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VICARIANCE AND DISPERSAL EFFECTS ON PHYLOGEOGRAPHIC STRUCTURE AND SPECIATION IN A WIDESPREAD ESTUARINE INVERTEBRATE

DAVID W. KELLY,[1,2] HUGH J. MACISAAC,[1] AND DANIEL D. HEATH[1]
[1]*Great Lakes Institute for Environmental Research, University of Windsor, Ontario N9B 3P4, Canada*
[2]*E-mail: dwkelly@uwindsor.ca*

Abstract.—Vicariance and dispersal can strongly influence population genetic structure and allopatric speciation, but their importance in the origin of marine biodiversity is unresolved. In transitional estuarine environments, habitat discreteness and dispersal barriers could enhance divergence and provide insight to evolutionary mechanisms underlying marine and freshwater biodiversity. We examined this by assessing phylogeographic structure in the widespread amphipod *Gammarus tigrinus* across 13 estuaries spanning its northwest Atlantic range from Quebec to Florida. Mitochondrial cytochrome *c* oxidase I and nuclear internal transcribed spacer 1 phylogenies supported deep genetic structure consistent with Pliocene separation and cryptic northern and southern species. This break occurred across the Virginian–Carolinian coastal biogeographic zone, where an oceanographic discontinuity may restrict gene flow. Ten estuarine populations of the northern species occurred in four distinct clades, supportive of Pleistocene separation. Glaciation effects on genetic structure of estuarine populations are largely unknown, but analysis of molecular variance (AMOVA) supported a phylogeographic break among clades in formerly glaciated versus nonglaciated areas across Cape Cod, Massachusetts. This finding was concordant with patterns in other coastal species, though there was no significant relationship between latitude and genetic diversity. This supports Pleistocene vicariance events and divergence of clades in different northern glacial refugia. AMOVA results and private haplotypes in most populations support an allopatric distribution across estuaries. Clade mixture zones are consistent with historical colonization and human-mediated transfer. An isolation-by-distance model of divergence was detected after we excluded a suspected invasive haplotype in the St. Lawrence estuary. The occurrence of cryptic species and divergent population structure support limited dispersal, dispersed habitat distribution, and historical factors as important determinants of estuarine speciation and diversification.

Key words.—Amphipod, cryptic speciation, dispersal, estuaries, glacial refugia, marine biodiversity, phylogeography.

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Widespread taxa, specifically those occupying historically glaciated and nonglaciated areas, may prove useful for assessing how vicariance and dispersal limitation influence phylogeographic patterns (Hewitt 2000; Wares and Cunningham 2001; Zamudio and Savage 2003; Marko 2004). Numerous studies have revealed cryptic diversity within such species (Taylor et al. 1998; Knowlton 2000; Rocha-Olivares et al. 2001), and failure to appreciate this pattern can undermine efforts to reconstruct evolutionary processes and speciation patterns.

Habitat and population fragmentation during glaciation are important factors in determining present-day patterns of genetic variation and allopatric speciation (Walker and Avise 1998; Hewitt 2000). For instance, glacial advance both pruned and promoted divergence in freshwater and terrestrial habitats in North America (Bernatchez and Wilson 1998; Holder et al. 1999; Avise 2000; Hewitt 2000; Weisrock and Janzen 2000). In contrast, the apparent lack of physical barriers, coupled with high dispersal potential, suggests that evolutionary mechanisms differ in diverse marine communities (Palumbi 1994; Myers 1997). However, as history, mode of development, and biogeographic zones appear to explain population structure in coastal areas, they may also play an important role in the determination of patterns of marine biodiversity (Engle and Summers 1999; Collin 2001; Wares 2002; Graham et al. 2003). Indeed, glaciation has caused increased diversification and lineage extinction in northern coastal habitats (Hewitt 2000; Edmands 2001; Wares and Cunningham 2001; Marko 2004).

At the freshwater-marine interface, estuaries have characteristics that are well suited to tests of the effects of vicariance and dispersal limitation on population genetic structure (Bilton et al. 2002). Recurrent historical variations in sea level isolated populations (Reeb and Avise 1990), while estuarine colonization has promoted ecological shifts and rapid adaptive divergence of freshwater animals from marine ancestors (Lee and Bell 1999; Beheregaray and Sunnucks 2001; Reusch et al. 2001). Furthermore, spatial isolation and physiological barriers should limit gene flow among estuaries (Cognetti and Maltagliati 2000; Dawson et al. 2001; Bilton et al. 2002), which would be reinforced by selection under different salinity and temperature profiles across latitudinal gradients (Lee 1999; Lee and Bell 1999; Rynearson and Armbrust 2004). Despite their unique ecological and evolutionary attributes, estuaries, as sources of diversification, have received little attention (Bilton et al. 2002).

Gammarus tigrinus is an endemic North American gammaridean amphipod that occurs principally in estuaries of the northwestern Atlantic at salinities up to 20‰ (Bousfield 1958, 1973). The species is distributed from the St. Lawrence River in Quebec to Florida (Bousfield 1958, 1973) and is common or dominant in intertidal and subtidal benthic habitats including reeds, *Enteromorpha*, hard substratum, and sand (Bousfield 1958, 1973; Van Maren 1978; D. Kelly, pers. obs.). Unlike some epiphytic amphipods, *G. tigrinus* is unlikely to disperse by algal rafting (see Myers 1993). *Gammarus tigrinus* is an ideal species with which to examine vicariance and dispersal effects on estuarine phylogeographic patterns. First, its direct development should reduce gene flow and increase population differentiation as compared to

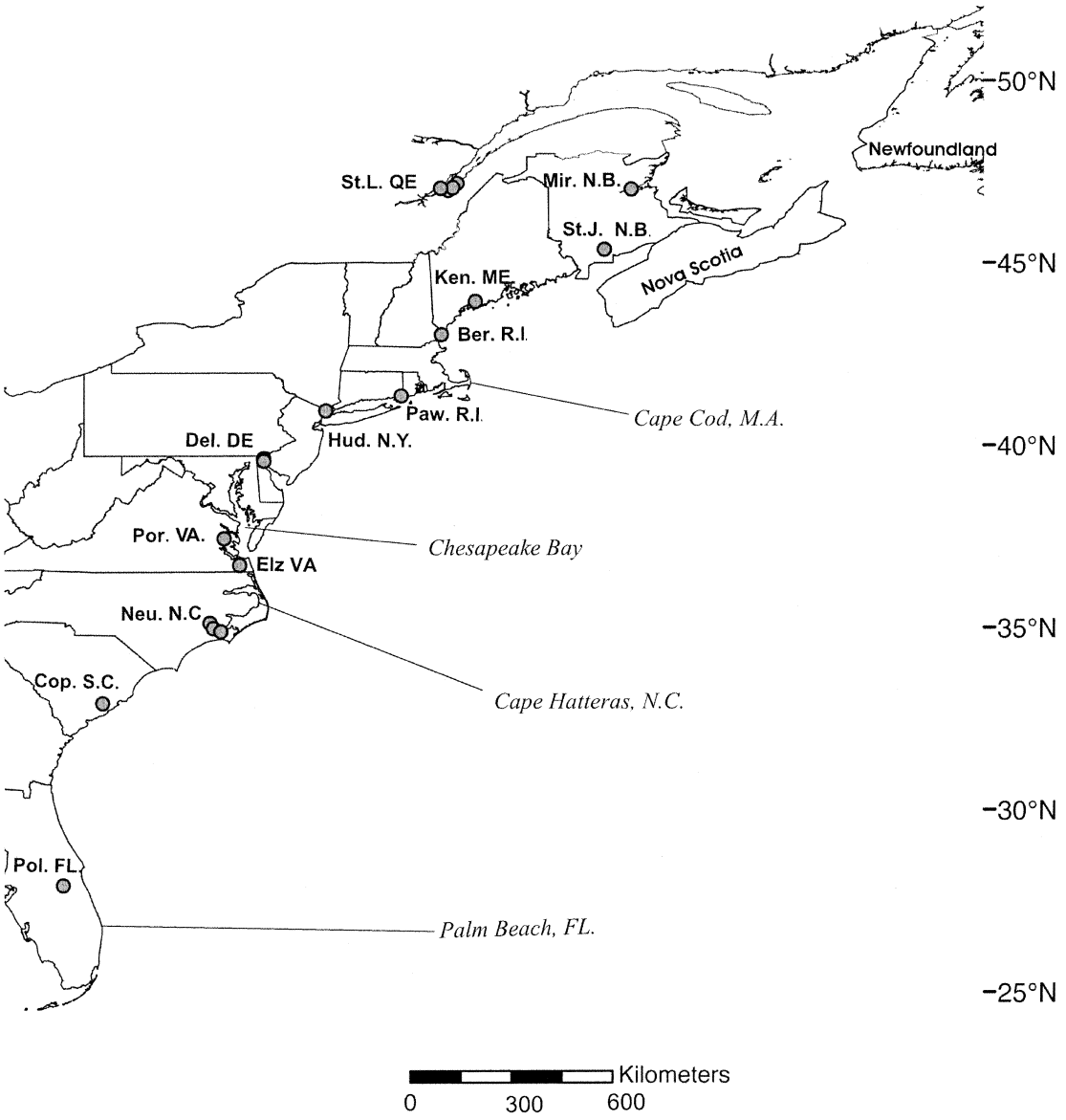

FIG. 1. Location of collection sites for *Gammarus tigrinus* along the northwestern Atlantic coast (see also Table 1). Major landmarks are labeled (see text for details).

vagile or planktonic larval dispersers (Myers 1993; Bilton et al. 2002; Cristescu et al. 2003). Second, its oligomesohaline salinity distribution should preclude propagule dispersal in saline coastal environments, even though its distribution encompasses different zoogeographic zones (Engle and Summers 1999; Avise 2000; Wares 2002). Finally, the species is morphologically similar over its entire Northwest Atlantic range, one that includes formerly glaciated areas (Bousfield 1958, 1973). Based upon these patterns, we expect deep levels of population structure among *G. tigrinus* populations, consistent with ancient vicariant events. Populations in previously glaciated regions should exhibit lower genetic diversity than those in nonglaciated areas; and dispersal should be restricted, even among neighboring estuaries, leading to considerable among-population genetic differences and an isolation-by-distance model of divergence.

MATERIALS AND METHODS

From January to July 2004, *G. tigrinus* was collected from 19 estuarine sites within 13 river systems of the northwestern Atlantic coast, from the St. Lawrence south to Florida (Fig. 1, Table 1). Sites encompassed the full native range as described in Bousfield (1958, 1973). All collection sites were subject to tidal regimes and salinity fluctuations. *Gammarus tigrinus* specimens were preserved in 95% ethanol and identified using the keys of Bousfield (1958, 1973).

Genomic DNA was extracted from a tissue sample of the dorsal pleon following the standard isolation from animal protocol in the Wizard DNA extraction kit (Promega, Madison, WI). A 542-bp fragment of the cytochrome *c* oxidase subunit I gene (COI) was amplified using polymerase chain reaction (PCR) and species-specific primers, forward primer

TABLE 1. Collection sites for *Gammarus tigrinus* populations and number of individuals sequenced per population for the COI gene. Estuary and provincial or state codes are used in Figures 1 and 3.

Location	Site code	Province/State code	*n*	Latitude	Longitude
St. Lawrence estuary, Montmagny, Quebec	StL	QE	7	46°58′23″	−70°60′14″
St. Lawrence, Islet sûr Mer, Quebec	StL	QE	4	47°10′51″	−70°16′23″
St. Lawrence, Cap Brulé, Quebec	StL	QE	8	47°06′58″	−70°42′24″
St. Lawrence, north channel, Quebec	StL	QE	6	47°02′57″	−70°45′36″
Miramichi estuary, New Brunswick	Mir	NB	12	47°01′25″	−65°30′03″
St. John estuary, New Brunswick	StJ	NB	9	45°22′01″	−66°14′10″
Kennebac estuary, Maine	Ken	ME	5	43°56′36″	−69°47′58″
Berrys Creek, New Hampshire	Ber	NH	11	43°02′35″	−70°44′03″
Pawcatuck estuary, Rhode Island	Paw	RI	10	41°20′04″	−71°49′48″
Hudson estuary, New York	Hud	NY	10	40°57′22″	−73°53′51″
Delaware estuary, Deemers beach, Delaware	Del	DE	8	39°38′41″	−75°35′32″
Delaware estuary, Reedy point, Delaware	Del	DE	5	39°34′22″	−75°34′50″
Poropotank estuary, Virginia	Por	VA	10	37°27′16″	−76°40′04″
Elizabeth estuary, canal locks, Virginia	Elz	VA	9	36°43′24″	−76°14′44″
Neuse estuary, New Bern, North Carolina	Neu	NC	7	35°08′22″	−77°03′36″
Neuse estuary, Neuse Harbor, North Carolina	Neu	NC	2	34°59′38″	−76°57′47″
Neuse estuary, Clubfoot Creek, North Carolina	Neu	NC	2	34°54′25″	−76°45′45″
Goose Creek, Cooper River, South Carolina	Cop	SC	7	32°56′33″	−80°03′39″
Poley Creek, Florida	Pol	FL	11	27°57′00″	−81°58′00″

5′-TGC TTG AGC AAG TGC CTT AG-3′, reverse primer 5′-CTC TAG GGT CAA AGA AGG AAG-3′. We initially used the universal COI primer pair LCO1490 and HCO2198 (Folmer et al. 1994), but only a few populations from the St. Lawrence to the Pawcatuck were successfully sequenced, generating a fragment length of 620 bp. Forward and reverse specific primers were then designed by aligning these sequences and searching for conserved primer annealing regions at the 5′ and 3′ ends, respectively. Each 25-μl reaction consisted of 2.5 μl of 10 × PCR buffer (20 mM Tris-HCl, pH 8.0, 100 mM KCl), 2.5 mM $MgCl_2$, 0.2 mM of each oligonucleotide, 2 μM of each primer, 0.5U of *Taq* DNA polymerase (Sigma-Aldrich, St. Louis, MO), and 1–3 μl of template DNA. Amplification involved initial denaturing at 94°C for 3 min, then 40 cycles of 94°C for 1 min, 60°C (COI) for 1 min, and 72°C for 1 min; and a final extension at 72°C for 5 min. PCR products from a total of 143 amphipods were sequenced using the DTCS Quick Start cycle sequencing kit and CEQ8000 automated sequencer (Beckman Coulter, Fullerton, CA), following the manufacturers' instructions. Using the same protocol, and with individuals collected in the Delaware River, COI sequences were obtained from *Gammarus daiberi*, another North Atlantic estuarine species.

Because levels of phylogeographic structuring suggested cryptic northern and southern species (see Results) apparently driven by historical limits to gene flow, an independent assessment of phylogeny was carried out (e.g., Schizas et al. 1999). A subset of individuals spanning the distribution of *G. tigrinus* was assessed for variation in the nuclear ribosomal internal transcribed spacer 1 (ITS1) region using the forward primer 5′-CAC ACC GCC CGT CGC TAC TAC CGA T-3′, specific for the small ribosomal subunit, 18S, and reverse primer 5′-GCG GCA ATG TGC ATT CGA CAT GTG A-3′, specific for the 5.8S ribosomal subunit. PCR and sequencing conditions were as for COI but with an annealing temperature of 58°C. ITS1 may exhibit intra-individual variation (Harris and Crandall 2000); thus, only individuals with single well-defined PCR bands were chosen for sequencing. As an outgroup in the analysis, an ITS1 sequence was obtained from GenBank (accession no. AY004852) for the amphipod *Ampithoe longimana*.

Phylogenetic Analysis

All sequences were aligned using the Clustal W alignment algorithm in Omiga 1.2 (Oxford Molecular Ltd., Oxford, U.K.) and confirmed by eye. No insertions or deletions (indels) were found in the COI alignment. To test for mutation saturation in the COI gene fragment, transitions (Ts) were plotted against transversions (Tv; determined in MEGA 3.0; Kumar et al. 2004), and the resulting scatterplot examined visually for evidence of saturation. Patterns of phylogeographic divergence in the distance matrix were assessed using the neighbor-joining (NJ) algorithm in MEGA and estimated using Kimura's two-parameter distance model with nodal support calculated using 10,000 bootstraps (Tamura et al. 2004). Maximum parsimony (MP) heuristic searches were also conducted on all unique COI haplotypes in PAUP* (ver. 4.0b10; Swofford 2001) using the branch-swapping algorithm, tree bisection reconnection (TBR) with 100 random stepwise additions. Branch support was obtained with 1000 bootstrap replicates. For the ITS1 dataset, sequence differences among populations were characterized both by point mutation substitutions and indels (see Results). Thus, indels were also included in the analysis because they contain important phylogenetic information (Vogler and DeSalle 1994; Girabet and Wheeler 1999; Simmons and Onchoterena 2000). Single gaps were initially treated as fifth character states in PAUP*. However, because indels may occur as single evolutionary events regardless of size (Girabet and Wheeler 1999), simple indel coding was used as a conservative approach implemented using the program GapCoder (Young and Healy 2003), where each indel with a different start and end position is considered as a single character in the data matrix (Simmons and Onchoterena 2000). The converted data matrix was then imported to PAUP* and heuristic searches conducted using the same parsimony criteria as for COI.

Estimates of population divergence employed the COI molecular clock of Knowlton et al. (1993) and Knowlton and Weight (1998), where sequence divergence for snapping shrimp ranged from 1.4% to 2.6% per million years. Thus, we cautiously applied an intermediate molecular clock of 2% sequence divergence per million years (see also Cox and Hebert 2001; Cristescu et al. 2003).

Population Genetic Structure

Population structure was assessed in Arlequin 2.0 (Schneider et al. 2000). To characterize structure among estuaries we used an analysis of molecular variance (AMOVA; Excoffier et al. 1992) conducted on sequence divergences, where the total variance is partitioned into components analogous to *F*-statistics. These were tested using 10,000 permutations of haplotypes between populations and an alpha value of 0.05 (Schneider et al. 2000). Levels of phylogeographic divergence were deep and suggestive of two cryptic species, one distributed across the eight northern populations and the other across the three southern populations (see Results). Thus, AMOVA was performed on populations of the northern phylogroup only. We used a hierarchical approach to assess the partitioning of molecular variation both within estuarine samples and among estuaries. We also tested the importance of more ancient vicariant effects by a priori assigning populations to one of two assumed vicariance groups: estuaries of latitude $<41°$N (includes the Elizabeth north to the Hudson) or estuaries of latitude $>41°$N (includes the St. Lawrence to the Pawcatuck south of Cape Cod, see Fig. 1). The grouping was used for the following reasons. First, phylogenetic breaks have been reported for several intertidal species in this latitudinal range, which occurs in an area just south of Cape Cod (Wares 2002). Second, Bousfield (1973) identified a 120-km radius around the Cape Cod region where the range of known amphipod species with more northerly distributions overlaps with those species having more southerly distributions. Third, ice advanced as far south as latitude 41°N in North America and had a profound influence on genetic population structure (Bernatchez and Wilson 1998; Hewitt 2000). Finally, this region spanned a geographic break identified in NJ and MP analyses (see Results).

To determine if geographic patterns existed with respect to the distribution of genetic diversity, we assessed nucleotide diversity (π; see Nei and Kumar 2000) and its variance and haplotype diversity (H_e) for each clade and population of the northern phylogroup using Arlequin. Latitude was regressed against nucleotide and haplotype diversity ($\log[x + 1]$ transformed) for each population.

A Mantel test assessed the correlation between genetic and geographic distance for populations of the northern phylogroup, and whether the form of dispersal conformed to an isolation-by-distance model (see Slatkin 1993). We regressed log geographic distance—measured in ArcGis version 9.0 (ESRI, Redmonds, CA) using the shortest coastal route—against estimates of population pairwise F_{ST}-values generated from genetic distances. The significance of the correlation was assessed using 10,000 permutations (Schneider et al. 2000).

RESULTS

Phylogenetic Analysis

Forty-four haplotypes were detected in the 143 individuals assayed for the 542-bp region of the COI gene (GenBank accession numbers DQ300208–DQ300251). In total, we detected 94 polymorphic sites, of which 79 were phylogenetically informative. There were four haplotypes detected in the outgroup species *G. daiberi* (GenBank accession numbers DQ300252–DQ300255).

Pairwise sequence comparisons showed no evidence of leveling off in a plot of Ts versus Tv, indicating a lack of transitional mutation saturation (see Kocher and Carleton 1997). When only transversions were used in the NJ analysis, we uncovered a toplogy similar to that using all mutations in the NJ and MP analyses (Fig. 2a,b). Populations were strongly structured spatially, with both trees revealing two reciprocally monophyletic and deeply divergent phylogroups (Fig. 2a,b). The first, designated ''N,'' includes populations from 10 estuaries distributed from the Elizabeth and Chesapeake Bays north to the St. Lawrence River estuary (Fig. 3). The second, designated ''S,'' includes populations from the remaining three systems from the Neuse estuary, North Carolina, south to Poley Creek, Florida.

Phylogroups were also highly substructured. Two clades, S1 and S2, were recovered in the S phylogroup, both receiving high bootstrap support using MP and distance methods (Figs. 2a,b). In the N phylogroup, four clades (N1–N4) received high bootstrap support, although support for the subdivision of clades N1–N3 was higher using MP. Clades N1 and N2 had Acadian Atlantic distributions, the former from the St. Lawrence to Berrys Creek (north of Cape Cod, MA), and the latter, more restricted, occurring in the Pawcatuck estuary south of Cape Cod as well as in the Miramichi estuary. Clade N3 occurred in the Hudson, Delaware, and Elizabeth estuaries of the mid-Atlantic, whereas clade N4 occurred in the Delaware, Poropotank, and Elizabeth estuaries (Figs. 2a,b, 3). Admixture zones were apparent: one in the Miramichi estuary for clades N1 and N2 and two more in the Delaware and Elizabeth estuaries for clades N3 and N4. Haplotypes from N1 and N3 were sympatric in the St. Lawrence estuary and highly discontinuous geographically relative to the other admixtures (Fig. 3). A closer look at the phylogenetic trees showed that each estuary was highly differentiated since, apart from the St. Lawrence–Hudson and Delaware-Poropotank, haplotypes in all estuaries were unique (Fig. 2a,b).

Mean sequence divergence for the major N and S phylogroups was high, ranging between 8.9% and 12% (Table 2), with the molecular clock estimating common ancestry 4.4–6.0 million years ago in the mid-Pliocene. Clades S1 and S2 diverged by 5.1%, with estimates of common ancestry in the late Pliocene–early Pleistocene. Clades N1 to N4 were also relatively ancient, diverging from between 1.2% and 3.1%, 0.60–1.55 million years ago during the Pleistocene (Table 2).

For the ITS1 region, sequences varied in length across populations due to indels (northern region: St. Lawrence, 550–552 bp; Pawcatuck, Poropotank, and Delaware, 552–556 bp; southern region: Neuse and Poley, 562 and 563 bp, re-

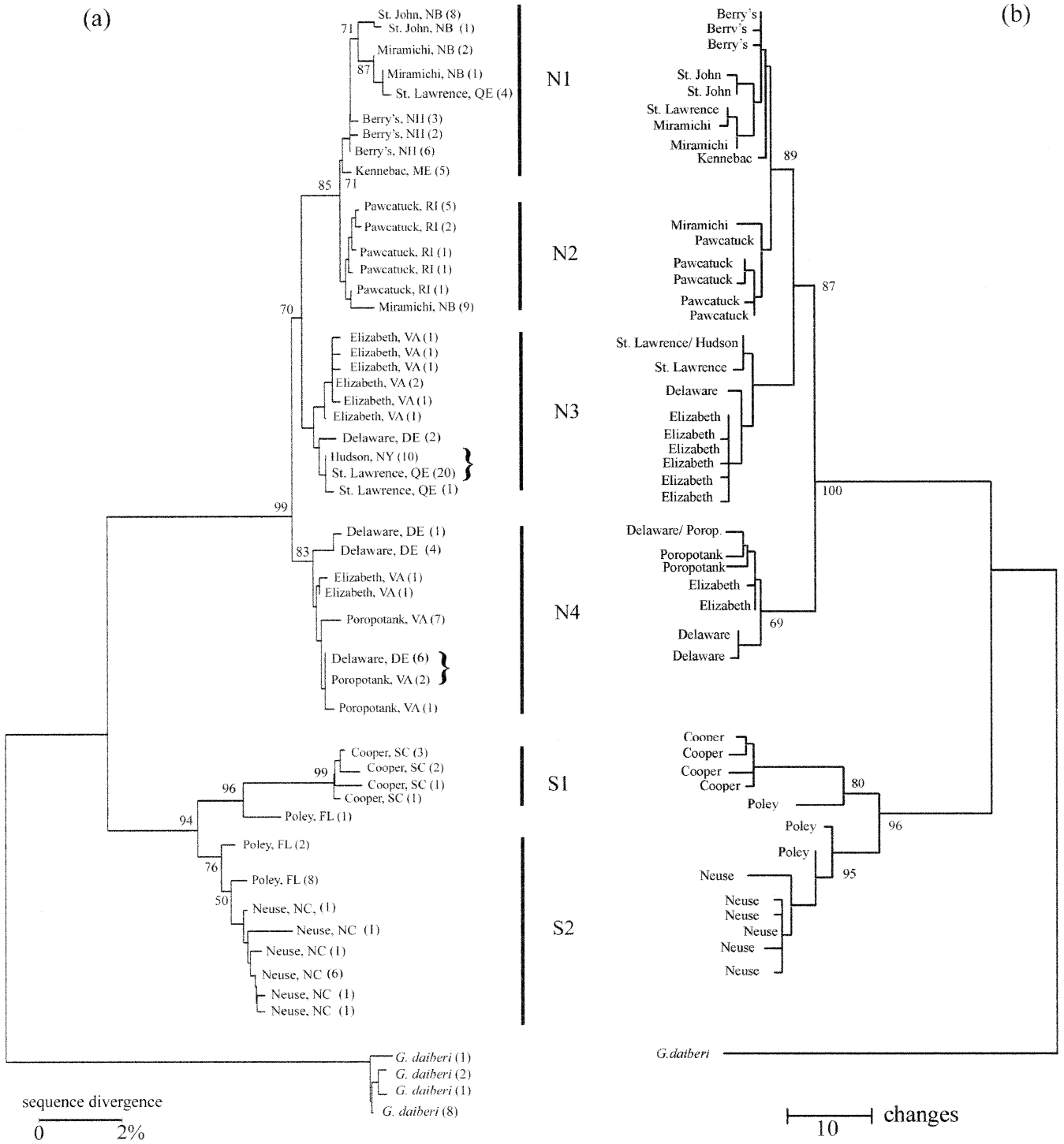

FIG. 2. (a) Neighbor-joining and (b) maximum parsimony trees, based on sequence variation for a 542-bp region of the COI gene across 13 estuarine populations of *Gammarus tigrinus*. The distance matrix used Kimura's two-parameter model of nucleotide substitution. Counts of each haplotype are indicated in parentheses. Brackets denote haplotypes that occurred in more than one estuary. Parsimony analysis used heuristic searches of all unique haplotypes and the tree is based on 50% majority-rule consensus. Nodal support is indicated by bootstrap values (1000 pseudoreplicates). Solid vertical bars delineate each of the main lineages. *Gammarus daiberi* sequences were used to root both trees.

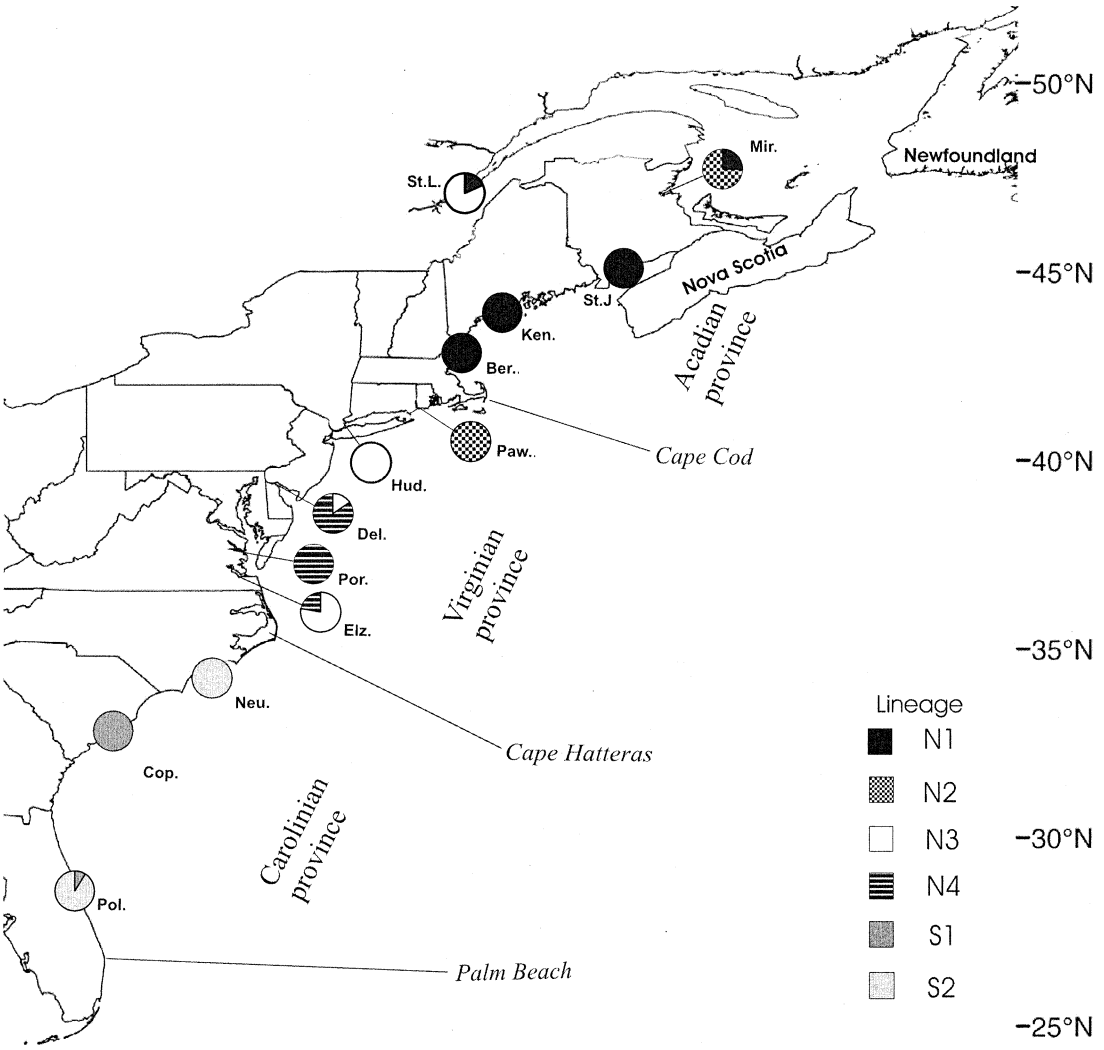

FIG. 3. Distribution of *Gammarus tigrinus* lineages identified in the neighbor-joining analysis (see Fig. 2a). Pie charts represent lineage frequencies. The three major coastal biogeographic provinces and their end points are shown.

spectively). Fixed differences also distinguished populations in the northern from those in the southern regions. An adenine base substitution occurred at position 453 in both Poley and Neuse populations, but as thymine in all northern populations (GenBank accession numbers DQ300256–DQ300260). There were also three fixed indel differences in Poley and Neuse populations: two insertions at positions 284 and 315 and a deletion at position 332. These differences were supported in the two methods of indel coding, as both resulted in similar tree topologies, though here we present results for only the more conservative simple indel coding method (Fig. 4). The ITS1 phylogeny was congruent with reciprocal monophyly of N and S phylogroups in the COI analyses. The limited resolution of external nodes in comparison to the COI analyses may reflect a much larger effective population size (N_e) of nuclear genes (Hudson and Turelli 2003).

TABLE 2. Mean pairwise sequence divergence (Kimura two-parameter) between *Gammarus tigrinus* lineages identified in the neighbor-joining analysis for a 542-bp region of the COI gene. Sequence divergence is shown above the diagonal, and standard errors from 1000 bootstrap replicates estimated in MEGA are below the diagonal.

Lineage	N1	N2	N3	N4	S1	S2	*G. daiberi*
N1		0.012	0.022	0.031	0.113	0.093	0.171
N2	0.004		0.022	0.030	0.113	0.091	0.169
N3	0.006	0.006		0.018	0.11	0.086	0.167
N4	0.007	0.007	0.005		0.12	0.89	0.165
S1	0.017	0.017	0.016	0.017		0.051	0.156
S2	0.014	0.014	0.014	0.014	0.01		0.163
G. daiberi	0.024	0.024	0.024	0.023	0.022	0.023	

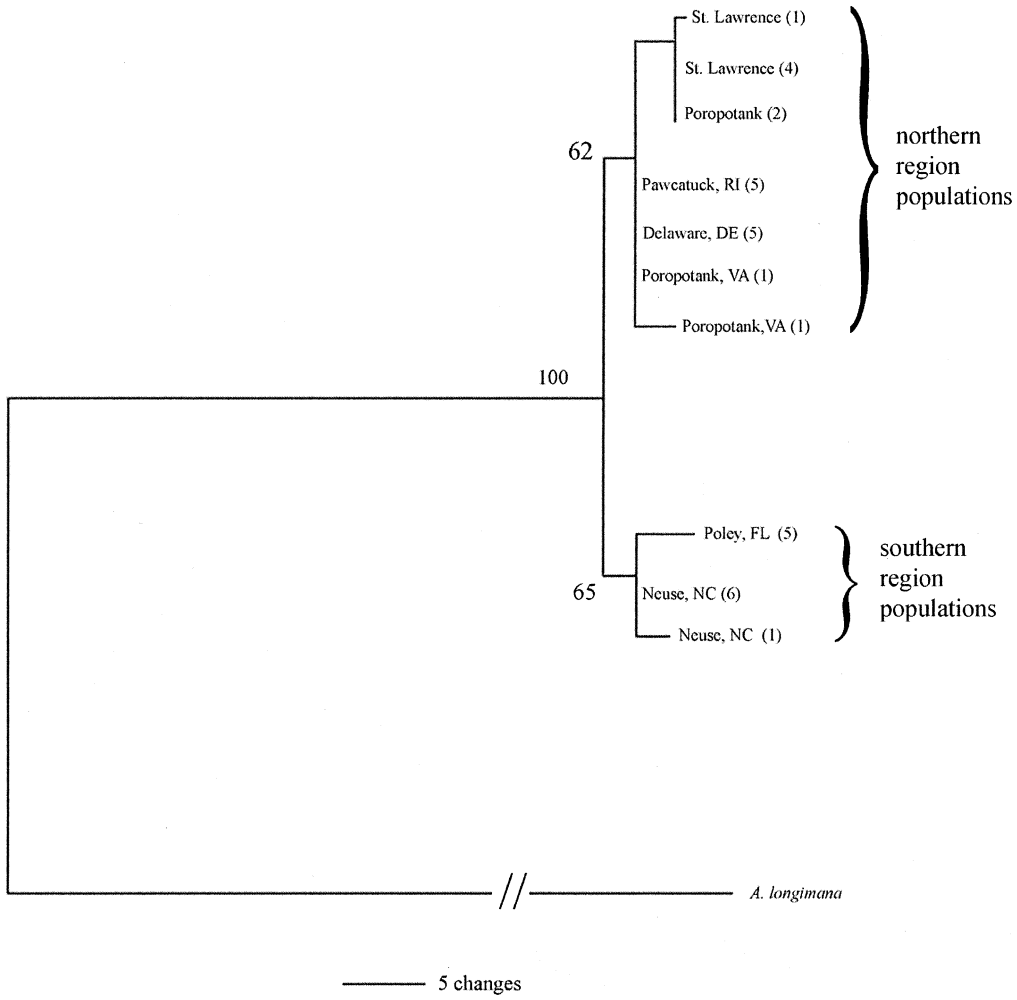

FIG. 4. Maximum parsimony tree based on sequence variation in the nuclear ribosomal ITS1 region across four northern and two southern estuarine populations of *Gammarus tigrinus*. Analysis used heuristic searches and the tree is based on 50% majority-rule consensus. Nodal support is indicated by bootstrap values (1000 pseudoreplicates). The tree shown was generated using a sequence alignment with simple indel coding of gap characters (see text). Frequencies for each haplotype are indicated in parentheses.

Population Genetic Structure

The AMOVA showed significant genetic structuring in the N phylogroup, with 44% of the genetic variation partitioned among estuaries (Table 3). However, geographic grouping explained 36% of genetic variation and was attributable to differences between estuarine populations north (N1, N2) as compared to those south (N3, N4) of latitude 41°N (Table 3; Figs. 2, 3). As the AMOVA incorporated the disjunct St. Lawrence haplotype from the N3 clade in the regional grouping north of 41°N, it may be an inappropriate regional grouping. However, we discuss a possible role for human-mediated dispersal of this haplotype (see Discussion), and, after its removal, the AMOVA showed an increase in the percentage of variation attributable to differences north and south of 41°N. Thus, genetic variation explained between regions in the initial AMOVA likely underestimates divergence.

There was no geographic pattern with respect to the distribution of diversity among clades of the N phylogroup. The highest diversity occurred in the most northern (N1) lineage (π = 0.0061, SD = 0.003, H_e = 0.872), followed by the most southern one (N4: π = 0.0059, SD = 0.003, H_e = 0.794). In the N2 lineage, which occurred north of N3, diversity was also higher (N2: π = 0.0058, SD = 0.003, H_e = 0.73; N3: π = 0.003, SD = 0.002, H_e = 0.678). Regression

TABLE 3. Analysis of molecular variance results of population structure in the *Gammarus tigrinus* northern phylogroup with among group variation based on those populations north and south of 41°N (see text for details).

Source of variation	df	Sum of squares	Variance component	Variation	*P*
Among groups	1	156.5	2.27	35.99%	<0.005
Among populations within group	8	255.1	2.78	43.95%	<0.0001
Within populations	104	132.0	1.27	20.06%	<0.0001

analyses were consistent with this finding, as neither population estimates of nucleotide nor haplotype diversity were related to latitude ($r = -0.106$, $P = 0.77$; $r = -0.343$, $P = 0.33$, respectively). Exclusion of the N3 St. Lawrence haplotype from the regressions after did not alter these results ($r = -0.31$, $P = 0.41$; $r = -0.47$, $P = 0.17$, respectively).

A MANTEL test demonstrated that only 2.1% of genetic variation was explained by geographic distance ($P = 0.8$); thus, we observed no evidence of isolation-by-distance effects. After excluding the St. Lawrence outlier, a slightly better relationship explaining 8% of variation was obtained ($P = 0.047$).

Discussion

Studies of geographic range and life history may overlook patterns of diversity and underlying evolutionary processes, particularly in diverse marine communities where barriers to gene flow are not readily apparent (Palumbi 1994; Bastrop et al. 1998; Knowlton 2000; Rocha-Olivares et al. 2001; Hebert et al. 2003a). Estuaries are excellent systems with which to address this issue, though studies have failed to explicitly identify the geographic background against which this might occur (Bilton et al. 2002). Our study revealed deep phylogeographic structure, indicative of allopatric separation, in *G. tigrinus*, a widespread species of the northwest Atlantic (Figs. 2a,b, 3; Table 2). Mitochondrial data supported the existence of major northern and southern phylogroups, which shared a common ancestor 4.4–6.0 million years ago, despite their separation over a small geographic distance. COI divergence levels between these phylogroups are similar to that reported in other studies of cryptic species (Knowlton et al. 1993; Witt and Hebert 2000; Rocha-Olivares et al. 2001) and are within the range determined for congeneric pairs in 13,000 animal species (Hebert et al. 2003b). However, Hudson and Turelli (2003) warned against species inference based solely on monophyly and divergence in mitochondrial markers. Only in cases where nuclear loci provide similar levels of divergence and monophyly can species status be confidently invoked (Schizas et al. 1999; Knowlton 2000). We identified a concordant nuclear phylogeny and, thus, propose a taxonomic revision to *Gammarus carolina* for populations formerly considered *G. tigrinus* south of Cape Hatteras, reflecting a distribution coincident with the Carolinian biogeographic zone.

The mid-Atlantic deep divergence of the phylogroups is surprising given the much shallower breaks reported for other species of this region (see Wares 2002), but it is similar to those observed across the Florida peninsula (Avise 1992; Vogler and DeSalle 1993). Formation of the Labrador Current 3.0 million years ago may have displaced temperate faunas south and isolated northern populations (see Nilsson 1983; Wares 2001), which then would be reinforced by dispersal limitation or selection due to near-shore hydrodynamics (see Palumbi 1994; Dawson et al. 2001; Wares 2002). For example, our study spanned three zoogeographic provinces (Engle and Summers 1999; Fig. 3) with the break occurring across Cape Hatteras, where steep temperature gradients and currents converge at the Virginian and Carolinian provincial boundaries (Palumbi 1994; Engle and Summers 1999). Here, breaks or range point ends in other species are linked to dispersal ability and the physical conditions (Saunders et al. 1986; Bastrop et al. 1998; Jones and Quattro 1999; Collin 2001), which likely preclude gene flow through vicariance or selection (Palumbi 1994; Sotka et al. 2004; Stevens and Hogg 2004).

However, even within phylogroups, divergent clades were consistent with late Pliocene to mid-Pleistocene separation. Inter- and intraspecific divergence in northern latitudes may arise from historical fragmentation due to glaciation (Walker and Avise 1998; Hewitt 2000; Grunwald et al. 2002), but such a process seems less likely in estuarine species due to the ephemeral nature and generally short geological histories of such habitats (see Bilton et al. 2002). Focusing only on the northern species, clades N1 and N2 were distributed in formerly glaciated areas and diverged markedly when compared with clades N3 or N4 (both of which occurred in nonglaciated areas). This break coincides with the Acadian–Virginian transition zone, where glaciation and physical gradients have been invoked to explain a similar discontinuity in coastal species (Engle and Summers 1999; reviewed by Wares 2002; Fig. 3). We expected reduced diversity in *G. tigrinus* populations of formerly glaciated areas, as is the case for many coastal and freshwater species (see Bernatchez and Wilson 1998; Wares 2002; Marko 2004; McMillen-Jackson and Bert 2004). The lack of significant latitude-diversity correlations contradicts that expectation, though our small within-estuary sample sizes may have limited our detection power. Inclusion of individuals from multiple estuarine populations resulted in much larger clade sample sizes, yet northern clades N1 and N2 still showed no evidence for reduced genetic diversity relative to N3 and N4. The high nucleotide and haplotype diversities in clades N1 and N2 are thus consistent with northern refugial survival and suggest large long-term N_e or a mixture of individuals from historically separated populations (sensu Avise 2000). Such a pattern could be explained by the isolation and extirpation of lineages followed by remixing during the Pleistocene cyclical marine intrusions of large estuarine embayments (see Dawson et al. 2001; Jacobs et al. 2004).

Although clade N3 occurred farther north than N4, these clades were sympatric in the Chesapeake and Delaware estuaries. Here, N3 may have a relict distribution caused by an isothermal contact zone that occurred farther south during the Pleistocene (Cronin 1988). This concurs with a pattern in the salt-marsh killifish *Fundulas heteroclitus*, where the gradual northward shift of the isotherm may have caused extirpation or entrapment of individuals from northern lineages in both bays (Smith et al. 1998). This could explain the high haplotype diversity but low nucleotide diversity of the N3 clade, if, for example, the process resulted in an ancestral population of small N_e followed by rapid population growth given sufficient time for mutations to elevate haplotype diversity alone (Avise 2000).

Historical environmental shifts and resulting complex population dynamics coupled with large-scale coastal hydrography are important factors in estuarine population divergence, but it has been suggested that isolation, habitat characteristics, and larval retention should reinforce this (Wares and Cunningham 2001; Bilton et al. 2002). In this study,

AMOVA and the occurrence of private haplotypes in most estuaries (including in the southern species) is consistent with the importance of isolation as well as *G. tigrinus* life history. Indeed, unlike algal rafting species, long-distance dispersal is probably rare in estuarine amphipods and other species that brood their young (see Myers 1993; Thomas et al. 1998; Thiel 2002; Sotka et al. 2003). For example, in the nest-brooding tidewater goby, *Eucyclogobbius newberryi*, populations along the Californian coast experience little gene flow (Dawson et al. 2001). Likewise, studies of sessile estuarine polychaetes demonstrated strong genetic structure along North American and European coasts (Bastrop et al. 1998; Virgilio and Abiatti 2004). It should be noted, however, that population structure can evolve even without the prerequisite of low dispersal potential. For example, Perrin et al. (2004) linked variation in gene flow among fjord populations of vagile sea stars *Coscinasterias muricata* to differences in internal estuarine circulation patterns. In the holoplanktonic copepod *Acartia tonsa*, Caudill and Bucklin (2004) found some evidence for genetic differentiation across four Atlantic coast estuaries. They suggested that these patterns could be caused either by retention of individuals through behavioral mechanisms or lineages within benthic diapausing egg banks.

Evidence exists that salinity alone may explain patterns of population divergence in some vagile species. A survey of coastal fish species showed that those presumed capable of inhabiting estuarine and marine habitats were more divergent than population pairs of the same species from marine habitats (Watts and Johnson 2004). Thus, salinity gradients may impose physiological limits to dispersal or impose strong selection pressures on dispersing individuals (Lee and Bell 1999; Bilton et al. 2002). Indeed, Lee (2000) suggested that selection might even occur across salinity gradients within individual estuaries. However, data from this study do not support this contention for *G. tigrinus*. In several of our sampled estuaries, multiple samples were obtained at sites crossing salinity gradients. For example, sites in the St. Lawrence estuary (see Table 2) ranged by up to 7‰, whereas those in the Delaware and Neuse varied by 8‰ and about 12‰, respectively. Preliminary phylogenetic analysis showed that haplotypes across sites within estuaries were identical or occurred in the same subclades. Across all estuaries, we also failed to detect *G. tigrinus* at sites of salinity in excess of 25‰, while laboratory studies have observed high mortality above 20‰ (Dorgelo 1974; Savage 1982). These data suggest that high coastal salinity could impede or prevent dispersal and contribute to allopatric diversification (sensu Bilton et al. 2002).

If populations are structured by dispersal limitation, an isolation-by-distance geographic pattern is expected (Slatkin 1993). Our initial analysis failed to identify such a pattern, possibly owing to human-mediated dispersal (see Cohen and Carlton 1998; Darling et al. 2004). *Gammarus tigrinus* has an extensive human-mediated dispersal history in Europe (see Jażdżewski et al. 2004), which could explain the St. Lawrence haplotype of the N3 clade as an outlier in our analysis (see Figs. 2, 3). Several lines of evidence support human-mediated dispersal and the Hudson estuary as the source. This haplotype was identical to that found in the Hudson estuary, while other estuarine populations in the region grouped in the N3 clade, indicating that the Hudson estruary is likely the source of this clade. Also, ballast transfers from past commercial shipping and current recreational boating connect the Hudson and St. Lawrence via navigational canals (Mills et al. 1996; Daniels 2001). When we excluded this questionable haplotype, a weak but significant isolation-by-distance effect was detected. However, greater spatial sampling and larger sample sizes are required to confirm whether the Hudson estuary was the source of the St. Lawrence population.

Mechanisms contributing to the evolution of marine diversity and population structure continue to challenge researchers (Palumbi 1994; Wares and Cunningham 2001). Coastal studies have made important contributions by identifying the respective roles of history, physical barriers, and environmental gradients (Knowlton 2000; Wares 2002). Our study shows that estuarine environments are important in the creation of genetic diversity—and hence in the speciation process. While vicariance and habitat distribution clearly contribute to cryptic speciation in *G. tigrinus*, low intrinsic dispersal and high salinity may enhance population segregation in a manner analogous to that observed in discrete habitats such as lakes (see Taylor and McPhail 2000; Witt and Hebert 2000; Cristescu et al. 2003). Furthermore, ecological and genetic divergence in freshwater populations recently derived from estuarine habitats suggests that these transitional zones may promote freshwater diversity (e.g., Lee 1999; Beheregaray and Sunnucks 2001). Estuaries may thus be hotbeds of speciation, though their evolutionary potential could depend on their transient nature or the homogenizing influence of human-mediated species introductions (Bilton et al. 2002; Jacobs et al. 2004; Hauswaldt and Glenn 2005). If deep population structure is a general feature of estuaries, management of threatened and or commercially important coastal species must include such population structure as a critical factor for effective conservation (e.g., see Pierce et al. 2000; Grunwald et al. 2002; McMillen-Jackson and Bert 2004). To understand the interplay of dispersal, historical processes, and transitional environmental zones in estuarine diversification, future studies of species that vary in vagility and life history are needed. If population genetic studies, like this one, are undertaken in tandem with studies of habitat use and ecology, a more complete understanding of the evolutionary mechanisms operating across aquatic habitats and species will result.

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Corresponding Editor: K. Crandall