Hydrobiologia
DOI 10.1007/s10750-010-0097-y

COPEPODA: BIOLOGY AND ECOLOGY

Phylogeography of the copepod *Acartia hudsonica* in estuaries of the northeastern United States

Peter J. Milligan · Eli A. Stahl · Nikolaos V. Schizas · Jefferson T. Turner

Abstract Copepods of the genus *Acartia* dominate zooplankton assemblages in northwestern Atlantic estuaries, many of which originated after the last glacial maximum 10,000–18,000 years ago. *Acartia hudsonica* occurs, at least seasonally, in estuaries from Chesapeake Bay to Labrador/Newfoundland. We sequenced the mitochondrial gene Cytochrome B (CytB) of 75 individuals of *A. hudsonica* from 26 estuaries from New Jersey to Maine, covering four biogeographic regions, and 11 individuals of *Acartia tonsa* from four of these estuaries in the southern part of the sampling range. *A. hudsonica* exhibited exceptionally high intraspecific DNA sequence variation. Uncorrected *p*-distances between sequences ranged from 0.3 to 31%. Five highly divergent sequence groups differed in frequencies across populations and biogeographic regions. One sequence group dominated northern localities, and two sequence groups were found at intermediate to high frequencies in two southern biogeographic regions. Ages of the sequence groups were estimated to be 11, 13, 30, and 37 million years, by applying a molecular clock calibrated by divergence in *Alpheus* snapping shrimps across the Isthmus of Panama. These ages were compared with independent biogeographic paleoceanographic data, and may have coincided with periods of global climate change over the past 40 MY.

Keywords *Acartia hudsonica* · Genomics · Phylogeography · Mitochondrial DNA

Guest editors: L. Sanoamuang & J. S. Hwang / Copepoda: Biology and Ecology

P. J. Milligan · J. T. Turner (✉)
School of Marine Science and Technology, University of Massachusetts Dartmouth, New Bedford, MA 02744, USA
e-mail: jturner@umassd.edu

E. A. Stahl · J. T. Turner
Biology Department, University of Massachusetts Dartmouth, North Dartmouth, MA 02747, USA

N. V. Schizas
Department of Marine Sciences, University of Puerto Rico, Mayagüez, PR 00681, USA

Introduction

Most offshore oceanic plankters exhibit widespread cosmopolitan distributions and extensive gene flow because parameters that determine distributions, such as temperature, salinity, and food concentration, may be similar over vast geographic distances. Conversely, in estuarine embayments, plankton may remain physically isolated from more offshore populations, and retain distinct assemblages more influenced by "embayment" parameters such as low salinity from runoff, warmer temperatures in summer, and shallow depth which may influence resting egg life cycles. If estuarine plankton remain isolated for

Published online: 04 February 2010

sufficient periods, then reduced gene flow with conspecific populations of adjacent estuaries would be expected to increase genetic variability between populations.

Copepods of the genus *Acartia* often dominate mesozooplankton assemblages in estuaries of northeastern North America. Two congeners of *Acartia* may co-occur in the same estuaries, albeit sometimes with seasonally offset occurrences, due to different periods of population dormancy in resting eggs (Sullivan et al., 2007, and references therein).

Acartia hudsonica Pinhey is typically found between Chesapeake Bay and Cape Cod, mainly during the colder months of the year. North of Cape Cod, *A. hudsonica* occurs year-round, with highest abundances in summer and fall (Lee & McAlice, 1979). South of Cape Hatteras, *A. hudsonica* generally does not occur (Turner, 1981). Although this species was described in 1926 from the Strait of Belle Isle between Labrador and Newfoundland, until the last three decades or so, on the western side of the North Atlantic, *A. hudsonica* was confused with the Eurasian species *Acartia clausi* (Bradford, 1976).

Acartia tonsa Dana occurs primarily during the warmer months between Cape Hatteras and Cape Cod, with year-round presence in estuarine waters south of Cape Hatteras, but only ephemeral seasonal occurrence, primarily in summer, from north of Cape Cod to the Baie-des-Chaleurs, New Brunswick (Turner, 1981). McAlice (1981) has argued that occurrences of *A. tonsa* in estuarine waters north of Cape Cod are isolated glacial relicts of a population that was continuous from Cape Hatteras to the Northumberland Strait (southern Gulf of St. Lawrence) in warmer waters during post-glacial periods 15,000–5,000 years before present (BP), which subsequently became isolated from source populations to the south due to rising sea level and continental cooling by 3,000 years BP. The seasonal reappearance of *A. tonsa* during summer in warm shallow embayments is likely due to hatching of resting eggs. Indeed, *A. tonsa* occurs in warm shallow waters of Shediac Bay, New Brunswick, but not further out into the Northumberland Strait between New Brunswick and Prince Edward Island (Citarella, 1982).

The *Acartia* congeners are primarily found in protected estuarine embayments, rather than in open coastal waters. When found in coastal or continental shelf waters, they are less frequent and less abundant than in estuaries (Turner, 1994, and references therein). Possible reasons include requirements for sufficient temperature and salinity ranges for completion of naupliar development (Tester & Turner, 1991), and shallow depths to allow completion of resting egg cycles (Marcus et al., 1994).

The requirement of embayment habitats, coupled with the fact that northeastern North American estuaries were recently glaciated, might suggest that populations of *A. hudsonica* and *A. tonsa* are more isolated, with possibly greater genetic divergence than in offshore copepods with presumed greater gene flow. Indeed, Caudill & Bucklin (2004) found that populations of *A. tonsa* from four estuaries on the Atlantic and Gulf of Mexico coasts of the United States exhibited substantial genetic diversity in DNA sequence variation of the 16S rRNA gene.

The high diversity within *A. tonsa* may be related to its substantial range compression and southern displacement during glaciations over the last 20,000 years. Estuarine and coastal habitats of eastern North America would have been drastically impacted by recent glaciations (Davis & Shaw, 2001; Lambeck & Chappell, 2001). This might have caused extinction of numerous estuarine organisms. In addition, long-lived diapause eggs may have accumulated in "egg banks" in estuarine sediments (Marcus et al., 1994), allowing for survival of multiple ancient lineages of *A. tonsa* to the south of glaciated areas. This combination of sequestration of some lineages from southern egg banks, together with extinction of intermediate clades in glaciated estuaries, may have led to deeply diverged lineages that re-colonized new estuarine habitats as the glaciers retreated (Caudill & Bucklin, 2004).

The high diversity in estuarine *A. tonsa* is unlike the low genetic diversity demonstrated for other offshore copepods. *Calanus finmarchicus* appears to exhibit a single interbreeding population with extensive gene flow throughout the region from the Gulf of St. Lawrence, through the Gulf of Maine, to Georges Bank and waters to the west of Georges Bank (Bucklin et al., 1996).

Although Caudill & Bucklin (2004) examined the genetic variability of *A. tonsa* in four estuaries of the eastern United States, and compared these to *A. hudsonica* from a single location (Narragansett Bay), to our knowledge, there has never been a similar extensive study of genetic variation in *A. hudsonica*.

Since *A. hudsonica* is primarily found in such estuaries at temperatures lower than 15–16°C (Sullivan et al., 2007), the main season when it would be found throughout most of its present range would be in winter. Also, since this copepod is usually active in the plankton in colder waters than its congener *A. tonsa*, and has a temperature-dependent diapause egg cycle that is the seasonal reverse of that in *A. tonsa*, the biogeographic history of *A. hudsonica* in relation to glaciation might be different from that hypothesized for *A. tonsa* (Caudill & Bucklin, 2004; McAlice, 1981). Accordingly, we sampled 37 embayments from northeastern Maine to southern New Jersey in winter, and examined the Cytochrome B mitochondrial gene (CytB) from adult female *A. hudsonica* which occurred in 35 of these embayments. We also performed similar analyses on adult females of *A. tonsa* which co-occurred in four of these embayments at the southern end of the sampling range. Our expectation was that since the embayments we sampled had only been formed since the last glaciation, and since *A. hudsonica* are characteristic of sheltered embayments rather than open coastal waters, recent isolation in estuaries might cause the genetic diversity of *A. hudsonica* to be high compared to open-ocean copepod species, and that divergence between populations would be relatively recent.

Methods

Sample collection

Zooplankton samples containing *Acartia* spp. were collected from 35 estuarine habitats (Fig. 1) between northeastern Maine and southern New Jersey during December 28, 2005–January 20, 2006. Collections were made with a 333-μm mesh net, and preserved in 95% EtOH. Salinity was measured using a refractometer, and temperature was measured with a thermometer. Adult females of *A. hudsonica*, and if present, *A. tonsa* were sorted from collections and retained in 95% EtOH. *A. hudsonica* were present in 35 of 37 samples, with genetic material sufficient for sequencing obtained from 26 locations (Fig. 1). *A. tonsa* were collected from four locations in Long Island Sound and New Jersey (Fig. 1). A total of 75 *A. hudsonica* and 11 *A. tonsa* females were analyzed.

DNA extraction

DNA was extracted from individual female copepods with 100 μl of DNAzol® and 2.0 μl of polyacrylamide, followed by repeated centrifugation and aspiration in 100 and 75% EtOH. Pelletized samples were then dehydrated in a vacuumed centrifuge, 15 μl of TE 10:1 buffer was added, and the samples were refrigerated for storage.

DNA amplification and sequencing

A 362 base pair segment of CytB was amplified using the polymerase chain reaction (PCR). This gene was chosen because it has been shown to be variable at the population level in copepods (Schizas et al., 1999; Papadopoulos et al., 2005). For the amplification of CytB, we used the forward 151F (5′-TGTGGRGCNACYGTWATYACTAA-3′) and reverse 270R (5′-AA NAGGAARTAYCAYTCNGGYTG-3′) primers of Merritt et al. (1998). The Basic Local Alignment Search Tool (BLAST) algorithm (Altschul et al., 1990) was used to search the GenBank database for the closest matches for each sequence (http://www.ncbi.nlm.nih.gov/blast).

Eighty-six individuals were aligned using MacClade 4.0 (Maddison & Maddison, 2000). A multiple sequence alignment of 311 base pairs included 75 *A. hudsonica* sequences and 11 *A. tonsa* sequences (which were missing 33 bases at the 3′ end). The outgroup *Eucalanus bungii* (GenBank accession number AB091772, bases 4712–4402; Machida et al., 2004), an oceanic calanoid copepod, was included in phylogenetic analyses. Sample and haplotype sequence information have been submitted to GenBank (accession numbers FJ916905–FJ916931).

Phylogenetic analyses

Population differentiation, and the geographical pattern of variation were examined by hierarchical analyses of molecular variance (AMOVA). AMOVA (Excoffier et al., 1992, 2005) used pairwise comparisons to decompose variation into components within populations, between pairs of populations within groups, and between pairs of populations between groups. Fixation indices, or *F*-statistics, represent the proportion of each component of variation, relative to

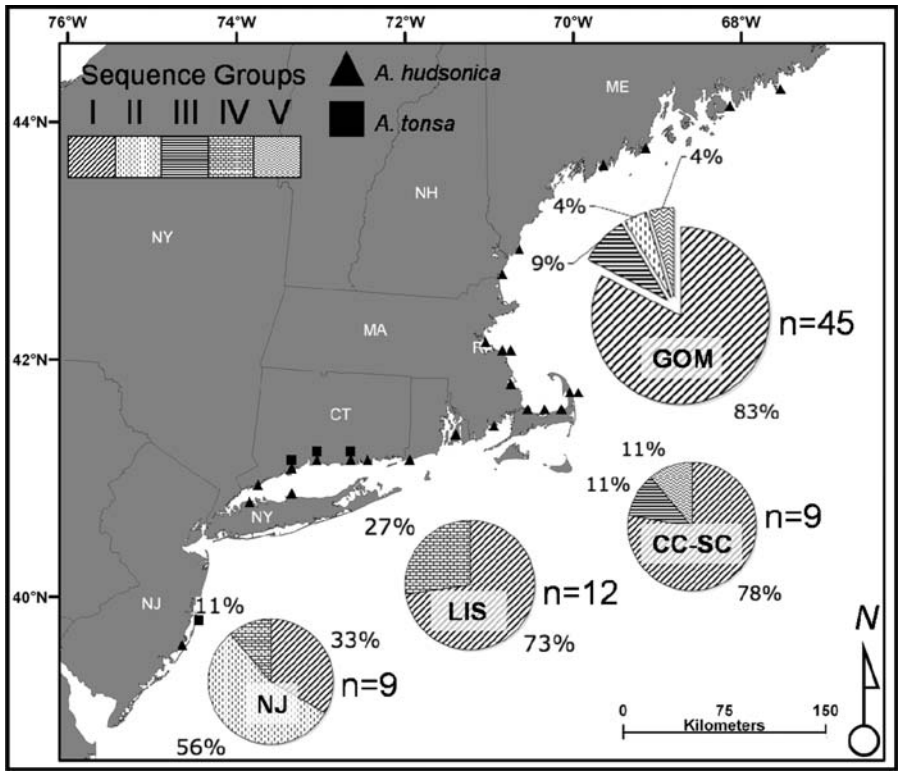

Fig. 1 Map of the sampling area with sampling locations marked with *triangles* (*A. hudsonica*), and *squares* (*A. tonsa*). Pie diagrams depict the percent frequency of sequence types found in the four areas: Gulf of Maine (GOM), outer Cape Cod, the south coast of Massachusetts and Rhode Island (CC-SC), Long Island Sound (LIS), and New Jersey (NJ). Sample size (n = number of individual copepods) in each of the four areas is shown next to the pie diagrams. The five *A. hudsonica* sequence types (I–V) are represented by the different patterns referenced in the key. The *A. tonsa* sequence group is not represented in a pie diagram

total variation (Weir, 1996). F_{ST} represents the proportion of variation among populations, F_{SC} represents the proportion of variation among groups, and F_{CT} represents the proportion of variation among populations within groups.

Hierarchical likelihood ratio tests were used to examine similarities and differences between sequences. These tests (Modeltest 3.6, Posada & Crandall, 1998) were to determine the appropriate model of nucleotide substitution. Within the substitution model we used maximum likelihood (ML) and Bayesian analysis to construct CytB gene genealogies in PAUP* v4.10 (Swofford, 2003) and MrBayes (Ronquist & Huelsenbeck, 2003), respectively. The robustness of clades produced using ML were evaluated with 100 bootstrap replicates (Felsenstein, 1985). The conditions for the Bayesian analysis were the following: four independent runs were conducted with 2 million generations, starting with random trees, employing four simultaneous chains and sampling every 100th tree. The initial 10% of the sampled trees were discarded as burn-in before stationarity was reached. Additional analyses were conducted in PAUP, DNAsp 4.10 (Rozas et al., 2003), and MEGA 3.1 (Kumar et al., 2004).

Hierarchical likelihood ratio tests yielded a six-parameter model with Gamma-distributed rates and a proportion of invariant sites (GTR + G + I). We estimated DNA base frequencies (A = 0.26, C = 0.20, G = 0.15, and T = 0.39), substitution rate ratios of 8.17 for transitions and 2.20 for A ↔ T and C ↔ G transversions, relative to A ↔ C and G ↔ T transversions, a Gamma-distribution shape parameter of 1.568, and the proportion of sites invariant to be 0.387. A ML pairwise distance matrix was generated for use in population analyses and to estimate divergence times through the application of a molecular clock.

Divergence times between sequence groups were estimated using a rate of 2.25% difference per million years (2.25%/MY) for CytB. This rate was based on 1.4%/MY for cytochrome oxidase subunit I (COI) of snapping shrimps (*Alpheus* spp.) (Knowlton & Weigt, 1998), multiplied by 1.61 from estimates of substitution models and rates from the *Calanus helgolandicus* and *Calanus euxinis* data of Papadopoulos

et al. (2005), in which some individuals of haplotypes H6 and H7 were sequenced at both CytB and COI.

Results

The 75 individual *A. hudsonica* sequenced at the CytB gene were sampled from 26 locations from New Jersey to Maine, which spanned portions of two biogeographic provinces, separated by Cape Cod (Ekman, 1953). All but the southernmost portion of our sampling range was glaciated during the last glacial period. Thus, we sampled estuarine populations from four regions: (1) New Jersey (NJ in Fig. 1); (2) Long Island Sound (LIS in Fig. 1); (3) outer Cape Cod, the south coast of Massachusetts and Rhode Island (CC-SC in Fig. 1); and (4) the Gulf of Maine (Cape Cod Bay northward through Maine—GOM in Fig. 1). The Beach Haven, NJ site was the only *A. hudsonica* population sample from New Jersey. We also included individuals of *A. tonsa* from four locations in Long Island Sound and New Jersey, which is near the northern end of their winter range (Turner, 1981).

Acartia hudsonica sequences exhibited substantial DNA sequence polymorphism. Uncorrected *p*-distances between sequences from *Acartia* (including *A. hudsonica* and *A. tonsa*) ranged from 0.3 to 31.0%. The *Acartia* and the *E. bungii* sequences differed by an average of 31.8% (SE = 0.23%).

There were 25 unique sequence haplotypes for *A. hudsonica* (Fig. 2). In the multiple sequence alignment of 311 nucleotides, 141 sites varied among *A. hudsonica* sequences, including 30 out of 32 fourfold degenerate third positions. Among the 141 variable sites, 84 had two segregating nucleotides while 57 had three or four; 25 of 30 fourfold degenerate variable sites had more than two segregating DNA bases. No stop codons were detected in any sequence, and coding sequence polymorphism analyses (Nei & Gojobori, 1985) estimated 106 synonymous and 12 non-synonymous mutations at 82 polymorphic sites (excluding sites in 24 codons at which complex mutation pathways obscure the amino acid mutation pathway).

Pairwise distances among *A. hudsonica* CytB sequences revealed five highly divergent sequence groups (numbered I–V, Figs. 1, 2). For our data set of unique *A. hudsonica* sequence haplotypes, average

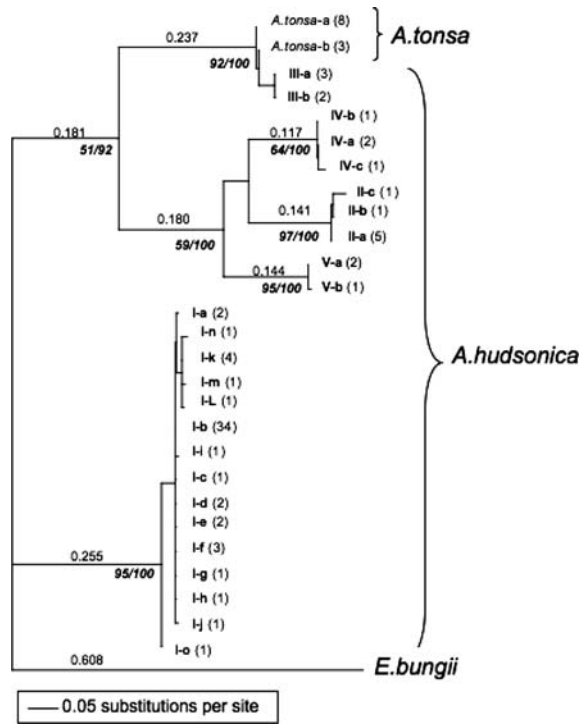

Fig. 2 Maximum likelihood tree was composed of all 27 unique sequence types from both *A. hudsonica* and *A. tonsa*. Bootstrap values and Bayesian posterior probabilities are in *bold italics* below, and branch lengths are above lines denoting branches. The five groups of *A. hudsonica* sequence types are indicated by *Roman numerals* (I, II, III, IV, and V) and unique sequences within those five groups are denoted by *lower case letters*, with their respective sample size in parentheses. There was one group of *A. tonsa* with two unique sequences, which fell within the sequence variation of the *A. hudsonica* groups when *E. bungii* was used as an outgroup

uncorrected pairwise *p*-distance is 0.180 overall, and 0.011 within and 0.254 between sequence groups. The largest average uncorrected pairwise *p*-distances are 0.293 within *A. hudsonica* between Sequence Groups II and III, and with an average of 0.304 between *A. hudsonica* sequences and the outgroup. After correction using our inferred substitution model, ML pairwise distances averaged 0.483 for the data set, 0.015 within, and 0.766 between sequence groups. The largest average ML pairwise distances were 0.976 within *A. hudsonica* between Sequence Groups I and II, and 0.965 between all *A. hudsonica* sequences and the outgroup. A saturation curve (not shown) revealed that substitutions among groups approached saturation indicating that the largest estimated divergences carry substantial

Table 1 AMOVA results

Source of variation	Using sequence differences		Using only frequencies	
	Percentage of variation (*P*-value)	Fixation indices	Percentage of variation (*P*-value)	Fixation indices
Among groups	18.81 (0.05)	$F_{SC} = 0.12$	3.26 (0.20)	$F_{SC} = 0.033$
Among populations within groups	9.45 (0.06)	$F_{CT} = 0.19$	13.41 (0.005)	$F_{CT} = 0.139$
Within populations	71.74		83.33	

Total population differentiation using maximum likelihood DNA sequence pairwise distances, $F_{ST} = 0.28$ ($P = 0.02$), and using haplotype frequencies, $F_{ST} = 0.17$ ($P = 0.0008$)

uncertainty. Nonetheless, divergence among groups ranged from 20 to 65 times the average within-group [non-zero] sequence divergence.

Geographic analysis

The percent frequencies of the five sequence groups across biogeographic regions are shown in Fig. 1. *A. hudsonica* exhibited a widespread group (Sequence Group I) that encompassed locations from Maine to New Jersey (56 of 75 sequences), and three geographically distinct smaller groups. The latter included Sequence Group V (4 of 75 sequences) from Rhode Island/South Coast Massachusetts/Cape Cod to southern Maine, Sequence Group IV (3 of 75 sequences) from southern Connecticut/Long Island Sound, and Sequence Group II (7 of 75 sequences) from southern New Jersey plus a single copepod from Maine. Our southern New Jersey population sample, 56% of which was Sequence Group II, was the only population sample in our study that was not dominated by Sequence Group I. The smaller widespread Sequence Group III (5 of 75 sequences) ranged from Rhode Island to northern Maine. *A. tonsa* from four locations between Connecticut and New Jersey formed a single group that fell within the sequence variability of *A. hudsonica*.

AMOVA revealed that populations were significantly different in their DNA sequence haplotype frequencies, and between-population variation among the four biogeographic regions was greater on average than variation within regions (Table 1). AMOVA based on haplotype frequencies only (identity/difference of DNA sequence haplotype, as opposed to DNA sequence distances) revealed significant population differentiation ($F_{ST} = 0.17$, $P < 0.001$), significant differentiation among populations within groups ($F_{SC} = 0.139$, $P = 0.002$), and a lack of differentiation among groups ($F_{CT} = 0.033$, $P = 0.2$). However, AMOVA using the ML DNA sequence pairwise distances between haplotypes revealed significant population differentiation ($F_{ST} = 0.28$, $P = 0.02$), and marginally significant differentiation both among populations within groups ($F_{CT} = 0.19$, $P = 0.06$), and among groups ($F_{SC} = 0.12$, $P = 0.05$). Increased differentiation among groups in the latter analysis reflected the differentiation among groups, particularly for highly diverged sequence types, which were heavily weighted by including pairwise sequence divergence.

Phylogenetic analyses of unique sequences

There was substantial divergence among the five different sequence groups of *A. hudsonica* (Fig. 2). All sequence groups were recovered as monophyletic in the majority of bootstrap replicates. Sequence Groups I–III and V were highly reproducible, exhibiting bootstrap values of 95 or greater, while Sequence Group IV was recovered in 64% of bootstrap replicates. The Bayesian analysis resulted in an identical topology as the ML method with overall higher levels of nodal support as indicated by the posterior probabilities (Fig. 2). The use of *E. bungii* as the outgroup in the phylogenetic analysis was based on the tremendous divergence found between the groups of *Acartia* species. The inclusion or exclusion of the outgroup did not change the topology of the tree, nor did it significantly change the DNA substitution model.

Additional samples of *A. tonsa* from four sampling locations (Fig. 1) were included in the phylogenetic

Table 2 Divergence and divergence time estimates for *A. hudsonica* sequence groups, and between *A. tonsa* and *A. hudsonica* Sequence Group III: (A) maximum likelihood divergences are averaged for the appropriate pairwise comparisons according to the phylogeny (Fig. 3), standard errors, and estimated divergence times (millions of years BP) are shown; (B) results for all pairs of sequence groups are shown, with divergence in the lower wedge and divergence times in millions of years in the upper wedge

(A)

A. hudsonica sequence group (comparison(s))	Average pairwise divergence	SE	Estimated divergence time (MY BP)
I (vs. II–V)	0.834	0.0644	37.0
III (vs. II, IV, V)	0.674	0.0639	29.9
V (vs. II, IV)	0.295	0.0005	13.1
II and IV	0.251	n/a	11.1
A. tonsa (vs. III)	0.027		1.19

(B)

A. hudsonica	I	II	III	IV	V	*A. tonsa*
I	–	43.32	29.92	35.43	39.34	35.80
II	0.976	–	34.70	11.13	12.87	30.10
III	0.674	0.782	–	24.87	30.15	1.18
IV	0.798	0.251	0.560	–	13.28	23.19
V	0.886	0.290	0.679	0.299	–	27.78
A. tonsa	0.806	0.678	0.027	0.522	0.626	–

analyses. *A. tonsa* exhibited two unique sequences out of 11 total, which were one change apart from each other (Fig. 2). The *A. tonsa* sequences grouped together and were closely related to *A. hudsonica* Sequence Group III. Divergences between *A. hudsonica* sequence groups vastly exceeded the divergence between *A. tonsa* and *A. hudsonica* Sequence Group III (Table 2).

Discussion

Acartia hudsonica from 26 estuaries from New Jersey to Maine revealed very high levels of intraspecific DNA sequence variation at the mitochondrial CytB gene, with greater variation than previously recorded for other copepod species. Five highly divergent sequence groups were identified, which differed in frequency across populations and biogeographic regions.

Geographical differences

The four geographic regions, and populations within regions, were different in their frequencies of sequence groups (Table 1). Differentiation among regions increased when pairwise DNA sequence divergences were included in the analysis. This differentiation between regions was marginally significant only when the New Jersey samples were included, because our Beach Haven, NJ sample (56% Sequence Group II, 33% Sequence Group I, 11% Sequence Group IV—Fig. 1) was the only population in which Sequence Group I was not the most common type. Interestingly, New Jersey was the only region sampled that was south of the last glacial maximum, whereas all other locations have emerged since the last glacial retreat.

The frequency of Sequence Group I increased systematically from south to north. Thus, one might speculate that the widespread Sequence Group I could be associated with post-glacial estuarine colonization, for historical reasons (i.e., from Sequence Group I-dominated source population(s)) and/or in association with genotype(s) adapted to environments in the post-glacial habitats.

These sequence groups may relate to cycles of diapause. With a single exception, Sequence Group II was found only in New Jersey, and Sequence Group IV was found only in New Jersey and Long Island Sound (Fig. 1). *A. hudsonica* undergoes summer/fall

diapause in waters south of Cape Cod, Massachusetts (Sullivan et al., 2007). Thus, Sequence Groups II and IV could represent *A. hudsonica* genotypes which undergo diapause, whereas Sequence Group I may be associated with non-diapausing genotypes.

Nearshore currents may disperse *Acartia* spp. between estuaries. Since the Gulf Stream flows away from the North American coast near Cape Hatteras, it likely has a minimal influence on the dispersal of copepods between estuaries along the mid-Atlantic coast between Cape Hatteras and Cape Cod. However, since the Maine Coastal Current flows from north to south close to shore in the Gulf of Maine, this current may assist dispersal of copepods between adjacent estuaries north of Cape Cod. Therefore, Sequence Group I could be of northern (Canadian/subarctic) origin, with its dispersal facilitated by the Maine Coastal Current. Conversely, the lack of similar alongshore currents between Cape Hatteras and Cape Cod may contribute to isolation of estuarine populations in the southern part of *A. hudsonica*'s range (New Jersey and Long Island Sound), facilitating the endemism of minor sequence groups in these regions.

Deep divergences within *Acartia* species

The levels of divergence for *A. hudsonica* (0.3–31% uncorrected *p*-distances between sequences) are high compared to reported values for other species of marine calanoid copepods (Bucklin et al., 1995, 1999, 2003), and may represent a cryptic species assemblage. In most other calanoids, interspecific variation is typically 10–25%, whereas intraspecific variation is typically <1–2%. However, congeners of *Acartia* exhibit higher intraspecific variation than most other calanoids, typically 10–14% (Caudill & Bucklin, 2004; Chen & Hare, 2008; Durbin et al., 2008). Some of this variation might be due to our measurement of CytB rather than other genes, because CytB has a higher substitution rate than either COI or 16S rDNA, but since we used the higher substitution rate in our divergence-time estimates, the high variation within *Acartia* species found in our study, as well as those of Caudill & Bucklin (2004), Chen & Hare (2008), Durbin et al. (2008) may relate to the general restriction of *Acartia* spp. to isolated estuarine, rather than more open coastal or oceanic habitats.

Molecular clock estimates

Possible divergence times between sequence groups were estimated based on an adjusted molecular clock. We used the divergence rate for the COI gene of snapping shrimps of the genus *Alpheus* of 1.4% per million years (Knowlton & Weigt, 1998), adjusted by the ratio (1.61) of CytB and COI substitution rates in the data of Papadopoulos et al. (2005). This ratio was based on ML estimation using data from individual copepods for which both genes were analyzed concurrently. Our CytB/COI substitution rate ratio is also consistent with the data of Bucklin et al. (1997) for euphausiids. This molecular clock analysis provides divergence-time estimates between *A. hudsonica* sequence groups ranging from 11.1 to 37.0 million years ago (Table 2). These estimates should be treated with caution as small variations in estimated substitution rates can imply large differences in time of divergence.

The maximum divergence time among sequence groups was for the origin of Sequence Group I versus the other sequence groups, with a mean of 37 million years ago (four comparisons, range = 29.92–43.32 million years ago—Table 2). Thus, the maximum divergence time among *A. hudsonica* sequence groups was 37.0/1.19 = 31.1 times greater than between *A. tonsa* and the closest *A. hudsonica* group (Sequence Group III). Using our aforementioned molecular clock procedure, we estimated a divergence time between *A. hudsonica* sequences and the outgroup *E. bungii* sequence of 42.9 million years (results not shown). Thus, divergence within *A. hudsonica* was as great as 37.0/42.9 = 86.2% of the divergence between *A. hudsonica* and *E. bungii*. These divergence estimates, although uncertain, strongly suggest that variation within *A. hudsonica* pre-dates the Pleistocene glaciation that produced the northeastern US estuarine habitats. Similarly, divergence-time estimates for other calanoid copepods (>10 million years) also pre-date Pleistocene glaciation (Bucklin et al., 1992, 1995; Goetze & Bradford-Grieve, 2005).

In the absence of interbreeding between species, the divergence between *A. hudsonica* and *A. tonsa* should be represented by the divergence time between *A. tonsa* and *A. hudsonica* Sequence Group III. This estimated time is 1.19 million years (Table 2). Given the close relation of *A. tonsa* and

A. hudsonica in the data set of this study, as well as the divergent sequence haplotypes identified in the previous studies of *A. tonsa* (Caudill & Bucklin, 2004; Chen & Hare, 2008), *A. tonsa* may harbor some of the same sequence groups found in *A. hudsonica*. Such ancient trans-specific polymorphism, shared between *A. hudsonica* and *A. tonsa* would be older than the separation of these two species.

Estimates of molecular clocks have been used extensively in phylogeographic studies, but they are uncertain due to a variety of factors (Graur & Martin, 2004; Welch & Bromham, 2005; Ho & Larson, 2006; Pulquerio & Nichols, 2006; Donoghue & Benton, 2007). These include estimating age from genetic distance assuming constant rates of genetic change, estimates of times of divergence for taxa that have little or no fossil record, calibration of molecular clocks based on ages of fossils, or earth-history events of uncertain age, different rates of genetic change for different types of organisms, differences in rates of mutations (changes in nucleotides) compared to rates of substitutions (mutations becoming fixed in populations), multiple duplications of portions of DNA ("pseudogenes") which artificially increase apparent diversity in sequences, and calibration against rates of divergence of other organisms that are not closely related.

We inferred a complex DNA substitution model with rate heterogeneity, which could contribute to higher estimated divergence times than in previous studies. Our molecular clock calibration comes from the snapping shrimp *Alpheus* distributed on both sides of the Isthmus of Panama (Knowlton & Weigt, 1998). The Isthmus arose approximately 3 MYA. However, there is disagreement regarding the timing of the separation of the habitats of *Alpheus* by the Isthmus (Hickerson et al., 2003). If the actual DNA divergence rate of our copepods is greater than that of *Alpheus*, then our sequence group ages might be overestimates. Although copepods and *Alpheus* are both crustaceans, they are only distantly related. Nonetheless, usage of *Alpheus* as a reference in molecular clocks is standard in the growing copepod molecular literature.

Our estimated origination times of *Acartia* CytB lineages invite comparisons of historical biogeographic events/scenarios with independent paleoceanographic data. *A. hudsonica* requires colder inshore waters, such as north of Cape Cod, to persist year-round without laying resting eggs. This might make *A. hudsonica* particularly affected by climatic fluctuations. Episodes of cooling might facilitate range expansions and diversification of *A. hudsonica* lineages.

The major divergences between *A. hudsonica* sequence types occurred at approximately 37 MYA (Sequence Group I), 30 MYA (Sequence Group III), and 11–13 MYA (Sequence Groups II, IV, and V). Interestingly, these ages coincide with periods of oceanic cooling and glacial production in both hemispheres (Eldrett et al., 2007, and references therein). Range shifting of an ancestral *Acartia* species during these cooling events, or perhaps vicariance events caused by glaciers spreading across coastlines in an irregular manner, could have created the opportunity for origination of novel *Acartia* genotypes or cryptic species.

Cryptic species

Various species of *Acartia* appear to prosper in estuarine habitats, which are highly fluctuating in physical conditions such as salinity and temperature, and may exclude less euryhaline and eurythermal species. Conversely, dependence on narrow ranges of temperature and salinity for completion of naupliar development (Tester & Turner, 1991), shallow depths for completion of resting egg cycles (Marcus et al., 1994; Sullivan et al., 2007), and/or other factors seem to limit *Acartia* species to estuarine waters. If *Acartia* species have difficulty dispersing through coastal waters between adjacent estuaries, then over time different estuaries may become isolated and therefore, develop genetically distinct populations of *Acartia*. However, some level of dispersal is then required to re-introduce the diverged genotypes into the surrounding estuaries. This could be either a low but persistent level of normal dispersal, or during episodic dispersal, such as recolonization after glacial retreat. The origin and maintenance of divergent lineages could be explained by cryptic species facilitated by ecological adaptation and/or partial reproductive isolation among genotypes. The seasonally offset cycles of resting eggs exhibited by some congeners of *Acartia* in the same estuaries might serve as such an isolating mechanism.

Copepods of the genus *Acartia* appear to be particularly amenable to origination of cryptic

species (Caudill & Bucklin, 2004; Chen & Hare, 2008). Chen & Hare (2008) found concordant mitochondrial and nuclear phylogenies, providing evidence for cryptic species within *A. tonsa*. Chen & Hare's (2008) mitochondrial divergences for *A. tonsa* (13.7%) appear comparable to those among our Sequence Groups II–V in *A. hudsonica* (Table 2). Our even-more-divergent Sequence Group I, the most common lineage in *A. hudsonica*, which co-occurred with the other sequence groups, suggests that *A. hudsonica* may include the most ancient cryptic species discovered to date in copepods.

Evaluation of hypotheses

Our initial hypotheses were that (1) genetic diversity of *A. hudsonica* would be high because the embayments where it is found are relatively isolated, and (2) divergences in *A. hudsonica* would be relatively recent because these embayments had only been formed since the last glaciation. Our expectations were partially met on both accounts. Genetic diversity in *A. hudsonica* was high, but the widespread occurrence of Sequence Group I, at least north of Cape Cod and throughout the Gulf of Maine, and the mixing of sequence haplotypes within estuaries, argue against isolation of these estuaries. Recent studies of red tide blooms in the Gulf of Maine have emphasized the importance of springtime snow melt and runoff from numerous rivers flushing out Maine estuaries into coastal waters (Anderson et al., 2005) as a dispersal mechanism for plankton. This mechanism, together with the southward-flowing Maine Coastal Current, could serve to re-distribute estuarine copepod haplotypes in the Gulf of Maine. Even more surprising, the deep divergences suggested by our molecular clock estimates (11–37 MYA), which long pre-date the most recent episode of Pleistocene glaciation, argue against our hypothesis of recent divergence of these copepods in recently formed estuarine embayments. Indeed, Sequence Group I, which dominated the glaciated estuaries in the Gulf of Maine, was the oldest of the five sequence groups. In conclusion, there is much remaining to be learned of the phylogeography of estuarine copepods in glaciated eastern North America.

Acknowledgments This project was partially funded by a NSF-EPSCoR grant awarded to NVS. DNA sequencing was performed in the Sequencing and Genotyping facility of the University of Puerto Rico-Río Piedras, which is supported in part by NCRR AABRE Grant #P20 RR16470, NIH-SCORE Grant #S06GM08102, the University of Puerto Rico Biology Department, and NSFCREST Grant #0206200.

References

Altschul, S. F., W. Gish, W. Miller, E. W. Meyers & D. J. Lipman, 1990. Basic local alignment search tool. Journal of Molecular Biology 215: 403–410.

Anderson, D. M., B. A. Keafer, W. R. Geyer, R. P. Signell & T. C. Loder, 2005. Toxic *Alexandrium* blooms in the western Gulf of Maine: the plume advection hypothesis revisited. Limnology & Oceanography 50: 328–345.

Bradford, J. M., 1976. Partial revision of the *Acartia* subgenus *Acartiura* (Copepoda: Calanoida: Acartiidae). New Zealand Journal of Marine and Freshwater Research 10: 159–202.

Bucklin, A., B. W. Frost & T. D. Kocher, 1992. DNA sequence variation of the mitochondrial 16S rRNA in *Calanus* (Copepoda: Calanoida): intraspecific and interspecific patterns. Molecular Marine Biology & Biotechnology 1: 397–407.

Bucklin, A., B. W. Frost & T. D. Kocher, 1995. Molecular systematics of six *Calanus* and three *Metridia* species (Calanoida: Copepoda). Marine Biology 121: 655–664.

Bucklin, A., R. C. Sundt & G. Dahle, 1996. The population genetics of *Calanus finmarchicus* in the North Atlantic. Ophelia 44: 29–45.

Bucklin, A., S. B. Smolenack, A. M. Bentley & P. H. Wiebe, 1997. Gene flow patterns of the euphausiid, *Meganyctiphanes norvegica*, in the NW Atlantic based on mtDNA sequences for cytochrome b and cytochrome oxidase I. Journal of Plankton Research 19: 1763–1781.

Bucklin, A., M. Guarnieri, R. S. Hill, A. M. Bentley & S. Kaartvedt, 1999. Taxonomic and systematic assessment of planktonic copepods using mitochondrial COI sequence variation and competitive, species-specific PCR. In Zehr, J. P. & M. A. Voytek (eds), Molecular Ecology of Aquatic Communities. Hydrobiologia 401: 239–254.

Bucklin, A., B. W. Frost, J. Bradford-Grieve, L. D. Allen & N. J. Copley, 2003. Molecular systematic and phylogenetic assessment of 34 calanoid copepod species of the Calanidae and Clausocalanidae. Marine Biology 142: 333–343.

Caudill, C. C. & A. Bucklin, 2004. Molecular phylogeography and evolutionary history of the estuarine copepod, *Acartia tonsa*, on the northwest Atlantic coast. Hydrobiologia 511: 91–102.

Chen, G. & M. P. Hare, 2008. Cryptic ecological diversification of a planktonic estuarine copepod, *Acartia tonsa*. Molecular Ecology 17: 1451–1468.

Citarella, G., 1982. Le zooplankton de la baie de Shédiac (Nouveau-Brunswick). Journal of Plankton Research 4: 791–812.

Davis, M. B. & R. G. Shaw, 2001. Range shifts and adaptive responses to Quaternary climate change. Science 292: 673–679.

Donoghue, P. C. J. & M. Benton, 2007. Rocks and clocks: calibrating the Tree of Life using fossils and molecules. Trends in Ecology and Evolution 22: 424–431.

Durbin, E. G., M. C. Casas, T. A. Rynearson & D. C. Smith, 2008. Measurement of copepod predation on nauplii using a qPCR of the cytochrome oxidase I gene. Marine Biology 153: 699–707.

Ekman, S., 1953. Zoogeography of the Sea. Sidgwick and Jackson, London.

Eldrett, J. S., I. C. Harding, P. A. Wilson, E. Butler & A. P. Roberts, 2007. Continental ice in Greenland during the Eocene and Oligocene. Nature 466: 176–179.

Excoffier, L., P. E. Smouse & J. M. Quattro, 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131: 479–491.

Excoffier, L., G. Laval & S. Schneider, 2005. Arlequin ver. 3.0: an integrated software package for population genetics data analysis. Evolutionary Bioinformatics Online 1: 47–50.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783–791.

Goetze, E. & J. Bradford-Grieve, 2005. Genetic and morphological description of *Eucalanus spinifer* T. Scott, 1894 (Calanoida: Eucalanidae), a circumglobal sister species of the copepod *E. hyalinus* s.s. (Claus, 1866). Progress in Oceanography 65: 55–87.

Graur, D. & W. Martin, 2004. Reading the entrails of chickens: molecular timescales of evolution and the illusion of precision. Trends in Genetics 20: 80–86.

Hickerson, M. J., M. A. Gilchrist & N. Takebayashi, 2003. Calibrating a molecular clock from phylogeographic data: moments and likelihood estimators. Evolution 57: 2216–2225.

Ho, S. Y. W. & G. Larson, 2006. Molecular clocks: when times are a-changin'. Trends in Genetics 22: 79–83.

Knowlton, N. & L. A. Weigt, 1998. New dates and new rates for divergence across the Isthmus of Panama. Proceedings of the Royal Society of London Series B 265: 2257–2263.

Kumar, S., K. Tamura & M. Nei, 2004. MEGA3: integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Briefings in Bioinformatics 5: 150–163.

Lambeck, K. & J. Chappell, 2001. Sea level change through the last glacial cycle. Science 292: 679–686.

Lee, W. Y. & B. J. McAlice, 1979. Seasonal succession and breeding cycles of three species of *Acartia* (Copepoda: Calanoida) in a Maine estuary. Estuaries 2: 228–235.

Machida, R. J., M. U. Miya, M. Nishida & S. Nishida, 2004. Large-scale gene arrangements in the mitochondrial genomes of two calanoid copepods *Eucalanus bungii* and *Neocalanus cristatus* (Crustacea), with notes on new versatile primers for the srRNA and COI genes. Gene 332: 71–78.

Maddison, D. R. & W. P. Maddison, 2000. MacClade: Analysis of Phylogeny and Character Evolution, Version 4.0. Sinauer Associates, Sunderland, MA.

Marcus, N. H., R. V. Lutz, W. Burnett & P. Cable, 1994. Age, viability, and vertical distribution of zooplankton resting eggs from an anoxic basin: evidence of an egg bank. Limnology & Oceanography 39: 154–158.

McAlice, B. J., 1981. On the post-glacial history of *Acartia tonsa* (Copepoda: Calanoida) in the Gulf of Maine and the Gulf of St. Lawrence. Marine Biology 64: 267–272.

Merritt, T. J. S., L. Shi, M. C. Case, M. A. Rex, R. J. Etter & J. M. Quattro, 1998. "Universal" cytochrome *b* primers facilitate intraspecific studies in molluscan taxa. Molecular Marine Biology & Biotechnology 7: 7–11.

Nei, M. & T. Gojobori, 1985. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Molecular Biology and Evolution 3: 418–426.

Papadopoulos, L. N., K. T. C. A. Peijnenburg & P. C. Luttikhuizen, 2005. Phylogeography of the calanoid copepods *Calanus helgolandicus* and *C. euxinus* suggests Pleistocene divergences between Atlantic, Mediterranean, and Black Sea populations. Marine Biology 147: 1353–1365.

Posada, D. & K. A. Crandall, 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14: 817–819.

Pulquerio, M. J. F. & R. A. Nichols, 2006. Dates from the molecular clock: how wrong can we be? Trends in Ecology and Evolution 22: 180–184.

Ronquist, F. & J. P. Huelsenbeck, 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574.

Rozas, J., J. C. Sánchez-DelBarrio, X. Messeguer & R. Rozas, 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics 19: 2496–2497.

Schizas, N. V., G. T. Street, B. C. Coull, G. T. Chandler & J. M. Quattro, 1999. Molecular population structure of the benthic copepod *Microarthridion littorale* along the Southeastern and Gulf coasts of the United States. Marine Biology 135: 399–405.

Sullivan, B. K., J. H. Costello & D. Van Keuren, 2007. Seasonality of the copepods *Acartia hudsonica* and *Acartia tonsa* in Narragansett Bay, RI, USA during a period of climate change. Estuarine Coastal Shelf Science 73: 259–267.

Swofford, D. L., 2003. PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods), Version 4. Sinauer Associates, Sunderland, MA.

Tester, P. A. & J. T. Turner, 1991. Why is *Acartia tonsa* restricted to estuarine habitats? Proceedings of the 4th International Copepod Conference, Bulletin of the Plankton Society of Japan, Special Volume 603–611.

Turner, J. T., 1981. Latitudinal patterns of calanoid and cyclopoid copepod diversity in estuarine waters of eastern North America. Journal of Biogeography 8: 369–382.

Turner, J. T., 1994. Planktonic copepods of Boston Harbor, Massachusetts Bay and Cape Cod Bay, 1992. In Ferrari, F. D. & B. P. Bradley (eds), Ecology and Morphology of Copepods. Proceedings of the 5th International Conference on Copepoda, Baltimore, MD, June 6–12, 1993. Hydrobiologia 292/293: 405–413.

Weir, B. S., 1996. Genetic Data Analysis II. Sinauer Associates, Sunderland, MA.

Welch, J. J. & L. Bromham, 2005. Molecular dating when rates vary. Trends in Ecology and Evolution 20: 320–327.