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RAPID AND REPEATED INVASIONS OF FRESH WATER BY THE COPEPOD *EURYTEMORA AFFINIS*

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Abstract.—Invasions of fresh water by marine organisms have been of great interest to evolutionary biologists and paleontologists because they typically constitute major evolutionary transitions. Recent (< 200 years) invasions of fresh water by brackish or marine species offer an opportunity to understand mechanisms underlying these events, but pathways of invasion from salt water have not been confirmed using genetic data. This study employed mitochondrial DNA sequences (652 base pairs from the cytochrome oxidase I (COI) gene) to reconstruct the geographic and evolutionary history of freshwater invasion by the common estuarine and saltmarsh crustacean *Eurytemora affinis* (Copepoda; Poppe 1880). Phylogenetic analysis of populations from North America, Europe, and Asia revealed at least eight independent invasions of fresh water from genetically distinct lineages. At least five of these freshwater invasions most likely arose independently in different river drainages, recently from saltwater sources within each river drainage. An analysis of molecular variance (AMOVA) was performed at three geographic scales (among continents, among drainages, and within drainages) to assess the hierarchical distribution of genetic variance. Results indicated that 52% of the genetic variance was explained by differences among drainages, 43% by differences among continents, but only 5% by differences within drainages, thus supporting geographic patterns of invasions inferred from the phylogeny. Physiological experiments were performed to determine whether adults and larvae from saltwater populations could tolerate freshwater conditions. Transfer to zero salinity resulted in high mortalities, but with some survival to the second generation in one population. This study provides genetic evidence and physiological support for rapid transitions from a saline life history into fresh water, with repeated invasions on a global scale.

Key words.—Cytochrome oxidase I, dispersal, freshwater colonization, habitat transition, phylogeography, range expansion, salinity tolerance.

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The interface between saline and freshwater habitats presents a formidable barrier to dispersal that few species have been able to penetrate (Hutchinson 1957). This transition between habitats constitutes a dramatic shift in "adaptive zones" (Simpson 1944) that most notably requires overcoming large gradients in osmotic pressure and ionic concentration (Hutchinson 1957; Prosser 1973). Freshwater invasions from salt water have led to rapid morphological (Bell and Foster 1994), physiological (Harris and Aladin 1997), and life-history (Calow 1978; Anger 1995) adaptations, some of which may have facilitated the colonization of land (Little 1990; Schubart et al. 1998).

A few species appear to have the unusual ability to invade fresh water from marine or brackish habitats over rapid time scales (< 200 yr; Lee and Bell, 1999). Such freshwater invasions from salt water appear to be increasing in frequency (Semper 1881; Jazdzewski 1980; Spidle et al. 1994), disrupting food webs and causing local extinctions (Jazdzewski 1980; Van den Brink et al. 1993; Carlton et al. 1995; McMahon and Ram 1996). Such recent invasions of fresh water possibly arose repeatedly in numerous invertebrate species, such as hydroids, polychaetes, crustaceans, and the notorious zebra mussel, *Dreissena polymorpha* (Semper 1881; De Beaufort 1954; Jazdzewski 1980; Spidle et al. 1994). These recent invasions provide valuable opportunities for identifying evolutionary and physiological mechanisms involved in transitions between salt- and freshwater habitats (Lee and Bell 1999). Despite the evolutionary significance and ecological implications of these invasions, their pathways, timing, and sources have not been confirmed through genetic analyses of invading and source populations (Lee and Bell 1999; Marsden et al. 1996).

The copepod *Eurytemora affinis* provides a model system for examining how species cross boundaries between "adaptive zones." The propensity of *E. affinis* and its congener, *E. velox,* to appear in man-made bodies of fresh water was noted by G. Evelyn Hutchinson (1957), who described them as "rare examples of animals caught in the last scene of the act of adaptation to fresh water." *Eurytemora affinis* is a dominant planktonic or epibenthic grazer in many estuaries, saltmarshes, and brackish seas throughout the northern hemisphere. Primarily within the past 60 years, *E. affinis* has invaded many freshwater habitats in North America, Europe, and Asia. Published accounts have documented recent occurrences of *E. affinis* in more than 30 freshwater reservoirs and lakes (Table 1), and recent sampling uncovered this species in 10 additional reservoirs in the southeastern United States (Table 1). Prior to invasions within this century, the freshwater distribution of *E. affinis* was documented from a few coastal and oxbow lakes (Marsh 1912; Willey 1923).

Given rapid appearances of *E. affinis* in fresh water, how readily have transitions to fresh water taken place? Geographic patterns of invasion may have involved one or a combination of the following processes (Fig. 1): (1) independent invasions of freshwater sites from nearby saltwater sources; or (2) dispersal from previously colonized freshwater habitats. The null hypothesis is that freshwater populations had a common saltwater origin, with recent appear-

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TABLE 1. Freshwater distribution of *Eurytemora affinis,* including new observations.

Location (site no. from Figs. 1, 2)	Date of reservoir construction	Estimated invasion date[1]	Reference	Current status (1994–1998)[2]
Europe				
IJsselmeer, Netherlands (5)	1932	1932	De Beaufort 1954	Present
Friesian Lakes, Netherlands[3]	11th C[3]	1932–1970	Beattie et al. 1978	Present
Volkerak-Zoommeer, Netherlands	1987	1987–1990	Gulati and Doornekamp 1991	No information
Japan (Hokkaido)				
Ohnuma (7)	N/A	1952s–1981	Ban and Minoda 1989	Present
Akanko (8)	N/A	1950s–1975	Ban and Minoda 1989	Present
Baratoko oxbow (10)	N/A	1950s–1989	Ban and Minoda 1989	Present
Shikaribetsu	N/A	1950s–1981	Ban and Minoda 1989	No information
Chobushiko	N/A	1950s–1997	S. Ban, pers. comm. 1997	Present
Takkobuko	N/A	1950s–1997	S. Ban, pers. comm. 1997	Present
North America				
St. Lawrence River system				
Lac St. Jean, PQ, Canada (13)	N/A	–1923	Willey 1923	Present
Lake Ontario	N/A	1958	Anderson and Clayton 1959	No information
Lake Erie	N/A	1961	Engel 1962	No information
Lake Huron	N/A	1965	Faber et al. 1966	No information
Lake Michigan (15)	N/A	–1969	Gannon 1974	Present
Lake Superior	N/A	–1968	Patalas 1972	No information
East Coast				
John's Pond, MA	N/A	–1881	Wilson 1932	Extinct?
Sutton, NC	1972	1972–1985	Mallin 1989	Extinct?
L Lake, SC	1985	1987	De Biase and Taylor 1993	Extinct?
Cooper River system				
Lake Marion, SC (23)	1941	1941–1984	McIlvaine 1986	Present
Mississippi River system				
Oxbow lakes				
Horn, MS	N/A	–1973	Bowman and Lewis 1989	Present
Black Bayou, MS (27)	N/A	–1912	Marsh 1912	Present
Beulah, MS (28)	N/A	–1973	Bowman and Lewis 1989	Present
Providence, LA	N/A	–1946	Bowman and Lewis 1989	Not found
Reservoirs				
McAlpine Pool, KY (29)	1930	1930–1985	Bowman and Lewis 1989	Present
Heidecke (Collins), IL	1976	1976–1984	Saunders 1993	Not found
Merrisach, AR (30)	1967	1967–1995	J. E. Havel, pers. comm. 1995	New
Dardanelle, AR (31)	1964	1982–1989	Rickett and Watson 1983	Present
Nimrod, AR	1942	1942–1995	J. E. Havel, pers. comm. 1995 Saunders 1993	New
Kerr, OK (32)	1970	1970–1989	Saunders 1993	Present
Eufaula, OK (33)	1964	1964–1989	Saunders 1993	Present
Arcadia, OK	1987	1987–1995	J. E. Havel, pers. comm. 1995	New
Keystone, OK (34)	1964	1964–1967	Kochsiek et al. 1971	Present
Texoma, OK (35)	1944	1955–1970	Saunders 1993	Present
Murray, OK	1960	1960–1995	J. E. Havel, pers. comm. 1995	New
Arbuckle, OK (36)	1967	1967–1995	J. E. Havel, pers. comm. 1995	New
Waurika, OK (37)	1982	1982–1995	J. E. Havel, pers. comm. 1995	New
Clear Creek, OK	1948	1948–1995	J. E. Havel, pers. comm. 1995	New

TABLE 1. Continued.

Location (site no. from Figs. 1, 2)	Date of reservoir construction	Estimated invasion date[1]	Reference	Current status (1994–1998)[2]
Duncan, OK	1937	1937–1995	J. E. Havel, pers. comm. 1995	New
Humphreys, OK	1958	1958–1995	J. E. Havel, pers. comm. 1995	New
Taylor, OK	1960	1960–1996	J. Saunders, pers. comm. 1996	New
Meredith, TX	1968	1968–1981	Saunders 1993	No information
Colorado River system				
Travis, TX (39)	1941	1941–1981	Saunders 1993	Present
Rio Grande River system				
Avalon, NM (40)[4]	1888	1888–1936	Northcote et al. 1964	Present
San Francisco Bay				
Lake Merced, CA	1869–1894	1894	Miller 1958	Extinct?

[1] Range is based on dates of reservoir construction or last time *E. affinis* was not found and first observations. Single dates preceded by – indicate invasion before that date. Single dates without – indicate exact dates of invasion. Dates of reservoir construction were obtained from U.S. Army Corps of Engineers (Tulsa, OK, and Little Rock, AR) and Oklahoma Water Resources Board.

[2] Populations were determined extinct if *E. affinis* was not found during year-round sampling. Extinction is inconclusive because absent populations could be in indefinite diapause. "Not found" indicates absence of *E. affinis,* but insufficient sampling to conclude that the population has gone extinct.

[3] Water is pumped into these artificial lakes from the IJsselmeer.

[4] The reservoir Lake Avalon (site 40) was rebuilt repeatedly since its initial construction date of 1888.

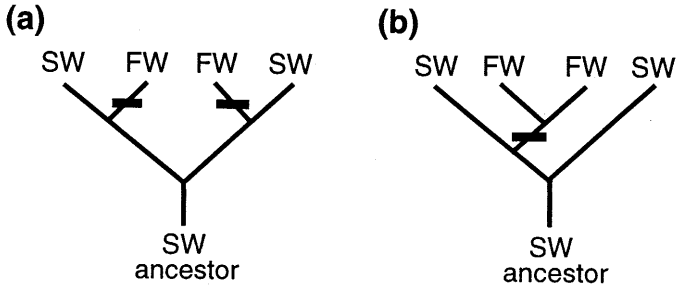

FIG. 1. Alternative hypotheses for the colonization of freshwater habitats, as represented by phylogenetic trees. (a) Independent colonizations of freshwater habitats. (b) A single colonization of a freshwater habitat followed by dispersal (or vicariance). FW, freshwater population; SW, saltwater population. Black bars represent habitat transitions.

ances reflecting dispersal from other freshwater habitats (Fig. 1b).

A phylogenetic analysis and an analysis of molecular variance (AMOVA), based on DNA sequences from the cytochrome oxidase I (COI) gene, were used to test the relative importance of these two invasion processes for *E. affinis*. Because the potential importance of these processes could depend on spatial scale and on mechanisms of transport, these tests were performed at three geographic scales (Fig. 2): (1) among continents (North America, Europe, and Asia); (2) among river drainages within a continent (St. Lawrence, Cooper, Mississippi, and Colorado); and (3) within river drainages (populations within the St. Lawrence, Cooper, and Mississippi drainages).

Physiologically, it seems implausible for most species to invade fresh water readily from saltwater habitats. Faunal compositions of fresh- and saltwater habitats are typically distinct. A few truly euryhaline species, such as the killifish *Fundulus heteroclitus*, can move instantaneously between salt- and freshwater habitats and reproduce in either habitat (Wood and Marshall 1994). Such species have specialized gills and other adaptations that allow rapid regulation of ion exchange when moving between habitats (Towle 1990; Wood and Marshall 1994).

Physiological mechanisms that would explain how *E. affinis* could colonize freshwater habitats are not known. For instance, to our present knowledge, the excretory structures of copepods are relatively simple and consist only of a pair of coelomic maxillary glands (Lowe 1935–1936). To determine whether *E. affinis* could tolerate a rapid transition to fresh water, experiments were performed on adults and eggs from three saltwater populations. Copepods were transferred from 15 PSU to zero salinity to determine whether they could survive, produce eggs, and develop to metamorphosis in fresh water.

MATERIALS AND METHODS

Population Sampling and Habitat Characterization

Forty salt ponds, saltmarshes, estuaries, and brackish seas, and 197 freshwater lakes and reservoirs were sampled between 1994 and 1998. From ponds, lakes, estuaries, and seas, samples were collected using plankton nets, with mesh sizes

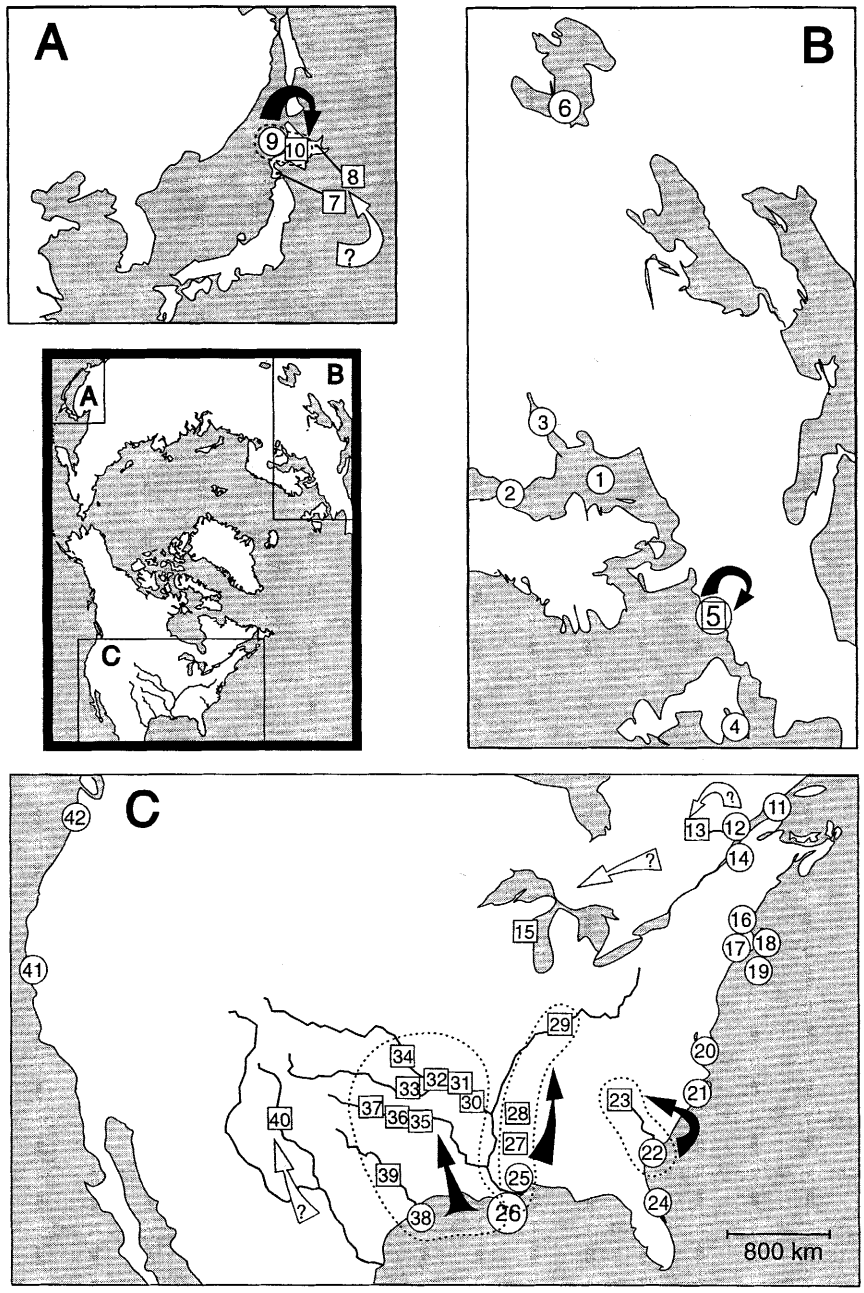

FIG. 2. Populations of *Eurytemora affinis* used for genetic analysis. Circled numbers, saltwater; boxed numbers, freshwater populations. Names of sites are shown in Figure 3. Arrows represent independent invasions of fresh water. Black arrows are invasions for which probable saltwater sources have been identified (Fig. 3), whereas white arrows are invasions from unknown sources. Dotted lines group populations that are genetically identical. (a) Hokkaido, Japan; sites 9 and 10 are in the same (Ishikari) river system. (b) Central Asia and Europe; site 5 is a freshwater lake (Ijsselmeer) that was created by impounding a brackish bay (Zuiderzee). (c) North America; the river systems are St. Lawrence (sites 11–15), Cooper (sites 22, 23), Mississippi (sites 26–37), Colorado (sites 38, 39), and Rio Grande (site 40). Maps a–c are roughly at the same scale with some distortion.

varying from 60 μm to 250 μm. From saltmarshes, samples were collected by hand from near the bottom using a turkey baster. Most sites known to contain freshwater populations of *E. affinis* were sampled for this study (Table 1). In 1995, 173 reservoirs and lakes within the Mississippi River drainage in Oklahoma, Arkansas, and Missouri were sampled by J. E. Havel. The identity of *E. affinis* used in this study was confirmed morphometrically by G. A. Heron and B. W. Frost. *Eurytemora hirundoides* and *E. hirundo* are synonyms of *E. affinis* (Busch and Brenning 1992).

Salinity of habitats was measured with an in situ YSI salinity meter or hand refractometer. Freshwater invasion is defined here as the establishment of a self-sustaining population of individuals that can reside in fresh water throughout an entire life cycle. In this paper, fresh water corresponds to 0.0–0.5 PSU, brackish water to 0.5–30 PSU, and sea water

to 30 PSU and greater (Remane and Schlieper 1971). "Saltwater" and "saline" refer to both brackish and marine water.

Phylogenetic Inference

DNA was extracted from individual copepods (live or ethanol-preserved) using a cell-lysis buffer with proteinase-K (Hoelzel and Green 1992). PCR primers COIL 1490 and COIH 2198 (Folmer et al. 1994) were used to amplify and sequence a 652-bp region of the mitochondrial COI gene. Three to five individuals were sequenced per population. Both light and heavy strands were sequenced for each copepod using an ABI373 automated sequencer. Sequences were aligned unambiguously by eye. Phylogenies were constructed using distance matrix and parsimony approaches (heuristic searches) with PAUP* 4.0 (Swofford 1998). One thousand bootstrap replicates were performed for both approaches. Distance trees were constructed using the neighbor-joining algorithm with branch-swapping (tree-bisection-reconnection). Maximum-likelihood distances were computed. Variation of evolutionary rates among sites (using shape parameter $\alpha = 0.184$ of a gamma distribution; Yang 1996) and transition: transversion ratio (4.7) were estimated using maximum likelihood. Consensus sequences were used for some of the saltwater populations that did not share haplotypes with other populations and were not closely related to any freshwater populations (such as the St. Lawrence estuary). Using consensus sequences did not affect the topology of the tree, while making the phylogenetic analysis less cumbersome. Sequences were obtained from congeners *E. americana* and *E. herdmani*, but were not used to root the tree because level of divergence with *E. affinis* was saturated and hierarchical relationships among clades could not be found.

To test the hierarchical distribution of molecular variance at different geographic scales, an AMOVA (Excoffier et al. 1992) was performed using the software package Arlequin version 1.1 (Schneider et al. 1997). The AMOVA used pairwise distances of haplotypes to estimate the relative contribution of molecular variance of at three geographic scales: among continents (ϕ_{CT}, correlation of haplotypes within continents relative to those from the whole species), among drainages (ϕ_{ST}, correlation of haplotypes within drainages relative to those from the whole species), and within drainages (ϕ_{SC}, correlation of haplotypes within drainages relative to those from each continent). Pairwise distances were computed using a Kimura two-parameter model with shape parameter $\alpha = 0.184$ of a gamma distribution. Significance tests for fixation indices were performed with 1000 permutations. Comparisons were not made within populations because intrapopulation variation was very small or nonexistent.

Freshwater Tolerance

Freshwater tolerance was examined for three North American saltwater populations with overlapping salinity ranges (10–25 PSU): Grays Harbor saltmarsh, Washington (Fig. 2, site 42); Waquoit Bay, Massachusetts (site 18); and Edgartown Great Pond, Martha's Vineyard, Massachusetts (site 19). Three populations were examined to detect variation among populations in their response to low salinity. Populations were cultured in a 13°C environmental chamber for at least two generations at 15 PSU prior to the experiments. All experiments were performed at 13°C. During the experiments all treatments and controls were fed an identical diet of freshwater algae (*Chlamydomonas* sp., *Scenedesmus* sp.) to avoid changing salinity of the medium and to prevent the copepods from gaining large amounts of salt from their diet.

Survivorship of Adults at Zero Salinity (Lake Water)

Salinity was changed at 2-PSU decrements from 15-PSU estuarine water to 0-PSU lake water over 7 h, and then held at 0 PSU for 7 days. Controls accounted for handling and senescence and were treated identically to treatments, except that salinity remained constant at 15 PSU. Treatment and controls contained 36–50 adult females, with three replicates per population, except for Waquoit Bay, where 137 females were transferred to 0 PSU without separate replicates or controls. Males were transferred to 0 PSU in the same manner for use in the egg-production experiment (see below). An unpaired *t*-test (two-tailed) was used to compare adult survivorship among populations.

Egg Production and Metamorphosis at Zero Salinity

Adults that survived zero salinity were separated into 10–20 mating pairs at 0 PSU. Adults from each population were also mated at 15 PSU as controls. The following percentages were measured: mating pairs that were able to produce eggs (out of all pairs), eggs within clutches that developed to metamorphosis (from nauplius to copepodid I), and clutches that yielded at least one offspring that developed to metamorphosis (out of all clutches). A Mann-Whitney *U*-test was used to compare number of metamorphs/clutch at 0 PSU relative to 15-PSU controls.

Transfer of Eggs to Zero Salinity

Eggs were transferred directly from salt to fresh water because their transport through gut-passage in birds has been hypothesized as a possible means for dispersal (Hutchinson 1957; Saunders 1993). Twenty clutches from two saline populations (Grays Harbor, WA, and Martha's Vineyard, MA) were split in half and 10–20 eggs were transferred to zero-salinity treatments and 15-PSU controls. Hatching rate and developmental time to metamorphosis (or mortality) were measured. A Wilcoxon signed ranks test was used to compare hatching rate between clutches that were split into freshwater treatments and controls. A statistical comparison was not performed on development time or survivorship because larvae in fresh water had 100% mortality before metamorphosis.

RESULTS

Distribution of Populations

Recent appearances of *E. affinis* tended to occur in reservoirs or altered (depauperate or polluted) lakes (Table 1). Within the Mississippi River drainage, *E. affinis* was found in 18 reservoirs connected to rivers and in four oxbow lakes closer to the river mouth. The distribution of *E. affinis* populations along rivers was patchy, with populations occurring in clusters (Table 1). Ten new freshwater populations of *E.*

affinis were found in 1995 and 1996, while some populations appear to have gone extinct (Table 1).

Within estuaries, *E. affinis* populations typically occurred between 5 PSU and 25 PSU at the "saltwedge," the abrupt interface between salt and fresh water. In marsh ponds, *E. affinis* was usually found near the bottom or on the banks during the day, in salinities as high as 40 PSU. Some studies may underestimate salinity distributions of *E. affinis* in brackish habitats because surface waters are often much lower in salinity than the depths at which *E. affinis* is found.

Phylogenetic Relationships

Trees constructed using distance-matrix and parsimony approaches were for the most part concordant. The two methods yielded a few differences in statistical support (bootstrap values) for some nodes. For the distance-matrix approach, bootstrap values were $> 50\%$ for a node grouping Ishikari River with the Caspian Sea in Asia (85%) and for a node grouping St. Lawrence River/Waquoit Bay with the Mississippi River drainage (86%). The parsimony approach yielded a bootstrap value of 59% for a node grouping all southeastern Atlantic populations, from Chesapeake Bay, Maryland, to St. John's River, Florida.

With some exceptions, the overall pattern was one of large sequence divergences among populations on different continents and among populations in different river drainages (Fig. 3; maximum sequence divergence of 17%), but low variation among haplotypes within drainages and within populations (typically 0–0.7% polymorphism). Genetic divergences were typically slight among populations within drainages (with the exception of St. Lawrence), irrespective of habitat type. Substitutions were generally at the third position and silent. Similarly, the AMOVA indicated that slightly more than half of the haplotype diversity occurred among river drainages (51.84%) and an appreciable amount among continents (43.34%), but very little within drainages (4.83%) (Table 2). Fixation indices, proportions of variance due to each component, were significant (Table 2).

Thus, it follows that freshwater populations were not monophyletic, but grouped into genetically distinct lineages (black and white arrows in Figs. 2, 3) that often corresponded to particular river drainages. For five of the freshwater lineages, genetically identical saltwater populations were found (black arrows in Figs. 2, 3). The saltwater origin of the Ijsselmeer population (site 5) is known because extensive monitoring documented that it survived the six-year transformation of the saline bay Zuiderzee into a freshwater lake (De Beaufort 1954).

On each continent, freshwater populations were more closely related to saltwater populations on the same continent than to freshwater populations throughout the world (Fig. 3). Within four river systems (St. Lawrence, Cooper, Mississippi, and Ishikari; Fig. 2), freshwater populations were genetically closest to saltwater populations in the same river drainage (Fig. 3) rather than to nearby freshwater populations from different drainages (such as sites 15 and 29). In three of the river systems (Cooper, Mississippi, and Ishikari), fresh- and saltwater populations were genetically indistinguishable (Fig. 3). Populations from Mississippi (site 26) and Colorado (site 38) estuaries could not be distinguished as potential sources for sites 30–37 and 39 because they shared the same haplotype (Fig. 3). Within the Mississippi River system, two regions were genetically distinct (Figs. 2, 3; sites 27–29 vs. 30–37). Two mtDNA haplotypes (1.8% COI divergence) were found in the Mississippi estuary (site 26), but only one haplotype was found in the eastern region (Fig. 2, sites 27–29), whereas a different haplotype was found in the western region (sites 30–37; Fig. 3).

Within the St. Lawrence River system two highly divergent (11% at COI) estuarine populations (sites 11 and 12) were found in close geographic proximity. The population in the St. Lawrence estuary (site 11) was genetically distant from all other populations within the drainage (sites 12–15), which were closely related to one another. Although their exact sources remain unknown, freshwater populations from Lake Michigan (site 15) and Lac St. Jean (site 13) were probably derived locally, given their genetic proximity to populations in nearby brackish (site 12) and hypersaline habitats (site 14).

For two freshwater populations, Lakes Ohnuma and Akanko on Hokkaido, Japan, the putative saltwater source populations were not sampled. These populations are thought to have originated from brackish lakes on the island of Honshu, through human-mediated transport of smelt eggs (Ban and Minoda 1989). Lake Avalon, New Mexico, contained a unique haplotype that is closely related to those found in the Mississippi River system. Whether Lake Avalon originated from the downstream estuary could not be tested because damming has reduced the riverine flow of the once large Rio Grande so that an estuary no longer exists.

Tolerance to Fresh Water

Adults from saltwater populations suffered high mortality when transferred to fresh water relative to 15 PSU controls (unpaired two-tailed t-test, $n = 3$ replicates, Grays Harbor: $t = -7.3$, $P < 0.005$; Martha's Vineyard: $t = -11.1$, $P < 0.001$; Fig. 4a). Of three populations tested, only individuals from Martha's Vineyard produced eggs in fresh water (Fig. 4b), despite the fact that females from Grays Harbor marsh and Waquoit Bay lived long enough to produce eggs (7.5 days $\pm$ 1.8 SE and 12.0 days $\pm$ 1.6, respectively). For the Martha's Vineyard population, matings at zero salinity and at 15 PSU resulted in similar numbers of eggs per clutch (13.3 $\pm$ 1.0 at 0 PSU, $n = 15$ clutches; 15.8 $\pm$ 1.5 at 15 PSU, $n = 13$ clutches; Mann-Whitney $U = 74$, $P = 0.3$). However, fewer eggs developed to metamorphosis in fresh water, both in terms of number of metamorphs per clutch (Mann-Whitney $U = 59$, $P < 0.05$; Fig. 4c), and proportion of all clutches that yielded metamorphs (Fig. 4d). Larvae lived for about five days (5.16 $\pm$ 0.96 SE) in fresh water, whereas at 15 PSU they metamorphosed after about 20 days (19.47 $\pm$ 4.17). From a starting number of 369 adults (159 F, 210 M), only four larvae metamorphosed into juveniles from parents that survived the zero-salinity transfer, of which two matured into adults.

When eggs from saltwater populations were transferred directly into fresh water, hatching rate was high in fresh water, but slightly higher in controls (Fig. 5a). This result was sig-

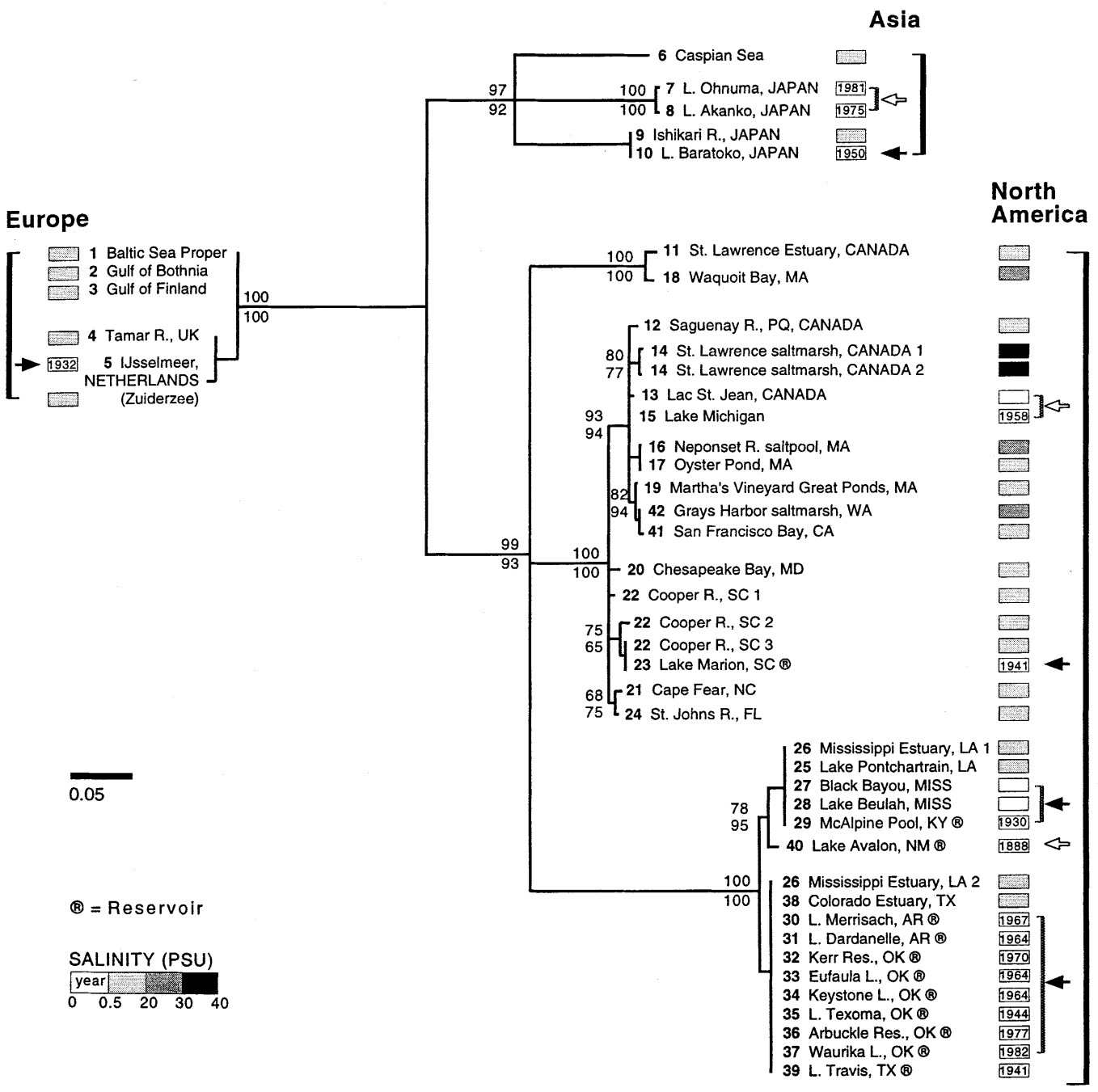

FIG. 3. Phylogeny (unrooted) of *Eurytemora affinis* populations using cytochrome oxidase I (COI, 652 base pairs). Locations of populations are shown at branch tips, with numbers representing populations shown in Figure 2. Shaded boxes indicate salinity ranges of populations. Numbers in boxes are date of detection in natural lakes (sites 7, 8, 15) or year of reservoir construction. The date shown for Lake Michigan (site 15) is the first observation of *E. affinis* in the Great Lakes. Black and white arrows correspond to independent invasions of fresh water shown in Figure 2. Branch lengths are maximum-likelihood distances that account for multiple substitutions per site; thus, long branches appear longer. Numbers next to nodes are bootstrap values based on 1000 bootstrap replicates (Felsenstein 1985) using distance matrix (above branches) and parsimony approaches (below branches) with the software package PAUP* 4.0 (Swofford 1998). Only nodes with bootstrap values > 65% are shown.

nificant for the population from Grays Harbor (Wilcoxon signed ranks test, $n = 20$, $t = -2.04$, $P < 0.05$), but not for that from Martha's Vineyard (Wilcoxon signed ranks test, $n = 20$, $t = -8.04$, $P = 0.42$). No larvae survived to metamorphosis in fresh water (Fig. 5b). Similar to results in the previous experiment, larvae lived for about five days at zero salinity (5.35 days ± 0.27 SE, Grays Harbor; 5.46 days ± 0.37, Martha's Vineyard) and metamorphosed in about 20 days at 15 PSU (19.82 days ± 2.08 SE, Grays Harbor; 19.05 days ± 1.12, Martha's Vineyard; Fig. 5).

TABLE 2. Hierarchical analysis of variance among haplotypes using AMOVA.

	Variance	% of total variation	Fixation indices[1]	P[2]
Source of variation				
Among continents	53.28	43.34	$\Phi_{CT} = 0.433$	0.000
Among drainages	63.73	51.84	$\Phi_{ST} = 0.952$	0.000
Within drainages	5.93	4.83	$\Phi_{SC} = 0.915$	0.000

[1] Excoffier et al. (1992).
[2] Probability that a random value from 1000 permutations is greater than or equal to the fixation indices found.

DISCUSSION

Pattern of Invasion

Freshwater populations were derived independently from eight distinct matrilineages (Figs. 2, 3). The fact that four of the freshwater lineages were genetically identical to saltwater populations strongly suggest that they were derived recently from saltwater sources (Figs. 1a, 3; also see next section).

At large scales of continents and of river systems within a continent (North America), a pattern of multiple independent invasions from salt water was supported by the fact that freshwater populations were most closely related to those in nearby saltwater habitats (Figs. 1a, 3). In contrast, dispersal among freshwater habitats may have been common at finer spatial scales (Fig. 1b, 3). Within the Mississippi River system, there is evidence for both independent invasions from salt water (Fig. 1a) and dispersal among freshwater sites (Fig. 1b). The western and eastern regions of the river system (Fig. 2C) appear to have been derived independently from the estuary because they contained different estuarine haplotypes (Fig. 3). The fact that all reservoirs in each region were fixed for one of two haplotypes suggests a history of dispersal among freshwater sites from an initial founding population within each region (Fig. 1b). Independent invasions of each reservoir from the estuary would not likely lead to fixation of the same haplotype in all reservoirs within a region. Although sample sizes per population (three to five) were small, leading to the possibility of sampling error and the underestimation of allele sharing, the maintenance of genetic distinction between the two regions is striking. Selection is unlikely to have caused fixation (assuming no mtDNA hitchhiking), given that substitutions did not lead to amino acid changes.

The AMOVA analysis (Table 2) revealed large amounts of genetic variance among drainages and continents, but very little within drainages. The greater variance among drainages than continents may at first seem puzzling. However, the result is congruent with the fact that genetic divergences are greater among drainages (such as Mississippi vs. St. Lawrence and Cooper drainages, and Caspian Sea vs. Ishikari River) than among ancestral nodes of different continents.

The predominance of different invasion processes at different spatial scales illustrates the importance of testing hy-

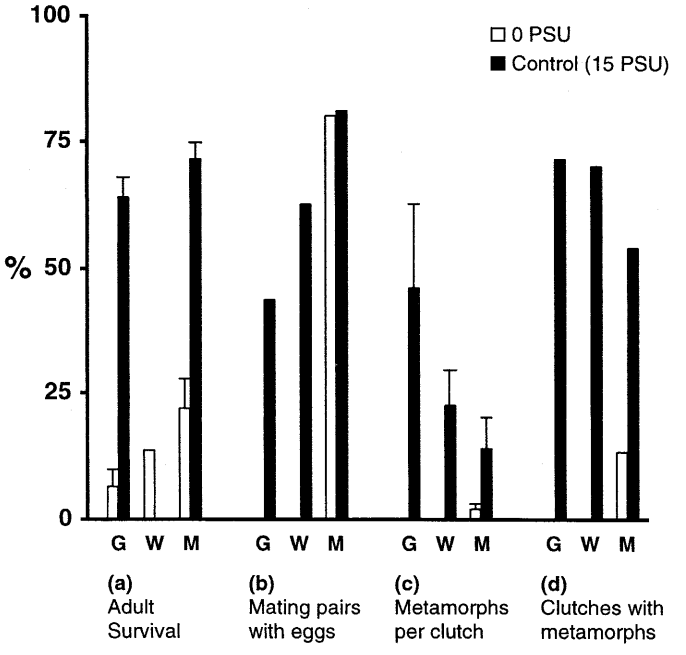

FIG. 4. Tolerance to zero (0 PSU) and control (15 PSU) salinities of adults and their offspring from saltwater populations. Populations are from Grays Harbor saltmarsh, Washington (G); Waquoit Bay, Massachusetts (W); and Martha's Vineyard, Massachusetts (M). Graph shows: (a) percentage of adult females that persisted for seven days; bars are means of three replicates ± SE (replication and controls are missing for Waquoit Bay); (b) percentage of all mating pairs that successfully laid eggs; (c) percentage of eggs within clutches that developed to metamorphosis; bars are means of 10–20 clutches ± SE; and (d) percentage of all clutches in which at least one larva metamorphosed.

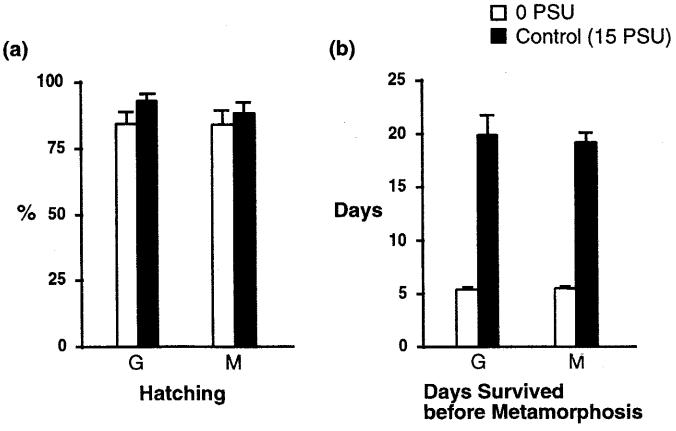

FIG. 5. Tolerance to zero (0 PSU) and control (15 PSU) salinities of larvae hatched from eggs from saltwater populations. Populations are from Grays Harbor saltmarsh, Washington (G), and Martha's Vineyard, Massachusetts (M). (a) Hatching rates of eggs at 0 PSU and at 15 PSU. (b) Days of survival before metamorphosis. Bars for 0-PSU treatment show days until mortality (mean ± SE). Bars for 15 PSU show days until metamorphosis (mean ± SE).

potheses of invasion at multiple spatial scales. For instance, no recent dispersal occurred between continents and little between rivers, but rapid movement was evident over great distances within river systems. Invasion from salt water and dispersal among freshwater habitats appear to have occurred primarily within river systems, from estuaries into lakes and reservoirs (Fig. 3).

Ancestral State and Time Scale of Invasions

The most parsimonious ancestral habitat of *E. affinis* is in saline rather than fresh water. Prior to recent reports, the known freshwater distribution of *E. affinis* was limited to few sites (Marsh 1912; Willey 1923), whereas saltwater occurrences spanned many estuaries and brackish lakes and seas throughout the world. There are no known preexisting freshwater populations that could have been sources for all salt- and freshwater populations.

The lack of genetic differentiation between freshwater populations and their putative sources does not allow an estimate of timing of invasions based on genetic data. A very gross upper bound can be estimated using rates of molecular evolution that have been calibrated for a different region of COI (using primers CO1a and f; Palumbi 1996) in other crustaceans, such as 1.4%/million yr for snapping shrimps (*Alpheus*; Knowlton and Weigt 1998) and 2.3%/million yr for grapsid crabs (*Sesarma*; Schubart et al. 1998). If these rates apply to COI in *E. affinis*, derivation of freshwater populations from saltwater sources had to occur within the past 500,000 to 700,000 years, but were probably much more recent. Relative to other studies using molecular markers, an upper bound of 700,000 years for freshwater invasions is still very recent. For instance, the radiation of grapsid crabs 4 million years ago into freshwater and terrestrial habitats in Jamaica is considered recent (Schubart et al. 1998). Molecular markers applied to two "glacial relict" species (Hutchinson 1957; Segerstråle 1982), the crustaceans *Mysis relicta* and *Pontoporeia affinis*, show that salt and freshwater "populations" are genetically highly divergent, with divergence times dating back possibly to the Tertiary (Väinölä 1990, 1994).

For most freshwater sites, invasions are known to have occurred within the past century (Table 1). It is possible that preexisting freshwater populations, such as those in oxbow lakes, served as intermediaries between estuaries and reservoirs, and that initial invasions of freshwater within drainages occurred long before this past century. However, even if oxbow or other lakes did serve as sources for recent expansions into reservoirs, the lack of genetic differentiation between reservoir and estuarine populations is consistent with relatively recent origins of freshwater populations.

Most notably, direct observations of survival of *E. affinis* during transformations of saline habitats into freshwater reservoirs (De Beaufort 1954) indicates that the transition to fresh water has in some cases occurred within a few years. Physiological experiments further support this possibility (see below).

Taxonomic Status of Eurytemora affinis

Although genetic divergences tend to be low within river drainages and within geographic regions, genetic divergences are extremely high among drainages and continents (Fig. 3). Despite morphological similarities among populations (B. W. Frost, in prep.), large genetic divergences and reproductive isolation (C. E. Lee, in prep.) between some of the populations suggest that *E. affinis* constitutes a species complex (Knowlton 1993). For instance, results from mating experiments suggest that the St. Lawrence/Waquoit Bay lineage (Fig. 3, sites 11 and 18) is reproductively isolated from other populations in the St. Lawrence River drainage (sites 12-15; C. E. Lee, in prep.). Morphological features not previously used for taxonomic purposes are able to distinguish some of the genetically divergent populations (B. W. Frost, in prep.). Relationships among genetically divergent *E. affinis* populations are explored further in another paper (C. E. Lee, in prep.). A pattern of high interpopulation variation at COI (20%) does occur in other copepod species (complexes), such as *Tigriopus californicus* (Burton 1998).

Low Salinity Tolerance

Saltwater populations differed in their response to zero-salinity water (Fig. 4), suggesting that the ability to invade fresh water varies among populations. Even in the most successful case, however, survival from adult to metamorphosed juvenile was only 1.1% (Fig. 4), suggesting that the transition to fresh water could occur in a single generation, but may result in a narrow population bottleneck. The few larvae that successfully metamorphosed may have survived because of either genetic or nongenetic factors (such as nutritional condition). When eggs were transferred to zero salinity, hatching rates were high (Fig. 5a), but none of the larvae survived to metamorphosis (Fig. 5b). Eggs from adults that passed through a freshwater bottleneck survived at a higher rate for the Martha's Vineyard population (Fig. 4d). Results suggest that the transfer of a few hundred eggs (in this case from 20 clutches) would not be sufficient to found a new population. These results are consistent with the possibility of rapid transitions to fresh water, but require large numbers of founders to form viable populations (Fig. 4).

Because it is impossible to replicate conditions of natural habitats, survival or mortality in the laboratory is not necessarily a true representation of tolerances in the wild. In addition, the tolerance experiments were limited in three ways: not all relevant populations were included, the role of acclimation was not explored, and the physiology of diapause eggs were not examined. At the time of the experiment, live cultures from several populations were not available because they were in diapause, and some of the populations not examined may have broader tolerances.

With longer acclimation times, populations may be able to enter fresh water with greater ease, and differences among populations may become less evident. For instance, survival of *E. affinis* during the six-year transformation of the saline bay into the freshwater IJsselmeer, Netherlands (site 5; De Beaufort 1954), demonstrates the ability of one population of *E. affinis* to make the gradual transition from saline to freshwater habitats over a period of a few years.

In some populations, diapause eggs are produced seasonally when the population disappears temporarily (Ban 1992), possibly to avoid stressful conditions. These diapause eggs

may be physiologically more resistant than adults or regular subitaneous eggs. Despite limitations of the experiments, results show that at least under a particular set of conditions, some populations may be able to survive transitions to fresh water in one or two generations.

Propensity and Opportunity to Invade

Until recently, reports of *E. affinis* in fresh water were extremely rare, in contrast to the preponderance of this species in saline habitats throughout the northern hemisphere. The remarkable spread of *E. affinis* to inland waters raises many questions about its physiology. For instance, why is *E. affinis* more invasive of freshwater habitats than most other estuarine and saltmarsh species, and why have invasions occurred primarily in modern times? Phylogenetic and physiological propensity, opportunities for transport, and availability of habitat are probably key factors.

The genus *Eurytemora* has a very broad habitat distribution (Heron 1976), with *E. affinis* having the broadest salinity distribution within the genus. Another copepod that is rapidly invading fresh water is the congener *E. velox*. Traits of *Eurytemora* that facilitate freshwater invasions are not understood.

Opportunities for transport into inland waters have been increasing with human activity. Whereas anadromous (migratory) fishes actively swim into freshwater habitats to reproduce (McDowall 1988), *E. affinis* is a passive disperser that requires a transport vector. Phylogenetic relationships indicate movement within river systems (Fig. 3). In addition, extensive sampling revealed that, with few exceptions, recent freshwater populations occurred in freshwater sites directly connected to river systems (Table 1), suggesting that rivers provided the routes for invasion. However, patchy distribution of populations along a river (Table 1), suggests that dispersal did not occur through passive river flow. For example, the reservoir Fort Gibson in Oklahoma, which lacked *E. affinis*, is situated between two reservoirs (Kerr and Keystone) that have contained *E. affinis* for at least 10 years (Saunders 1993). This disjunct distribution of populations suggests that transport within river systems have been mediated by episodic events, such as bilge water disposal from boats, transplantation of recreational fishes within river systems, or transport by waterfowl with ranges confined within river systems. Limited movement among river systems suggests that transport over land, such as by waterfowl (Saunders 1993), was not common. Lack of genetic overlap among continents (Fig. 3) suggests that transoceanic transport has not occurred recently, either between similar or distinct habitat types, in contrast to the case of zebra mussels, which were most likely brought into the Great Lakes from Europe (Hebert et al. 1989).

Increased availability of depauperate habitats may have enhanced opportunities for invasion. Recent freshwater invasions by *E. affinis* have taken place in reservoirs (> 90%) or habitats that had become altered or depauperate from pollution, such as the Great Lakes (Table 1). This pattern is also true for the copepod *E. velox* in Europe (Hutchinson 1957). These depauperate habitats may have been amenable to invasions because they were devoid of competition from freshwater species (Dayton 1971). Given the physiological challenges of invading fresh water, reservoirs could provide a haven for freshwater acclimation and adaptation and serve as stepping stones into natural communities. For instance, several saline species that had invaded or had been transplanted deliberately into reservoirs eventually expanded their ranges into natural lakes (Jazdzewski 1980).

Thus, *E. affinis* may have a propensity to invade fresh water that is enhanced through human intervention. Yet, will *E. affinis* persist in fresh water over evolutionary time? Colonization and extinction may characterize the pattern of freshwater invasion over evolutionary time. In fact, several freshwater populations appear already to have gone extinct (Table 1). Alternatively, modern-day opportunities may have resulted in evolutionary adaptations and persistence in freshwater habitats.

Comparisons with Other Species

Based on numerous observations, modern invasions of fresh water do not appear to be unique to *E. affinis*, given that recent impoundments and deliberate transplantations have resulted in freshwater populations of several saline species (Lee and Bell, 1999). However, no other study has systematically reconstructed evolutionary histories of recent freshwater invasions, other than studies of diadromous (migratory) fishes. In particular, genetic studies of anadromous (migratory) fishes are the only other cases in which independent derivations of freshwater populations from saltwater sources have been documented (Taylor et al. 1996; Taylor and McPhail, 1999). Frequent landlocking of anadromous fishes in freshwater lakes (McDowall 1988) is easier to understand than freshwater invasions by *E. affinis*, given that these fishes probably descended recently from freshwater ancestors (McDowall 1988), and breed and spend their early life history in freshwater habitats.

For zebra mussels, the null hypothesis (Fig. 1b) appears to characterize the dominant process of freshwater invasion, unlike the pattern evident for *E. affinis*. Genetic analysis has not yet been performed on potential source populations (Marsden et al. 1996), but based on shell pattern (Rosenberg and Ludyanskiy 1994) and historical observations (De Martonne 1927; McMahon and Ram 1996), recent (< 200 years) freshwater invasions by zebra mussels have been hypothesized to have originated from the saline Black and Caspian Sea region followed by rapid dispersal throughout inland Europe.

Conclusion

Parallel invasions by *E. affinis* over relatively short time scales suggests that these transitions occurred with relative ease, resulting in occurrences of closely related populations in hypersaline, brackish, and freshwater habitats in multiple river systems. The evolutionary history of freshwater invasion and tolerance to fresh water were examined as first steps toward understanding mechanisms involved in the habitat transition. Multiple independent invasions by *E. affinis* provide an opportunity to examine replicated tests of adaptation involved in habitat transitions.

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