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GLOBAL PHYLOGEOGRAPHY OF A CRYPTIC COPEPOD SPECIES COMPLEX AND REPRODUCTIVE ISOLATION BETWEEN GENETICALLY PROXIMATE ''POPULATIONS''

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Abstract.—The copepod *Eurytemora affinis* has a broad geographic range within the Northern Hemisphere, inhabiting coastal regions of North America, Asia, and Europe. A phylogenetic approach was used to determine levels of genetic differentiation among populations of this species, and interpopulation crosses were performed to determine reproductive compatibility. DNA sequences from two mitochondrial genes, large subunit (16S) rRNA (450 bp) and cytochrome oxidase I (COI, 652 bp), were obtained from 38 populations spanning most of the species range and from two congeneric species, *E. americana* and *E. herdmani*. Phylogenetic analysis revealed a polytomy of highly divergent clades with maximum sequence divergences of 10% in 16S rRNA and 19% in COI. A power test (difference of a proportion) revealed that amount of sequence data collected was sufficient for resolving speciation events occurring at intervals greater than 300,000 years, but insufficient for determining whether speciation events were approximately simultaneous. Geographic and genetic distances were not correlated (Mantel's test; $r = 0.023$, $P = 0.25$), suggesting that populations had not differentiated through gradual isolation by distance. At finer spatial scales, there was almost no sharing of mtDNA haplotypes among proximate populations, indicating little genetic exchange even between nearby sites. Interpopulation crosses demonstrated reproductive incompatibility among genetically distinct populations, including those that were sympatric. Most notably, two geographically distant (4000 km) but genetically proximate (0.96% 16S, 0.15% COI) populations exhibited asymmetric reproductive isolation at the F_2 generation. Large genetic divergences and reproductive isolation indicate that the morphologically conservative *E. affinis* constitutes a sibling species complex. Reproductive isolation between genetically proximate populations underscores the importance of using multiple measures to examine patterns of speciation.

Key words.—Biogeography, cryptic speciation, dispersal, *Eurytemora affinis*, hybrid breakdown, phylogeography.

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Sibling species are common in marine habitats, reflecting both inadequate study of morphological features and lack of divergence in morphology accompanying speciation events (Knowlton 1993). In addition, species boundaries are often difficult to define because of lack of data that link genetic and morphological diversity with patterns of reproductive compatibility. This study illustrates a case in which speciation was accompanied by neither detectable genetic nor morphological differentiation. Furthermore, this provides a rare case study on the intercontinental phylogeography and speciation of a widespread and passively dispersed estuarine species.

The crustacean order Copepoda, which represents the most abundant group of metazoans in the sea, is understudied with respect to its evolutionary history and genetic diversity. The relatively few studies on copepod biodiversity suggest numerous examples of cryptic species, as revealed by molecular markers, interbreeding, or detailed morphometrics (Carillo et al. 1974; Frost 1974, 1989; Fleminger and Hulsemann 1987; Boileau 1991; McKinnon et al. 1992; Cervelli et al. 1995; Ganz and Burton 1995; Einsle 1996; Reid 1998). These cryptic species appear to result from the prevailing pattern of morphological conservatism coupled with large genetic divergences (Frost 1974, 1989; Sevigny et al. 1989; McKinnon et al. 1992; Bucklin et al. 1995; Burton 1998). However, with few exceptions (Burton 1990; Ganz and Burton 1995; Edmands 1999), it is unknown whether the large interpopulation genetic distances correspond to reproductively compatible entities.

The copepod *Eurytemora affinis* is regarded as cosmopolitan, spanning a broad geographic range in the Northern Hemisphere from subtropical to subarctic regions of North America and temperate regions of Asia and Europe (gray shading in Fig. 1). This crustacean has been a focus of many ecological studies because of its dominance as a primary grazer in estuaries throughout the world (Fig. 1; Mauchline 1998). *Eurytemora affinis* is planktonic (or epibenthic) throughout its life and is considered a passive disperser because of its small size (1–2 mm) and inability to swim against ambient fluid flow. Because this species inhabits coastal waters, such as estuaries, salt marshes, and brackish lakes (and freshwater reservoirs in recent years), both open oceans and land might pose geographic barriers to dispersal. However, long-range dispersal has been hypothesized for *E. affinis*, through transport by birds and fish of adults and digestion-resistant eggs (Saunders 1993; Conway et al. 1994).

A previous study on freshwater invasions by *E. affinis* (Lee 1999) revealed unexpectedly high levels of intraspecific genetic divergence, thus casting doubts on its integrity as a single species. Interpopulation genetic divergences, estimated from DNA sequences of the mitochondrial cytochrome oxidase I (COI) gene (652 bp), were as a high as 17% with no evidence of genetic exchange among continents (Lee 1999) and little among drainage basins. However, morphological traits that can distinguish among lineages are not obvious, consisting of variation in body proportions between Europe and other clades and slight or no discernible differ-

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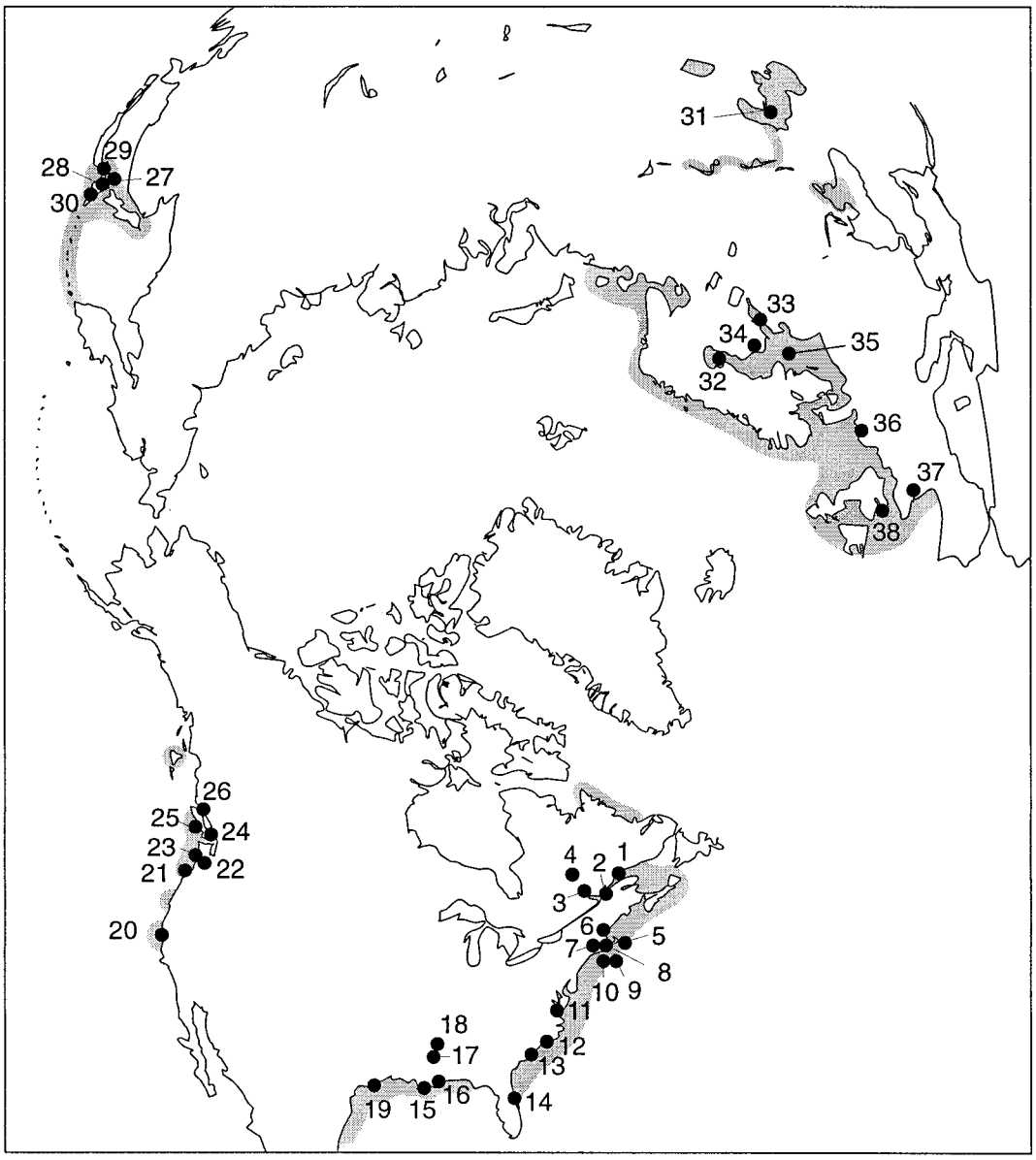

FIG. 1. Populations of *Eurytemora affinis* sampled for this study (represented by black dots on map with place names listed below). Gray shading shows the known distribution of *E. affinis*. Populations of *E. affinis* in northern Russia may be more widespread. (1) St. Lawrence estuary, Canada; (2) St. Lawrence marsh, Canada; (3) Saguenay River, PQ, Canada; (4) Lac St. Jean, PQ, Canada; (5) Waquoit Bay, MA; (6) Parker River pool, MA; (7) Neponset River pool, MA; (8) Oyster Pond, MA; (9) Edgartown Great Pond, MA; (10) Tisbury Great Pond, MA; (11) Chesapeake Bay, MD; (12) Cape Fear, NC; (13) Cooper River, SC; (14) St. John River, FL; (15) Fourleague Bay, LA; (16) Lake Pontchartrain, LA; (17) Black Bayou, MI; (18) Lake Beulah, MI; (19) Colorado Estuary, TX; (20) San Francisco Bay, CA; (21) Columbia River estuary, OR; (22) Chehalis River estuary, WA; (23) Grays Harbor Marsh, WA; (24) Nitinat Lake, BC, Canada; (25) Nanaimo River, BC, Canada; (26) Campbell River, BC, Canada; (27) Ishikari River, Japan; (28) Lake Baratoka, Japan; (29) Lake Ohnuma, Japan; (30) Lake Akanko, Japan; (31) Caspian Sea; (32) Gulf of Bothnia; (33) Gulf of Finland; (34) Sällvik Fjord, Finland; (35) Baltic Sea Proper; (36) IJsselmeer, Netherlands; (37) Gironde estuary, France; (38) Tamar estuary, England.

ence among the non-European clades (B. W. Frost, pers. comm.). In contrast to the morphological stasis evident among lineages, considerable plasticity exists within lineages, including variation in surface area, body size, and length/width ratio of the furca (tail) according to season or habitat type (Busch and Brenning 1992; Castel and Feurtet 1993).

While the previous study focused on reconstructing pathways of freshwater invasion from saltwater habitats (Lee 1999), the goals of the present study were to broaden both the geographic and genetic scopes of the initial survey to (1) more thoroughly examine geographic patterns of genetic variation; (2) gain rough estimates of timing of divergence among clades; and (3) determine reproductive compatibility among genetically distinct but sympatric and genetically similar but geographically distant populations. The first goal was accomplished by adding nine populations from previously unsampled geographic regions; by including 29 of 39 pop-

ulations from the previous study using COI (Lee 1999); and by sequencing an additional locus, the mitochondrial large subunit (16S) rRNA (450 bp) gene, for 30 populations. The second goal was accomplished by using 16S rRNA to obtain a rooted tree for dating speciation events and by comparing levels of divergence with those of other crustacean taxa (Cunningham et al. 1992; Avise et al. 1994; Bucklin et al. 1995). To achieve the third goal, four populations of varying degrees of genetic divergence were intermated to test whether the populations constitute a single biological species.

Materials and Methods

Population Sampling

Eurytemora affinis (Poppe 1880) was collected between 1994 and 1999 from 38 sites spanning much of the global range of the species (Fig. 1), including diverse habitats such as hypersaline marshes, brackish estuaries, and freshwater lakes. Populations from very recently invaded freshwater sites (mostly reservoirs within the past 60 years) were not included in this study, but were discussed in a previous paper (Lee 1999), except for populations from Lakes Ohnuma and Akanko from Hokkaido, Japan. These two recent populations were included because they contained unique haplotypes that were highly divergent. These populations are thought to have originated from a brackish lake on Honshu Island in Japan (Ban and Minoda 1989). Congeners, *Eurytemora americana* from the Duwamish River, Washington, and *E. herdmani* from Halifax, Nova Scotia, Canada, were collected for use as outgroup species in the phylogenetic analysis. The identities of *E. affinis*, *E. americana*, and *E. herdmani* were confirmed morphologically by G. A. Heron and B. W. Frost. Detailed morphometric studies have indicated that *E. affinis*, *E. hirundo* (Giesbrecht 1881), and the more slender *E. hirundoides* (Nordquist 1888) are morphological variants of the same species (Wilson 1959; Busch and Brenning 1992; Castel and Feurtet 1993). The varieties *E. affinis* and *E. hirundoides* were collected from the Gironde River, France (by the late J. Castel) for genetic confirmation that they belong to the same species.

Phylogenetic Reconstruction

Intraspecific phylogenies of *E. affinis* were constructed using the mitochondrial 16S rRNA (450 bp) and the more rapidly evolving COI (652 bp) genes. Genomic DNA from ethanol-preserved individual copepods was extracted using a cell-lysis buffer with proteinase K (Hoelzel and Green 1992). Polymerase chain reaction (PCR) primers 16Sar 2510 and 16Sbr 3080 were used to amplify sequences from 16S rRNA, and primers COIH 2198 (5′ TAAACTTCAGGGTGACCAAAAAATCA 3′) and COIL 1490 (5′ GGTCAACAAATCATAAAGATATTGG 3′; Folmer et al. 1994) were used to obtain sequences from COI. Primer pairs 16SA2 (5′ CCGGGT C/T TCGCTAAGGTAG) and 16SB2 (5′ CAACATCGAGGTCGCAGTAA) were designed specifically to amplify 340 bp of 16S rRNA from the Columbia River estuary population and from *E. americana*. Temperature profiles of five cycles of 90°C (30 sec), 45°C (60 sec), 72°C (90 sec) followed by 27 cycles of 90°C (30 sec), 55°C (45 sec), 72°C (60 sec) were used for PCR amplification. PCR product was run out on agarose gels, excised, and then purified using a Qiagen (Qiagen, Inc., Valencia, CA) gel extraction kit. Purified PCR product was sequenced using an Applied Biosystems Inc. 373 automated sequencer (Applied Biosystems, Foster City, CA). Both strands were sequenced to confirm accuracy of each haplotype sequence.

Phylogenies were constructed using distance matrix and parsimony approaches with the software package PAUP* 4.0 (Swofford 1998). For distance matrix reconstructions, the neighbor-joining algorithm (Saitou and Nei 1987) was used to construct the starting tree, followed by heuristic searches with the tree-bisection-reconnection (TBR) branch-swapping algorithm to optimize the tree. Parsimony reconstructions were based on heuristic searches with unweighted characters. For COI, parsimony reconstructions were performed using all codon positions, with the third codons removed. Sequences were aligned according to secondary structure for 16S rRNA and unambiguously by eye for COI. A consensus sequence for each population was used based on three to five individual sequences per population. Polymorphism within populations was either absent or very low ($< 1\%$). Congeners *E. americana* and *E. herdmani* were used as outgroups for 16S rRNA, but not for COI because substitutions were saturated among *Eurytemora* species (see Results on mutational saturation). Bootstrapping with 100 replicates (Felsenstein 1985) was performed to obtain a measure of robustness of tree topology. Maximum-likelihood distances were computed to account for saturation of substitutions. When obtaining distances, a maximum-likelihood approach was used to estimate transition:transversion ratio (ts:tv ratio; 1.45 for 16S rRNA and 4.7 for COI, taking into account saturation) and variation of evolutionary rates among sites (using shape parameter (α) of a gamma distribution of 0.181 for 16S and 0.184 for COI; Yang 1996).

Partition-homogeneity tests (Farris et al. 1995; Messenger and McGuire 1998) were performed using PAUP* 4.0 (Swofford 1998) to determine whether datasets were significantly incongruent and should not be combined for phylogenetic analyses and for the power test (described in next section on Hypothesis Testing). Partition-homogeneity tests were performed on (1) stem (paired) versus loop (unpaired) regions of 16S rRNA; (2) a combined dataset of 16S rRNA and COI; and (3) first, second, and third codon positions of COI. For 16S rRNA, tests on stem and loop regions were performed on 15 *E. affinis* populations (Fig. 1: sites 1, 2, 5, 7, 11, 12,15, 21, 24, 27, 29, 31, 32, 37, 38) and two outgroup species (*E. americana*, *E. herdmani*).

Degree of mutational saturation was estimated to determine whether particular sequences were appropriate for use in phylogenetic analyses and the power test (described below). Degree of mutational saturation was assessed by examining the correlation between ts:tv ratio and pairwise sequence divergence. A decrease in ts:tv ratio with increasing genetic divergence is an indication of mutational saturation (Kocher et al. 1995). Mutational saturation was determined for stem and loop regions of 16S rRNA and for codon positions of COI.

Hypothesis Testing

Mantel's test (Mantel 1967) was performed to test the correlation between genetic and geographic distance using The

TABLE 1. Geographic and genetic distances between crossed populations of *Eurytemora affinis.* See Figure 2 for key to clade assignments (in circles).

Population crosses (site)	Clade		Geographic distance (km)	% sequence divergence	
				16S	COI
Waquoit Bay, MA (5)	⊕	× Edgartown Great Pond, MA (9) ●	20	5.16	10.6
Edgartown Great Pond, MA (9)	●	× Grays Harbor salt marsh, WA (23) ●	4000	0.96	0.15
Grays Harbor salt marsh, WA (23)	●	× Columbia River estuary, OR (21) ○	55	7.66	17.1

R Package 3.0 (Legendre and Vaudor 1991). This test indicates whether differentiation among the major clades occurred through gradual isolation by distance. Pairwise geographic distances between populations were determined while accounting for the curvature of the earth (Geographic Distances in The R Package 3.0). Pairwise maximum-likelihood genetic distances between populations were computed using PAUP* 4.0 (Swofford 1998).

A power ($1 - \beta$) test (Walsh et al. 1999) was used to determine whether polytomies among clades resulted from actual simultaneous speciation events (hard polytomies) or from rapid cladogenesis (soft polytomies), along with lack of resolution in the data. The test was applied to sequences from 16S rRNA (450 bp), sequences from first and second codon positions of COI (435 bp) and then to a combined dataset of 16S rRNA and 1,2 codons of COI (885 bp). The third positions of COI were omitted for this analysis because substitutions were saturated (see Results). This method tests whether the amount of sequence data and the pairwise sequence divergence rate are sufficient to expect substitutions within a desired time interval. For instance, if there were only 500 bp with a substitution rate of 2.2%/million years, the probability of substitutions occurring within 100,000 years would be low. Thus, the data would be insufficient for resolving a polytomy where speciation events occurred within such a short time interval. The more conservative ''difference of a proportion test'' was applied rather than the ''difference of a mean test'' (Walsh et al. 1999).

The null hypothesis was that the major clades diverged roughly simultaneously, and the alternative hypothesis was that the major clades diverged over successive geological events. Resolution of less than 1 million years was desired, because level of genetic divergence suggested that the multifurcation had occurred around the Miocene/Pliocene boundary (see Results), when climatic fluctuations were probably occurring on a 1 million-year time scale (Crowley and North 1991). The test statistic, $h = 2^{1/2}(\Phi_1 - \Phi_c)$, represents the difference in proportion of substitutions between internodes of 1 million years (soft polytomy) and an internode of zero length (hard polytomy). Proportion (P) of bases expected to undergo substitution during an internode period (the ''effect size'') was arcsine transformed ($\Phi = 2\text{arcsine}[P]^{1/2}$). Significance level was set at 0.05 and power at 0.80 ($\beta = 0.20$). To compute the proportion (P), a substitution rate of approximately 0.9%/million years was used for 16S rRNA (Sturmbauer et al. 1996; Schubart et al. 1998). A rate of 0.4%/million years was assumed for the first two codons of COI, based on rates from another region of COI for *Sesarma* crab sequences taken from Genbank (Schubart et al. 1998). An average rate of 0.65%/million years was used for the combined dataset, weighted for the number of bases per locus. The number of bases required to resolve a given internode length (for a given value of h) was taken from table 1 in Walsh et al. (1999).

To compare levels and timing of divergence with those of other crustacean taxa using the same distance scale, an unweighted pair group method using arithmetic averages (UPGMA) was used to cluster distances based on a Kimura two-parameter model of evolution (Cunningham et al. 1992; Avise et al. 1994). The dendrogram based on 16S rRNA was used to estimate timing of events, because rates of evolution have been calibrated for 16S rRNA in other crustaceans (Bucklin et al. 1995; Sturmbauer et al. 1996; Schubart et al. 1998), whereas comparable molecular clock calibrations have not been made for the region of COI used in this study. A likelihood-ratio test (Felsenstein 1981; Huelsenbeck and Rannala 1997) was performed on the 16S rRNA data to determine whether the assumption that substitutions in the data evolved in a clocklike manner was violated and whether constructing a UPGMA tree (which assumes a clocklike substitution rate) was acceptable.

Interpopulation Mating

Interpopulation matings were performed between two genetically divergent clades (North Pacific vs. North America), between two genetically divergent North American subclades (Atlantic vs. North Atlantic), and within one subclade (Atlantic; Table 1). The populations from divergent clades and subclades were chosen from regions where they come into contact (Table 1, Fig. 1) to determine whether genetically divergent but geographically proximate populations are reproductively isolated. Additionally, two populations from a single subclade from opposite coasts of the North American continent (sites 9 and 23) were crossed (Fig. 1) to determine whether speciation has occurred between genetically proximate but geographically distant populations.

Populations were reared in the laboratory for at least two generations to standardize for environmental effects. Ten to 58 replicates were assembled in both reciprocal directions for each population cross (Table 2). For each replicate, individual male and juvenile female mating pairs were placed in 20-ml vials, in a 12°C environmental chamber on a 15:9 L:D cycle. These vials contained 15 parts per thousand of salt (PSU) water made from a mixture of water from Puget Sound, Washington (27 PSU), and Lake Washington (0 PSU). Populations originated from habitats with overlapping salinity ranges (Columbia River: 3–15 PSU; Grays Harbor marsh: 5–30 PSU; Edgartown Great Pond: found at 11 PSU; Waquoit Bay: found at 23 PSU). A mixture of three algal species,

TABLE 2. Results from interpopulation crosses among four populations of *Eurytemora affinis*, showing number of eggs produced per clutch, survivorship per clutch, percent clutches that produced adults out of all crosses, and development time to adulthood. P, parent; F_1, first generation; F_2, second generation; n/a, not applicable; and ?, no data.

Population cross (Female × Male)	No. replicate crosses		No. eggs/clutch ± SE (no. clutches)		Survival of adults/clutch (% ± SE) (no. clutches)		% clutches yielding adults		Development time (day ± SE) (no. clutches)		Type of isolation
	P	F_1	F_1	F_2	F_1	F_2	F_1	F_2	F_1	F_2	
(Sites 21 × 23)											
Columbia × Grays	58	14	15.6 ± 1.6 (25)	7.8 ± 0.9 (6)	13 ± 5 (27)	11 ± 12 (6)	17	7	31.4 ± 3.8 (10)	38.25 (1)	
Grays × Columbia	57	8	13.4 ± 2.0 (27)	13.6 ± 3.3 (5)	7 ± 3 (27)	0 (5)	9	0	25.7 ± 2.9 (7)		inviable F_2
Control: Columbia	20	n/a[1]	16.4 ± 2.5 (12)	n/a	38 ± 9 (12)	n/a	40	n/a	21.0 ± 0.8 (8)	n/a	
Control: Grays	17	n/a	19.3 ± 2.6 (10)	n/a	28 ± 8 (10)	n/a	41	n/a	20.2 ± 1.2 (7)	n/a	
(Sites 23 × 9)											
Grays × Edgartown	20	10	10.7 ± 3.3 (9)		27 ± 12 (11)	? (5)	20	30	20.1 ± 2.5 (4)	21.2 ± 1.9 (3)	
Edgartown × Grays	20	10	13.9 ± 4.4 (15)		27 ± 9 (18)	0 (5)	30	0	16.7 ± 1.5 (6)		inviable F_2
Control: Grays	10	n/a	44.9 ± 3.4 (8)	n/a	45 ± 12 (9)	n/a	70	n/a	18.7 ± 2.2 (7)	n/a	
Control: Edgartown	10	n/a	11.6 ± 1.9 (9)	n/a	40 ± 13 (9)	n/a	60	n/a	17.8 ± 1.7 (6)	n/a	
(Sites 9 × 5)											
Edgartown × Waquoit	30	1	13.9 ± 2.0 (17)		1.0 ± 0.8 (17)	no clutches	7	0	37.5 ± 10.5 (2)		sterile F_1
Waquoit × Edgartown	29	1	9.1 ± 1.9 (8)		0 (8)		0				inviable F_1
Control: Edgartown	32	n/a	12.6 ± 1.5 (24)	n/a	10 ± 4 (24)	n/a	31	n/a	39.2 ± 1.9 (10)	n/a	
Control: Waquoit	29	n/a	16.4 ± 1.3 (21)	n/a	24 ± 7 (21)	n/a	48	n/a	37.2 ± 2.9 (14)	n/a	

[1] Measurements were not made for controls beyond the parent generation because genetic composition does not vary among generations and controls can reproduce indefinitely in the experimental vials with no decline in survivorship.

Isochrysis galbana, *Thalassiosira pseudonana*, and *Rhodomonas* sp., was used as a food source. Number of eggs per clutch, percentage of survival to adult within a clutch, percentage of clutches that produced adults out of all replicate crosses, and development time to adulthood were recorded for F_1 and F_2 offspring.

Individuals were classified as adults when males developed geniculate right antennules, and females developed large wing-like processes on the posterior end of their prosome (body). Each mating experiment lasted for approximately 3 months and experiments were performed in sequence (Grays × Edgartown: summer/fall 1996, Columbia × Grays: winter/spring 1997, Edgartown × Waquoit: summer/fall 1997). Because the three mating experiments were performed sequentially at different times of the year, results from different crosses were not compared directly to one another, but to intrapopulation crosses (controls). Controlled intrapopulation matings were performed concurrently with each experiment. Allozyme data were collected to confirm the production of hybrids from the crosses using five loci (*Amy*, *Mpi*, *Pep*, *Pgi*, and *Pgm*).

RESULTS

Sequence Diversity

Phylogenetic analysis revealed deep splits among clades (Figs. 2, 3), with maximum pairwise divergences of 10% in 16S rRNA and 19% in COI. Topologies of the phylogenies based on 16S rRNA and COI were mostly concordant (Fig. 2), with COI providing greater resolution among closely related populations. Because a partition-homogeneity test (Farris et al. 1995) showed that sequences from 16S rRNA and COI were not significantly congruent ($P = 0.86$), the datasets were kept separate for phylogenetic reconstructions.

Sequences from stem and loop regions of 16S rRNA were significantly congruent ($P = 0.16$) and thus were combined. A separate phylogenetic analysis of stem (277 bp) and loop (173 bp) regions yielded similar tree topologies and proportion of polymorphic sites (loops: 50 bp, 29%; stems: 67 bp, 24%). Degree of mutational saturation, as revealed by declining ts:tv ratios with increasing sequence divergence, was similar for both stem and loop regions in 16S rRNA (Fig. 4). Mutational saturation was evident among congeneric species of *Eurytemora* (Fig. 4). There were 68 parsimony-informative sites for 16S rRNA, and consistency and retention indices were 0.67 and 0.74, respectively.

In contrast to the congruence between stem and loop regions of 16S rRNA, codon positions of COI were not significantly congruent ($P = 0.99$). Mutational saturation at the third codon position occurred with pairwise sequence divergences above 5%, whereas first and second codon positions of COI did not become saturated among populations (Fig. 5). A graph for the second codon position was not presented in Figure 5 because transversions were rare. Despite the fact that third codon positions of COI were saturated, they provided useful information for phylogenetic analysis. For instance, phylogenetic analyses using only the first two codons resulted in reconstructions with much lower bootstrap values, due to insufficient data. Saturation at the third position was accounted for by computing maximum-likelihood distances (see Methods, Fig. 2b). There

were 197 parsimony-informative sites for COI, and consistency and retention indices were 0.64 and 0.84, respectively. All substitutions in COI were synonymous, resulting in no amino acid substitutions. Third codon positions were omitted for the power test because mutational saturation would violate the assumption that sequence divergences reflect an even occurrence of substitutions over time.

Geographic Structure and Timing of Divergence

The four major clades of *E. affinis,* corresponding to Europe, Asia, North America, and North Pacific, formed a polytomy (multifurcation; Fig. 2a). A phylogenetic reconstruction based on the combined dataset of 16S rRNA and COI also yielded a polytomy among the major clades. A power test (Walsh et al. 1999) indicated that the 16S rRNA data were sufficient to resolve internodes of 500,000 years ($h = 0.190$, $\Phi_1 = 0.134$, $\Phi_c = 0.000$, $P = 0.0045$, 2.0 bases), whereas the combined dataset of 16S and COI (885 bp, third codon positions removed) was sufficient to resolve internodes of 300,000 years ($h = 0.125$, $\Phi_1 = 0.088$, $\Phi_c = 0.000$, $P = 0.00195$, 1.72 bases). Results from the test suggest that the polytomy represented speciation events occurring within 300,000 years, but the data were insufficient to determine whether the events were approximately simultaneous. Given rates of evolution of the loci examined, more than 1000 bp would be required to resolve internodes of 200,000 years or less.

The major clades, except for the European clade, contained highly divergent subclades. The North American clade consisted of three subclades, North Atlantic, Atlantic, and Gulf (Figs. 2, 3), having maximum divergences of 6% in 16S rRNA and 15% in COI. Even though only a few populations were sampled, nearly as much genetic divergence was present in the Asian clade (4% 16S, 13% COI), suggesting the potential for more genetic diversity with additional sampling. Similarly, genetic diversity within the North Pacific clade may not have been fully explored because population sampling was confined to a small area in this region (Fig. 3). In contrast, interpopulation genetic divergences were low in Europe, with maximum divergences of only 1% in 16S rRNA and 3% in COI. Morphological variants within Europe, designated as ''*E. affinis*'' and the more slender ''*E. hirundoides,*'' were genetically identical at both 16S rRNA and COI, in concordance with morphological studies that found *E. hirundoides* to be an invalid species (Wilson 1959; Busch and Brenning 1992; Castel and Feurtet 1993).

At finer spatial scales, there was almost no sharing of mtDNA haplotypes among geographically proximate (but nonidentical) populations, indicating a lack of genetic exchange among nearby sites (see Atlantic clade, Fig. 2b) and a completion of lineage sorting. Populations with identical haplotypes, such as those from Massachusetts, might reflect a recent common history rather than ongoing dispersal. Variation in sequence within populations was either absent or very low ($<$ 1% divergence).

There was a lack of correlation between genetic and geographic distances among populations (Mantel's test; $r = 0.023$, $P = 0.25$). This pattern was not surprising, given that highly divergent clades were distributed in close geographic proximity. A correlation was not present even when the clades most likely contributing to lack of correlation were removed from the analysis, such as the most divergent North Pacific clade and West Coast populations (sites 20, 23) belonging to the North American clade (Atlantic subclade; Figs. 2, 3).

Zones of contact between highly divergent clades were present on both coasts of the North American continent (Fig. 3). The highly divergent North American and North Pacific clades (17–19% COI divergence) both occurred on the West Coast of North America (sites 20 to 26). The two clades overlapped in range in Grays Harbor, Washington (Fig. 3), with one clade present in a salt marsh (Atlantic subclade; site 23) and the other in the Chehalis River estuary (North Pacific clade, site 22). On the East Coast of North America, populations from two subclades within the North American clade (Atlantic and North Atlantic) overlapped in range in the St. Lawrence River drainage and in Massachusetts (sites 1–10). An estuarine population from each subclade (~11% COI divergence; sites 1 and 3) coexisted within the St. Lawrence River drainage. Within this drainage, populations from the Atlantic clade were found in estuarine, salt marsh, and freshwater habitats (sites 2, 3, and 4). In contrast to the above scenarios, genetically proximate populations belonging to the same subclade (Atlantic) occurred on opposite coasts of the North American continent. West Coast populations in San Francisco Bay, California (site 20) and Grays Harbor salt marsh, Washington (site 23) were most closely related to East Coast populations from Martha's Vineyard, Massachusetts (Tisbury and Edgartown Great Ponds, sites 9 and 10).

Relative to other species of *Eurytemora,* populations of *E. affinis* were clearly monophyletic (Fig. 2a). While sequence divergences in 16S rRNA never exceeded 10% among *E. affinis* populations, divergences were 14–18% between *E. affinis* and *E. americana* and 17–21% between *E. affinis* and *E. herdmani.* These sequence divergences among species of *Eurytemora* were probably underestimates due to mutational saturation in 16S rRNA (Fig. 4).

Branch lengths from the UPGMA dendrogram (Fig. 6) suggest a separation among major clades (node B) of approximately 5.1 million years, dating to the time of the Miocene/Pliocene boundary. This estimate assumes a substitution rate of approximately 0.9%/million years in 16S rRNA, calibrated for fiddler crabs (*Uca vocator*; Sturmbauer et al. 1996) and Jamaican grapsid crabs (*Sesarma*; Schubart et al. 1998). Similarly, separation appears to have occurred approximately 19 million years ago between *E. affinis* and *E. americana* (node A) and approximately 23 million years ago between *E. americana* and *E. herdmani.* These estimates are extremely rough due to the uncertainties of the molecular clock and degree of mutational saturation among congeners (Fig. 4). Level of divergence among *E. affinis* ''populations'' was similar to that between sister species of the copepod *Calanus* (*C. glacialis* and *C. marshallae*; Bucklin et al. 1995) and was greater than that among species of grapsid crabs, *Sesarma* (Schubart et al. 1998). Divergences among recognized *Eurytemora* species was also large, equivalent to that among species of *Calanus* (Bucklin et al. 1995) and horseshoe crabs (Avise et al. 1994), and greater than that between king and hermit crabs (Cunningham et al. 1992). Nodes on the UPGMA tree that separate the major clades into two groups (nodes C and D, Fig. 6) were not statistically supported (see Fig. 2a).

The clustering method used to construct the UPGMA tree

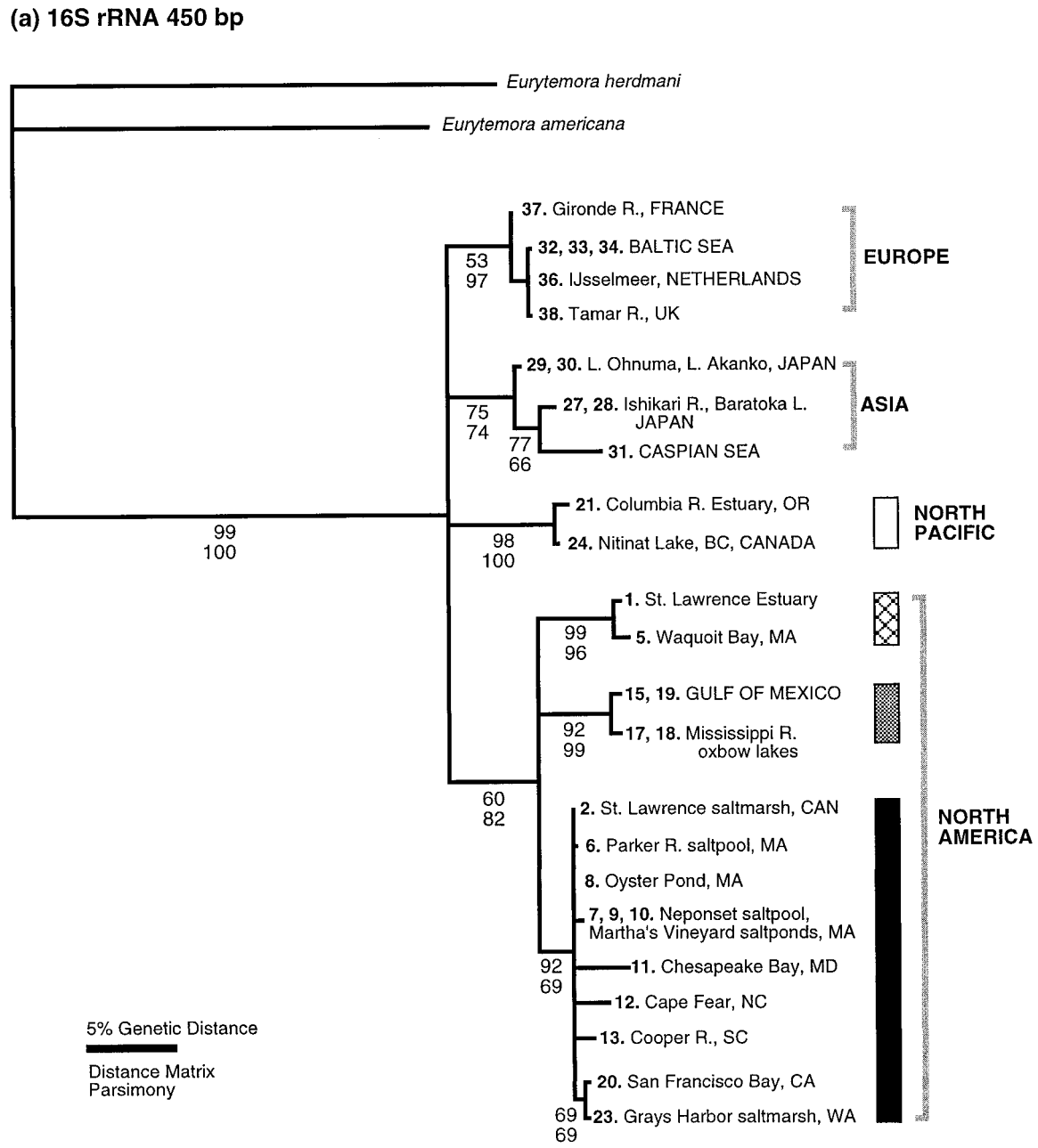

FIG. 2. Phylogeny of populations and sibling species of *Eurytemora affinis* using (a) 16S rRNA (450 base pairs) and (b) cytochrome oxidase I (COI, 652 base pairs). Locations of populations are shown at branch tips, with numbers designating populations as in Figure 1. Gray brackets indicate the four major clades, and thick patterned bars (a) and patterned circles (b) represent distinct clades and subclades within the North American continent (see Fig. 3 for key). The trees shown were constructed with a distance matrix approach using PAUP* 4.0. Branch lengths reflect genetic distances, with scale bar indicating 5% genetic distance (maximum likelihood). The maximum-likelihood distances attempt to account for saturation of substitutions. Numbers next to nodes are bootstrap values based on 100 bootstrap replicates using distance matrix (upper number) and parsimony approaches (lower number; Felsenstein 1985). Bootstrap values of ns indicate branches not supported by values greater than 50% for a given phylogenetic method. Congeners, *E. americana* and *E. herdmani*, were used as outgroup species for 16S rRNA but not for COI because level of divergence was saturated among congeners (i.e., COI tree is unrooted).

is based on the assumption that the data are ultrametric (have constant rate of substitutions). A likelihood-ratio test, applied to test this assumption, could not reject the null hypothesis that the tree is clocklike. Using 17 populations, the difference in log likelihoods between tree reconstructions with and without a clock enforced was $-1623.5 - (-1637.5) = 14.0$. This value was less than the χ^2 value of 24.996 (df = 15, α = 0.05), indicating that the likelihood values for the reconstructions were not significantly different.

Reproductive Incompatibility among ''Populations''

None of the crosses (Table 1) were able to produce F_2 offspring in both reciprocal directions (Table 2). Males did

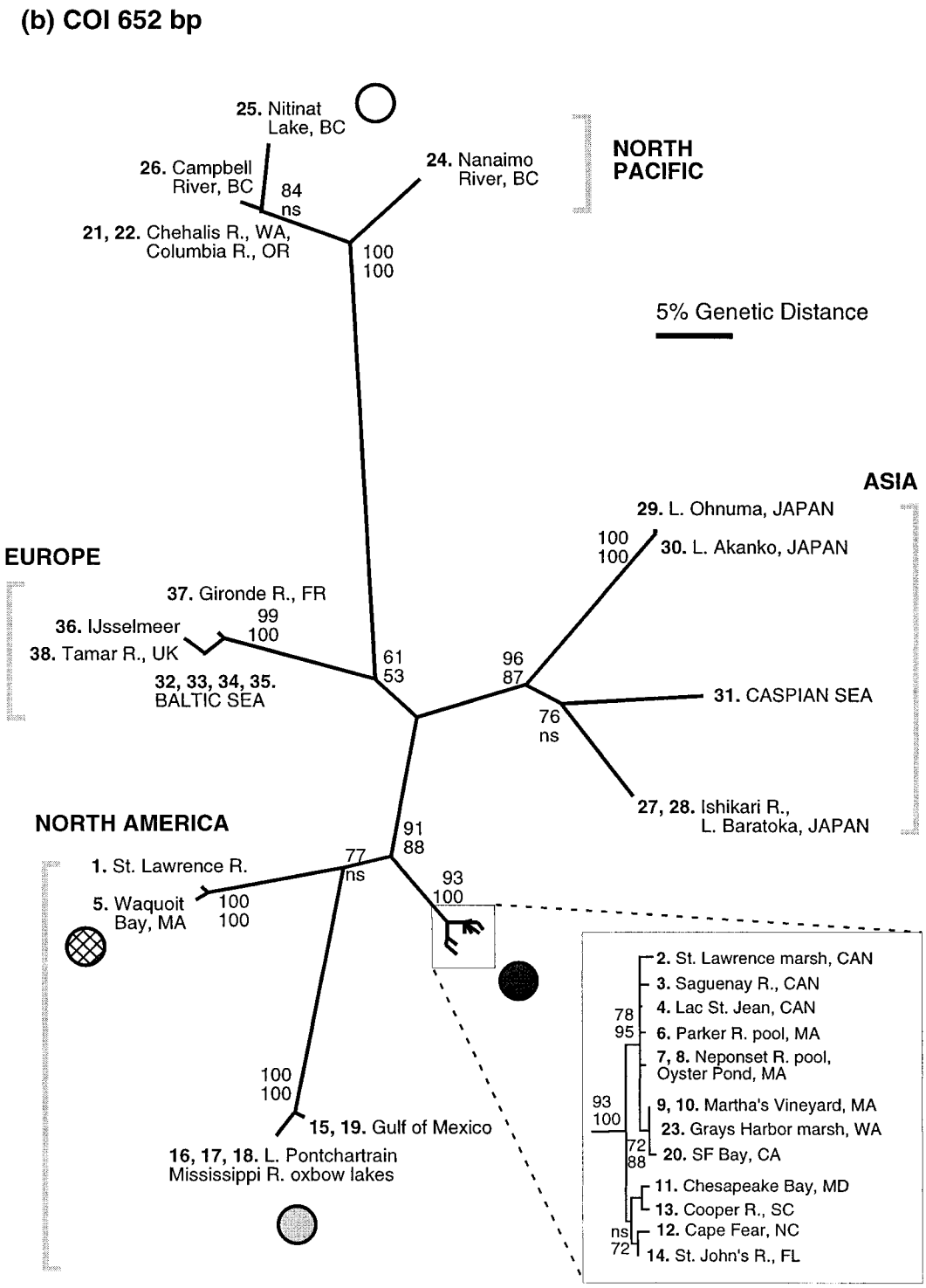

FIG. 2. Continued.

transfer spermatophores, carried by the fifth leg, to the genital pores of females with no apparent difficulty. Hybrid breakdown was evident not only from statistical differences in survivorship or development time, but from morphological deformities of some of the F_1 and F_2 offspring (see below).

Populations from the Columbia River (site 21) and Grays Harbor salt marsh (site 23) belong to genetically divergent clades (North Pacific vs. North America) that overlap in distribution (Table 1; Fig. 3). F_1 and F_2 offspring from these crosses were morphologically deformed, with antennules less

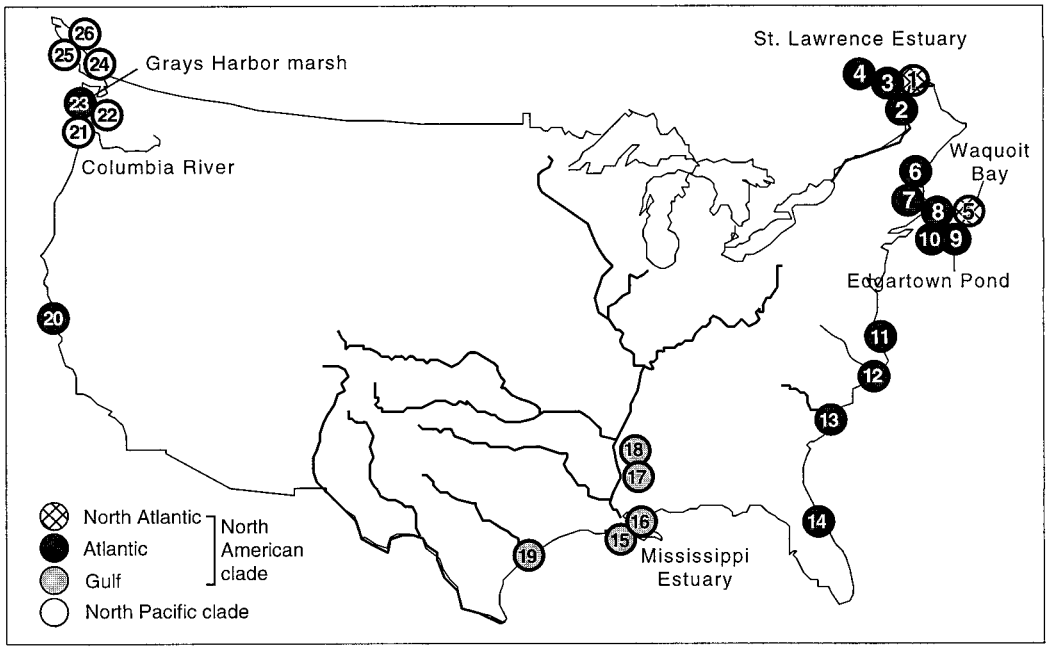

FIG. 3. Geographic distribution of three North American subclades and the North Pacific clade within the North American continent. The North Atlantic, Atlantic, and Gulf subclades belong to the North American clade, whereas the North Pacific clade is highly divergent from all the other clades (Fig. 2). Zones of contact between genetically divergent clades and subclades are in the Pacific Northwest and Atlantic Northeast regions of the North American continent near the U.S.-Canadian border.

than half the normal length, and occasionally with stunted bodies. Degree of isolation was asymmetric in that the F_2 offspring from Grays Harbor females and Columbia River males did not survive to the adult stage (Table 2). Survival of adults per clutch in the F_1 generation was significantly lower in the crosses relative to controls (Table 2; Mann-Whitney, $P < 0.05$) and proportion of clutches with offspring that developed to adults was lower (Table 2). F_1 development time to adulthood was significantly longer for crosses with Columbia estuary females ($P < 0.05$), but not for crosses with Grays Harbor females ($P > 0.1$), and variances were higher in crosses relative to controls. F_1 hybrids assayed for allozymes were heterozygous for alleles that were fixed (*Amy* and *Pgm*) in the parent populations.

Populations from Edgartown Great Pond and Waquoit Bay are from genetically divergent subclades (Atlantic vs. North Atlantic) that overlap in range (Table 1; Fig 3). Very few F_1 offspring were produced, with only two clutches of 30 replicates yielding survivors to adulthood for the Edgartown female cross and none surviving in the other cross. Lower survival and longer development times (Table 2; Mann-Whitney, $P < 0.05$) of Edgartown controls relative to those from the earlier experiment suggests overall lower performance of copepods in this experiment, which was performed last (see Methods). Still, the range of development times observed for controls were within or near the expected range for *E. affinis* at 12°C (Heinle and Flemer 1975). Moreover, interpopulation crosses were clearly less successful than the controls (Table 2).

The most surprising result emerged from the cross between the geographically distant but genetically proximate populations from Atlantic subclade (Table 1; Fig. 3). Crosses between these populations, Grays Harbor, Washington (site 23) and Edgartown Great Pond, Massachusetts (site 9), were much more successful than those between genetically divergent populations, but results showed clear evidence of hybrid breakdown (Table 2). Percentage of survival to the adult stage was lower in crosses than in controls, but was not significant (Mann-Whitney, $P > 0.05$; Table 2). Most notably, crosses between Edgartown females and Grays Harbor males were unable to produce F_2 offspring. Out of ten replicate F_1 crosses, five F_2 clutches were produced, but none hatched. The eggs were darker and more opaque than normal eggs and appeared malformed (with irregular shapes). Results indicate that speciation has occurred even between these seemingly closely related populations.

DISCUSSION

Clearly, *E. affinis* is a sibling species complex, composed of genetically divergent and reproductively isolated ''populations'' that are difficult to distinguish morphologically (Mayr and Ashlock 1991; Knowlton 1993). Long branch lengths on the phylogeny in Figure 2b suggest the presence of at least eight sibling species (North Pacific, Europe, three subclades within Asia, and three subclades within the North American clade). Such high levels of genetic divergences among morphologically indistinct clades of *E. affinis* were equivalent to those of morphologically distinct species in other crustacean groups (Fig. 6; Cunningham et al. 1992). Furthermore, the number of species within *E. affinis* may be even greater, given the reproductive incompatibility between two genetically proximate (0.15% divergence in COI) yet morphologically indistinct populations (Tables 1, 2b). Thus,

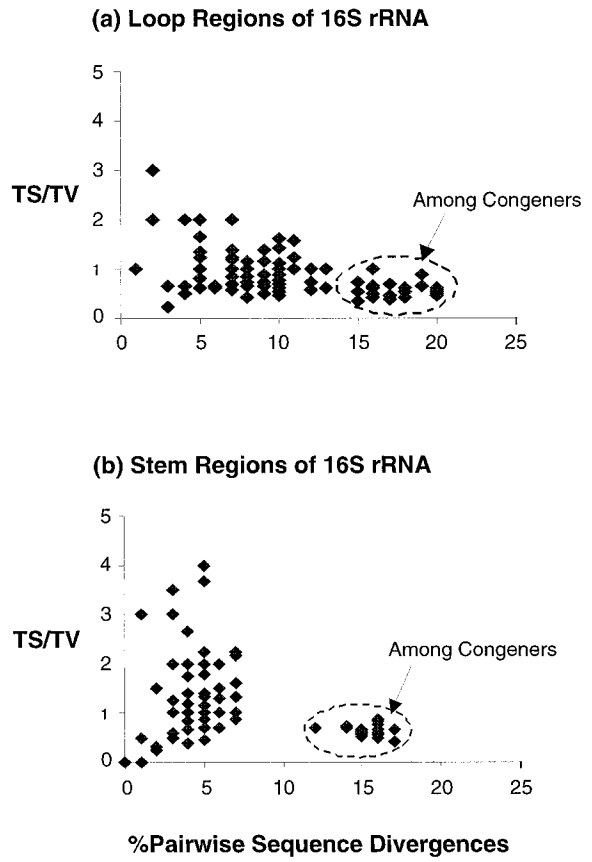

FIG. 4. Relationship between transition/transversion ratio (TS/TV) and percent pairwise sequence divergence for populations of *Eurytemora affinis* and congeners *E. americana* and *E. herdmani*. (a) Loop regions of 16S rRNA; (b) stem regions of 16S rRNA.

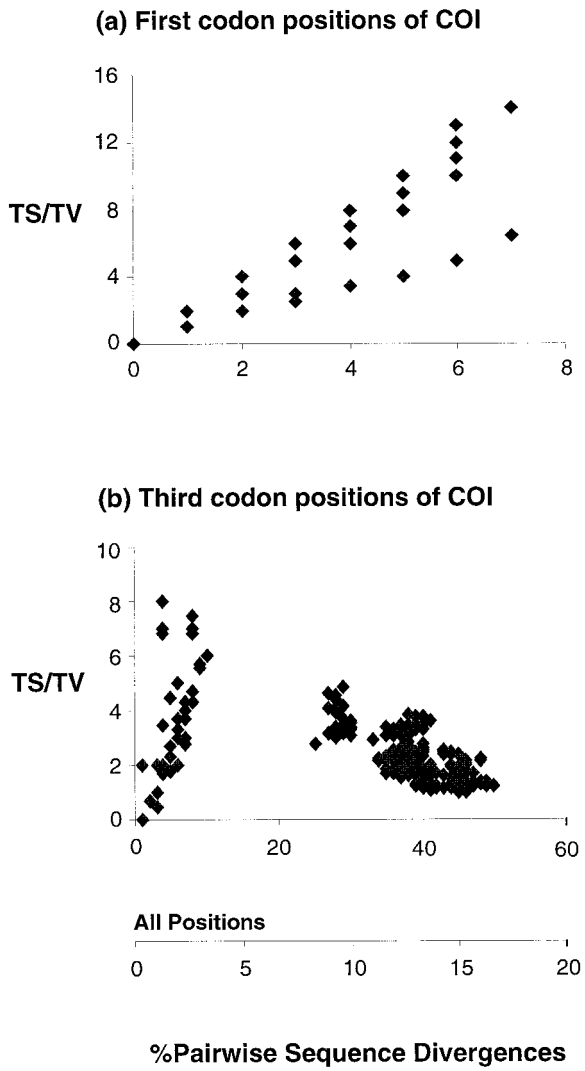

FIG. 5. Relationship between transition/transversion ratio (TS/TV) and percent pairwise sequence divergence for populations of *Eurytemora affinis*. (a) First codon position of COI, (b) third codon position of COI. Scale bar beneath the graphs represents the equivalent percent pairwise sequence divergence for all codon positions.

cryptic species that are genetically close but morphologically indistinguishable may be far more common than previously thought, yet difficult to detect because of difficulties of performing interpopulation crosses.

Phylogeography

The polytomy among clades suggests near-simultaneous divergence of major lineages (Fig. 2), with levels of divergence placing the event roughly 5.1 million years ago, during the late Miocene or early Pliocene (Fig. 6). This estimate is extremely rough, and could be an overestimate due to rapid rates of molecular evolution in *E. affinis* relative to other crustaceans, resulting from factors such as small body size (1 mm) and short generation time (about 20 days; Table 2; Martin and Palumbi 1993). Higher rates of substitution in *E. affinis* would place timing of speciation among the major clades closer to the Pleistocene epoch, which began 2 million years ago. Assuming that rates from other species are applicable, a possible scenario of speciation places *E. affinis* in the unglaciated Arctic region during the warmer Miocene, followed by geographic isolation and speciation during a southward migration resulting from a cooling period about 5 million years ago (Crowley and North 1991).

A power test (Walsh et al. 1999) revealed that the available sequence data was sufficient to resolve speciation events occurring at intervals of approximately 300,000 years or greater. Thus, speciation events appear too rapid to have been dependent on the lengthy 1 million-year climatic cycles of the Late Miocene/Early Pliocene (Crowley and North 1991). Because the power test depends on assumptions of accurate and even rates of substitution over time, confidence intervals for this test can be quite large. If the error for the molecular clock is $\pm 0.1\%$/million years, the confidence interval for the resolvable internode is about $\pm 50{,}000$ years. Mutational saturation can reduce the resolution of this method, by lowering the number of substitutions (P) relative to expectations and increasing the actual amount of sequence data required to resolve the nodes. Attempts to avoid this problem were taken by using unsaturated datasets. Even with large confidence intervals, results from the power test appear to support rapid speciation events among the clades that form a polytomy (Fig. 2a).

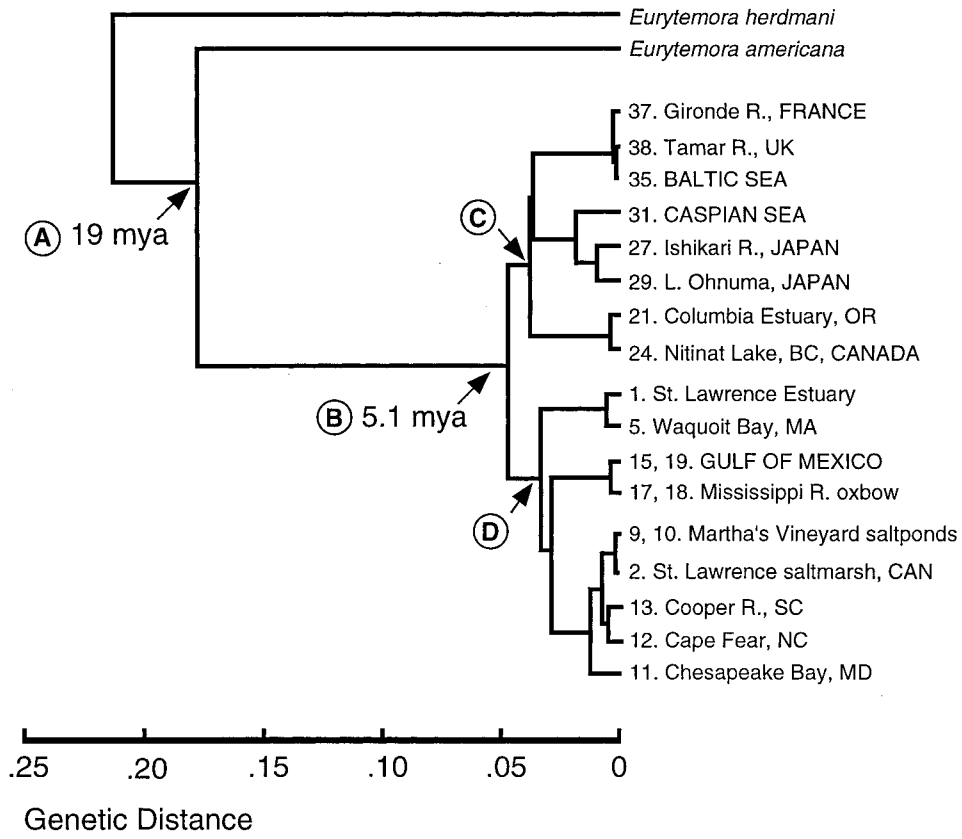

FIG. 6. UPGMA dendrogram for populations of *Eurytemora affinis* based on 16S rRNA gene sequences. The scale bar for genetic distance is based on a Kimura two-parameter model of evolution. Letters enclosed in circles represent major speciation events. B indicates a speciation event among major clades of *E. affinis*, when the polytomy in Figure 2a was formed.

Speciation among the clades probably occurred in allopatry, when populations radiated from the polar region (or some other region) and became geographically isolated. The presence of postmating, but not premating, reproductive isolation among sympatric clades (Table 2) is consistent with an allopatric model of speciation followed by secondary contact, given that the ability to copulate would have prevented speciation in sympatry. Thus, overlapping ranges between divergent clades and subclades in the Pacific Northwest and Atlantic Northeast regions of the North American continent (Fig. 3) most likely reflect secondary contact following speciation events. This secondary contact probably occurred recently, given that regions of contact were glaciated as recently as 15,000 years ago (Hocutt and Wiley 1986). This ice cover extended as far south as the Washington-Oregon border on the West Coast and Massachusetts on the East Coast of the North American continent (Hocutt and Wiley 1986). Sympatric sibling species of *E. affinis*, such as the genetically distinct estuarine populations that share a common drainage (sites 1 and 3), might conceivably be direct ecological competitors. Given the geographic juxtaposition of highly divergent clades, it is not surprising that geographic and genetic distances were not correlated on a global scale (Mantel's test, $r = 0.023$, $P = 0.25$).

Even though large-scale movements might have been necessary to colonize previously ice-covered regions, the lack of sharing of mtDNA haplotypes among closely related (but nonidentical) proximate populations indicates very low dispersal even between nearby sites (Fig. 2b, Atlantic clade). This lack of genetic exchange among drainages and continents argues against a preponderance of long-distance transport of adults or eggs (Conway et al. 1994; Flinkman et al. 1994) by birds or humans, although such transport could have been rare and episodic. Even in modern times, transport of *E. affinis* via any means (including humans) appears to have been restricted to movement upstream into reservoirs and lakes within drainages (Lee 1999).

The unusually close genetic proximity between West and East Coast populations of the Atlantic clade (Fig. 3) could be an example of such a rare episodic dispersal event. The Atlantic clade probably originated on the East Coast, which harbors most of the genetic diversity within the clade. Haplotypes were not shared between the coasts, and level of divergence at 16S rRNA places their common origin at 300,000 to 800,000 years ago, although this could be an overestimate. This divergence could reflect either the actual time of separation or failure to assay the source populations on the East Coast.

Levels of genetic diversity within the Asian clade were probably underestimated, given that the region between the Caspian Sea and Japan was not sampled for this study. The three long branches within the Asian clade probably represent sibling species of *E. affinis*, including two genetically divergent groups on the island of Hokkaido, Japan (Fig. 2, sites

27, 28 vs. 29, 30). The two divergent groups on Hokkaido did not speciate in close proximity, as the populations in Lakes Ohnuma and Akanko (sites 29 and 30) were thought to have been introduced from the island of Honshu in Japan (Ban and Minoda 1989).

Morphological Stasis and Plasticity

For the copepod *E. affinis,* morphological stasis has been maintained despite dramatic genetic divergences among lineages (Fig. 2). The pattern of morphological stasis across lineages of *E. affinis* coupled with high levels of morphological plasticity within lineages has been found in other microcrustaceans, such as within the genus *Daphnia* (Colbourne and Hebert 1996). The relatively large morphological variation within lineages has led to the erroneous subdivision of *E. affinis* into invalid species, such as *E. hirundo* and *E. hirundoides* (Busch and Brenning 1992), whereas the large genetic distances among lineages are not manifested in obvious phenotypic differences (B. W. Frost, pers. comm.). Not only are rates of morphological and genetic evolution uncoupled, but patterns of differentiation are discordant. For instance, two clades within the North American continent (North Pacific and North America) were morphologically very close (B. W. Frost, pers. comm.), but were genetically the most divergent (19% in COI; Fig. 2b). In contrast, the European clade was the only one that exhibited consistent and obvious morphological differences from other clades (with differences in proportions of the body and in the male fifth leg; B. W. Frost, pers. comm.), but was not more divergent genetically from other clades (Fig. 2).

Morphological conservatism in copepods has evidently led to a prevalent pattern of undersplitting of groups. An indication of undersplitting is the fact that copepod orders exhibit excessive levels of genetic divergence. For instance, branch lengths in orders of copepods in 18S rRNA is 2.5–5 times greater than those among branchiopod orders (brine shrimps, fairy shrimps, and cladocerans, such as *Daphnia*), and branch lengths for copepods are always longer than for other crustacean taxa (T. Spears, pers. comm.). Calibrating a molecular clock for copepods would help determine whether and to what extent rates of morphological evolution in copepods are retarded. Relationships among morphology, phylogeny, and habitat type will be addressed in a future study (C. E. Lee and B. W. Frost, unpubl. ms.).

Speciation within the Eurytemora affinis Complex

Results from this study emphasize that levels of genetic divergence and reproductive isolation are not comparable among species and that speciation events can be genetically cryptic. Reproductive incompatibility between genetically proximate, but geographically distant populations (sites 9 vs. 23; Table 2) was somewhat surprising. Such low levels of genetic divergence (Table 1) between populations from Edgartown Great Pond (site 9) and Grays Harbor Marsh (site 23) would not typically warrant species recognition. The lack of genetic or morphological divergences between these reproductively isolated ''populations'' may reflect slow rates of divergence or recent speciation (Knowlton and Weigt 1997). In this case, recent speciation is more likely, although the amount of time separating the two populations (or sibling species) is not known, but only roughly estimated (see above). It would be informative to examine reproductive compatibility between genetically and geographically proximate populations of *E. affinis* to determine whether reproductive isolation is widespread among closely related populations.

Reproductive incompatibility between sibling species from two different clades (sites 21 vs. 23), and subclades (sites 5 vs. 9) was not surprising, given their large genetic distances (Table 1; Fig. 2). However, reproductive success was greater for crosses between distant clades (sites 21 and 23; Table 1) than between subclades (Table 2), suggesting a lack of correlation between genetic distance and reproductive compatibility. However, the number of interpopulation (or interspecific) crosses was not sufficient for detecting a general trend, given that rates of divergence are stochastic. A large amount of noise accompanied a positive trend between genetic distance and reproductive isolation among species of *Drosophila* (Coyne and Orr 1989, 1997) and among populations of the splash pool copepod *Tigriopus californicus* (Edmands 1999).

Levels of reproductive incompatibility were much greater in *E. affinis* than in both *T. californicus* and *Daphnia.* Whereas hybridization was not possible among both genetically proximate and distant populations of *E. affinis* (Table 2), hybridization occurred among highly divergent (up to 22.3% in COI) populations of *T. californicus* (Edmands 1999) and among divergent (14% in 12S rRNA) species of *Daphnia* (Colbourne and Hebert 1996). The pattern for *E. affinis* suggests that reproductive incompatibility can evolve rapidly between populations.

Beneficial effects of hybridization, in terms of F_1 hybrid vigor, were not evident in this study, in contrast to results from a study conducted on the genetically distant copepod, *T. californicus* (Edmands 1999). Edmands (1999) found increases in F_1 hybrid vigor relative to parentals with no correspondence with genetic distance and a decline in F_2 hybrid fitness with increasing genetic (0.2–22.3% in COI) and geographic (5 m to 2007 km) distances. Patterns similar to those found for *T. californicus*, of F_1 hybrid vigor and F_2 hybrid breakdown, might occur for *E. affinis* with a large number of crosses among closely related populations.

Even if the genetically divergent sympatric clades of *E. affinis* diverged in allopatry followed by seconday contact, greater levels of prezygotic isolation is generally expected in sympatry due to reinforcement (of mating discrimination by natural selection against maladaptive hybrids; Dobzhansky 1937; Coyne and Orr 1997). Such reinforcement was not detected in this study (Table 2). Only postzygotic reproductive isolation was evident in the interpopulation (or interspecific) crosses, in the form of hybrid sterility or inviability (Table 2). An adequate test of prezygotic isolation was not performed in this study, given that mate choice was not allowed during the experiments. It is possible that *E. affinis* can discriminate among sibling species using chemical cues, given that it uses such cues to distinguish conspecifics from more distantly related copepods (Katona 1973). In addition, sibling species might actually occur in a state of microallopatry. For instance, the divergent clades in the St. Lawrence River drainage (sites 1 and 3) might be prevented from com-

ing into contact through niche partitioning, such as occupying reaches of the estuary that differ in flow speed or salinity.

The lack of concordance among geographic distance, genetic divergence, reproductive isolation, and morphological differentiation emphasizes the importance of using multiple measures for examining patterns and processes of speciation. At finer scales, within clades, species boundaries may prove to be nebulous, if reproductive isolation between genetically proximate ''populations'' and asymmetries in reproductive isolation (Table 2) prove to be the rule. The lack of genetic exchange among sites (especially among drainages) suggests that for the most part, populations are geographically isolated and are in the process of speciation. However, genetic exchange may become more prevalent in the future with increases in transport facilitated by humans (Lee 1999; Lee and Bell 1999).

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