencies has been observed for planktonic copepods in previous studies; Bucklin & Kocher (1996) hypothesized that apparent temporal variation may result from unresolved spatial patchiness in the genetic makeup of zooplankton populations. For *A. tonsa*, the cause of this variation may result from significant year-to-year variation in each estuarine population, although the diapause egg stage is thought to damp genetic changes between generations (Hairston & De Stasio 1988). If clades are ecologically or reproductively distinct, observed interannual variation may also be attributed to temporal patchiness and/or seasonal variation in the abundances of the clades, as is known for *Acartia* species (Jeffries 1962, Durbin & Durbin 1981). Such temporal variation represents a problem for phylogeographic analyses, and suggests that our sampling may be insufficient to accurately resolve all genetic variation of the *A. tonsa* of each estuary. Ongoing studies seek to address this question, and entail more frequent and more spatially intensive sampling, with analysis of larger samples from each estuary. This analysis will provide clearer resolution of temporal and spatial variation in the population genetic make-up of *A. tonsa*, but we

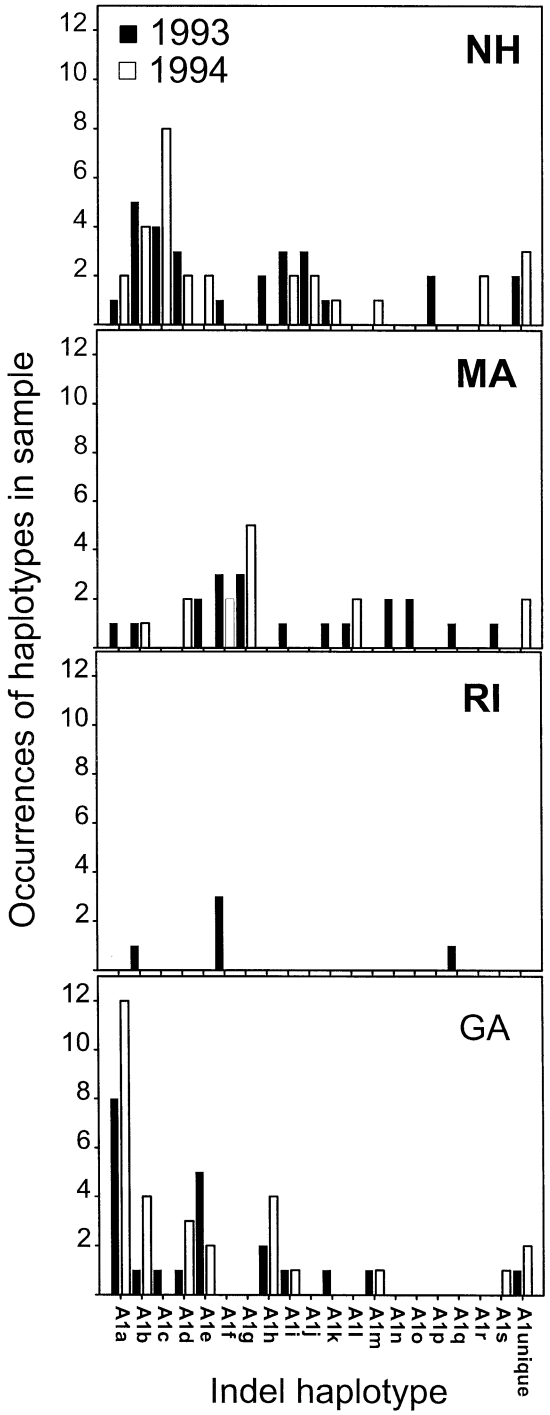

Figure 3. Occurrences of 'indel' haplotypes, defined by insertion/deletion events within haplotype A1, for samples of *A. tonsa* collected during 1993 (shaded bars) and 1994 (unshaded bars) from estuaries in New Hampshire (NH), Massachusetts (MA), Rhode Island (RI), and Georgia (GA). Twenty indel haplotypes occurring more than once in the study were arbitrarily designated A1a–A1s; haplotypes observed only once in the two-year study were pooled as A1 unique.

Table 3. Results of hierarchical Analysis of Molecular Variation (AMOVA) of mt16S rRNA for *Acartia tonsa* samples collected from four Atlantic estuaries in 1993 and 1994 for: (1) all clades – Euclidean; (2) clade A – Euclidean; (3) clade A – Equidistant, including indel haplotypes; and (4) clade B – Euclidean. Φ-statistics are explained in the text; molecular covariance is partitioned among estuaries (Φ_{ST}), among years within an estuary (Φ_{SC}), and within samples (Φ_{CT}); partitioning is shown as a percentage (% Variation); statistical significance of each covariance component is shown as a P-value (asterisks indicate $p < 0.0001$).

	Φ-statistic	% Variation	P-value
(1) All Clades – Euclidean			
Among estauries (Φ_{ST})	0.572	43%	0.0000***
Among years (Φ_{SC})	0.199	11%	0.0000***
Within samples (Φ_{CT})	0.466	46%	0.1046
(2) Clade A Euclidean[a]			
Among estauries (Φ_{ST})	0.229	71%	0.0000***
Among years (Φ_{SC})	0.079	6%	0.002
Within samples (Φ_{CT})	0.228	23%	0.1681
(3) Clade A Equidistant[a]			
Among estauries (Φ_{ST})	0.097	90%	0.0000***
Among years (Φ_{SC})	0	0%	0.4565
Within samples (Φ_{CT})	0.097	10%	0.0000***
(4) Clade B Euclidean[b]			
Among estauries (Φ_{ST})	0.228	77%	0.0323
Among years (Φ_{SC})	0.497	76%	0.0000***
Within samples (Φ_{CT})	0	−53%[c]	0.4751

[a]Sample RI94 not included; no haplotypes found.
[b]Samples NH94 and GA94 not included; no haplotypes found.
[c]Negative covariance components can occur in absence of genetic structure.

do not expect that it will alter our current findings of deeply-divided clades and significant geographic variation.

Comparisons with A. tonsa *on other coasts*

The genetic distinctiveness of the Gulf of Mexico sample of *A. tonsa*, with four of six haplotypes unique to that sample, was consistent with previous studies of marine fauna occurring in both regions. Some researchers suggest that the Florida Peninsula acts as a dispersal barrier for marine fauna of the Gulf of Mexico and the Atlantic coast (Reeb & Avise, 1990; Karl & Avise, 1992; Hare & Avise, 1996, 1998; reviewed by Avise, 1994). In a population genetic study of the oyster, *Crassostrea virginica*, Hare et al. (1996) hypothesized that the near-shore current flow in the Florida Straights limited the extent of suitable nearshore habitat for estuarine species, creating a barrier to gene

flow at the point of nearest contact of the current to shore. However, *A. tonsa* is known to occur outside estuaries of the South Atlantic Bight, from North Carolina to Florida (Paffenhöfer & Stearns, 1988). Thus, it is unclear whether the genetic differentiation of the Atlantic and Gulf of Mexico populations results from isolation by distance and/or geographic barriers to dispersal. Genetic analysis of samples collected from additional intermediate locations is needed to document gene flow patterns of *A. tonsa* between the Atlantic and Gulf of Mexico coasts.

The phylogenetic analysis presented here indicates that the Pacific sample of *A. tonsa* most likely represents a distinct species, with the likelihood that the new species is paraphyletic to *A. tonsa*. There were no haplotypes in common and sequence differences were in the same range as those between described species of *Acartia* (see Fig. 1 and Table 2). However, our single Pacific coast sample cannot provide definitive evidence, since only a few individuals were sequenced and the phylogenetic tree had a low bootstrap values at branchpoints among *A. clausi*, *A. hudsonica* and *A. longiremis*. Whether this lack of resolution at these branchpoints resulted from the sampling of too few characters (i.e., too short a DNA sequence), rapid evolution of the mt16S rRNA gene, or other causes is unclear. Analysis of additional molecular characters and more species is needed to clearly resolve the evolutionary relationships within the genus *Acartia*.

Other types of evidence also support the probable distinction between Atlantic and Pacific coast *Acartia* species. There are reported life history differences between the copepod on the two coasts: Atlantic populations of *A. tonsa* entered diapause as temperatures dropped to 9–14.5 °C (Zillioux & Gonzalez, 1972), while a Southern California population produced only subitaneous eggs at 6.5 °C (Uye & Fleminger, 1976). Also, a laboratory study by Enrique Carrillo et al. (1974) demonstrated failure of interbreeding between Atlantic and Pacific copepods identified as *A. clausi.*

The role of life history in causing population structure

The genetic differentiation among populations of *A. tonsa*, which is not known to occur outside the headlands in the Gulf of Maine (McAlice, 1981), may result from active behavioral mechanisms which aid its retention in estuaries (see Miller, 1983; Hough & Naylor, 1991). The benthic diapause egg stage of *A. tonsa* may also help retain the species in estuaries, and may act as an 'egg bank', retaining mtDNA lineages, damping genetic changes between generations, and slowing evolutionary changes in the species (Hairston & DeStasio, 1988; Marcus et al., 1994). Similar to freshwater organisms, estuarine species like *A. tonsa* may experience pulsed colonization events and have rapid reproductive rates. In combination, these traits can produce persistent genetic structure over small spatial scales in the face of high gene flow (De Meester et al., 2002).

The importance of historical climatic variation

The exceptional divergence of mtDNA lineages of *A. tonsa*, with the resolution of haplotypes among deeply-divergent clades, may be hypothesized to result from historical climatic variation associated with glaciation. The Atlantic coast of N. America was profoundly transformed over the geological periods consistent with the observed genetic divergence: sea level dropped ∼200 m and coastal estuaries emptied (Lambeck & Chappell, 2001); cold waters intruded southward and isotherms were compressed in the Northwest Atlantic (Cronin, 1988). We may hypothesize that the climatic and environmental changes caused *A. tonsa*'s range to be latitudinally displaced and compressed, with significant impact on patterns of molecular genetic diversity within the species (see Bucklin & Wiebe, 1998; Davis & Shaw, 2001).

The biogeographic displacement of the species may have caused the extinction of some mtDNA lineages, resulting in the sorting of lineages into deeply-diverged clades observed here. Coalescent or 'backward-looking' analysis of the molecular diversity of a species with this evolutionary history (see Hudson, 1990) might be expected to reveal the presence of a limited number of deeply-diverged clades, as was observed for *A. tonsa* in this study. Assuming usual rates of mtDNA evolution of 1.5% to 2.5% per Ma (e.g., Rand, 1994), the presence of deeply-diverged clades may be hypothesized to result from the loss of intermediate mtDNA lineages over geological time, with the accumulation of multiple haplotypes within each clade subsequent to these events.

Observed geographic patterns of genetic structure of *A. tonsa* are consistent with McAlice's (1981) hypothesis that recent sea level and climate variation resulted in the expansion, contraction, and isolation of populations of *A. tonsa* along the Northwest Atlantic coast. McAlice (1981) suggested that *A. tonsa* populations in the Gulf of Maine were nearly continuous during the Hypsithermal period (3 to 5.5 Ka); the

cooling climate at the end of this period caused a vicariance event, restricting populations north of Cape Cod to shallow, warm water estuaries, where summer water temperatures permit reproduction. The widespread distribution of many substitution haplotypes and the greater restriction of indel haplotypes would seem to be consistent with reduced exchange in the recent past.

Patterns of genetic differentiation between Atlantic and Pacific populations of *A. tonsa* may also be explained in terms of the geological history of the oceans. These biogeographic regions may have been isolated since the closure of the Panamanian isthmus ~3 Ma (Knowlton et al., 1993). It is possible that, as a cool temperate species, *A. tonsa* may have been either introduced or exchanged from the Pacific to the Atlantic during the trans-Arctic interchange ~3.5 Ma (Vermeij, 1991).

Anthropogenic impacts on distribution and dispersal

Alternate pathways of dispersal proposed for *A. tonsa*, including transport in ballast water (Carlton, 1985), seem unlikely to explain the observed geographic patterns of genetic diversity and structure of *A. tonsa* in the sampled estuaries. The presence of multiple haplotypes within each clade and the occurrence of many haplotypes in multiple estuaries is difficult to explain using an 'invasion pathway' scenario for *A. tonsa*. In this case, one might expect to find unique, highly divergent haplotypes that are identical to those of the population of origin (see Kooistra et al., 2001; Cristescu et al., 2001). The marked distinctiveness of NW Atlantic and NE Pacific coastal populations in mt16S rRNA haplotype frequencies also suggests long-term isolation. Ongoing studies are examining copepods identified as *A. tonsa* in samples from across the global range of the species, in order to evaluate circumglobal patterns of genetic diversity and structure in terms of the impacts of both the climatic and geological history of the oceans and the transport of organisms in ships' ballast water.

Conclusion

Estuarine populations of *A. tonsa* along the Atlantic and Gulf of Mexico coasts of the U.S.A. were genetically distinct in haplotype frequencies for the mt16S rRNA gene, with the exception of samples from closely adjacent estuaries in MA and RI. The degree of genetic differentiation suggested that dispersal of *A. tonsa* in coastal waters is restricted. Phylogenetic reconstruction of relationships among *A. clausi*, *A. hudsonica*, *A. longiremis*, and *A. tonsa* confirmed the distinctiveness of the described species but could not resolve species relationships. Phylogenetic analysis of *A. tonsa* revealed the assortment of mtDNA haplotypes among four deeply-diverged clades, within which haplotypes differed by <2% and between which differences approached those between species of calanoid copepods. Based on mt16S rRNA sequence divergence, samples of Atlantic and Pacific coast populations of *A. tonsa* almost certainly represent distinct species. We hypothesize that historical climatic variation, with consequent biogeographic range compression and displacement, may have caused the loss of some mtDNA lineages and resulted in the clade structure within *A. tonsa*. Understanding of the ecological and evolutionary forces at work in this species may help explain the exceptional diversity of the genus in estuarine and coastal habitats around the world.

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