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## Reconstructing the history of intercontinental dispersal in *Daphnia lumholtzi* by use of genetic markers

**Abstract**—After its appearance in 1989, the cladoceran *Daphnia lumholtzi* rapidly dispersed throughout the southern United States. In the current study, we used allozyme and mitochondrial deoxyribonucleic acid sequence data to infer the past dispersal of this species. Both genetic markers revealed the similarity among all U.S. populations and those from Uganda and Nepal but their divergence from Australian lineages. The extent of genetic divergence among populations, when coupled with estimates of rates of molecular evolution, suggests that the distribution of this species reflects a series of long-distance dispersal events over the last 4 million years.

Biogeographers attempt to explain the distributions of plants and animals from knowledge of ecology, systematics, and the geologic record. Species with disjunct intercontinental distributions are of particular interest because this pattern might arise either as a result of vicariance or through more recent dispersal from a center of origin (Brown and Lomolino 1998). Vicariance involves the appearance, over geologic time, of barriers that disrupt distributions that were originally continuous. For example, the division of Pangea (~200 million years ago [mya]) and the fragmentation of Gondwanaland (~65 mya) might explain the distribution of species now found on several continents. In contrast, the

center of origin hypothesis presumes the dispersal of modern lineages from a localized ancestral population. Conclusions about centers of origin are often controversial (Brown and Lomolino 1998) because dispersal corridors and mechanisms are necessary for this hypothesis to explain contemporary distributions, but these factors are usually difficult to investigate.

Freshwater cladoceran zooplankton provide an interesting opportunity to test biogeographic hypotheses. Fossil evidence indicates that the Cladocera originated in the Permian (280 mya) (Kerfoot and Lynch 1987), indicating that their origins predate the current position of the continents. Molecular data have additionally suggested that many of the species complexes in the cladoceran genus *Daphnia* are up to 100 million years old (Colbourne and Hebert 1996), suggesting the modern distributions of species might reflect the impact of vicariance events linked to the fragmentation of Gondwanaland. However, a much more recent exchange of lineages among continents, by dispersal, is also possible, given that cladocerans produce diapausing eggs, which are viable outside of water. In some cladoceran genera such as *Daphnia*, these eggs are held in an ephippial case that provides both additional protection and adherent structures (Benzie 1988; Dodson and Frey 1991; Hebert 1995), which

Table 1. List of populations sampled for allozyme and DNA analysis. Locations for U.S. populations indicate county and state.

Site and code	Population name	Location	Date sampled	Item sampled
Uganda				
AFR	Lake Victoria	Jinja	Jul 94*	A, DNA†
Nepal				
ASIA	Lake Phewa	Pokhara Valley	Mar 94*	A, DNA
Australia				
AUS-1	Centennial Lake	Sydney, New South Wales	Jan 90	A
AUS-2	Lake Parrametta	Sydney	Apr 96	DNA
AUS-3	Lyell Reservoir	Sydney	Apr 96	DNA
United States				
AZ-1	Canyon Lake	Maricopa, Arizona	Jul 95	DNA
FL-1	Parker Lake	Polk, Florida	Dec 92	A
FL-2	Lake Okeechobee	Palm Beach, Florida	Aug 96	DNA
IL-1	Lake Springfield	Sangamon, Illinois	Aug 95*	A
IL-2	Illinois River	Mason	Aug 96	DNA
IL-3	Lake Heidecke	Grundy	Oct 96	
KS-1	Clinton Lake	Douglas, Kansas	Aug 95*	A, DNA
KY-1	Barkley Lake	Trigg, Kentucky	Aug 93	A
KY-2	Kentucky Lake	Calloway	Aug 93, Aug 95*	A
LA-1	Atchafalaya Swamp	St. Martin, Louisiana	Sep 95*	A
MO-1	Pomme de Terre Lake	Hickory, Missouri	Sep 93	A, DNA
MO-2	Stockton Lake	Dade	Sep 93, Jul 94, Aug 94	A (Sep 93), A (Jul 94), A, DNA (Aug 94)
MO-3	Thomas Hill Lake	Randolph	Aug 95*	A
OH-1	Ohio River	Jefferson, Ohio	Aug 93	A
OK-1	Fort Gibson Lake	Cherokee, Oklahoma	Jul 95	A
OK-2	Grand Lake	Ottawa	Jul 94	A, DNA
OK-3	Humphrey Lake	Stephens	Jul 95	A
OK-4	Lake Texoma	Marshall	Jul 95, Jul 95*	A
SC-1	Lake Greenwood	Greenwood, South Carolina	Aug 96	DNA
TN-1	Douglas Lake	Jefferson, Tennessee	Dec 92	A
TX-1	Lake Waco	McLennan, Texas	Mar 96	DNA

\* Preserved by the trehalose method (Taylor et al. 1994). Other samples were flash frozen in liquid nitrogen.

† A, population sampled for allozymes; DNA, population sampled for DNA.

should allow attachment to migrating waterfowl. Although ehippia would seem readily dispersed among water bodies, genetic analyses on numerous species of *Daphnia* have shown marked regional divergence, suggesting that the exchange of migrants is ordinarily limited (DeMeester 1996). Furthermore, detailed taxonomic studies have revealed that individual species of cladocerans are less widely distributed than once believed (Frey 1982). In the genus *Daphnia*, most temperate species are restricted to single continents and many species have very restricted distributions (Hebert and Wilson 1994; Hebert 1995; Taylor et al. 1998).

Nevertheless, some cladocerans, such as *Daphnia lumholtzi*, have broad distributions. This species occurs naturally in Australia, the Indian subcontinent, and several regions of Africa (Benzie 1988), and also appeared recently in North America (Sorensen and Sterner 1992; Havel and Hebert 1993) where it spread rapidly across much of the eastern United States (Havel unpubl. data).

Genetic analyses can evaluate hypotheses concerning the origins of North American populations of *D. lumholtzi*. A strong genetic similarity between these populations and those from another continent would be consistent with that continent being the source of the invading populations. For example, allozyme analysis of the cladoceran *Bythotrephes*

*cederstroemi* indicated the similarity of Great Lakes populations and those from Finland, but their marked divergence from Swedish and German populations, suggesting Finland as the source of colonization (Berg and Garton 1994). A preliminary allozyme study of *D. lumholtzi* indicated a high similarity between two North American populations, but their marked divergence from an Australian population, suggesting that the source was not Australia (Havel and Hebert 1993). However, no information was available for populations from other segments of its distribution.

The current study uses both allozyme and mitochondrial deoxyribonucleic acid (mtDNA) sequence data from multiple populations in the United States and the three continents where the species is native to examine hypotheses on the history of invasion by *D. lumholtzi*. By coupling the DNA divergence data with the application of a molecular clock, our work also provides insight into the biogeography of the species. For instance, if the estimated divergence times of populations of *D. lumholtzi* from separate continents is on the order of 65 million years, this would suggest its early origin and subsequent isolation on fragments of Gondwanaland. Alternatively, if the estimated divergence times are much less, this would suggest its recent dispersal from a center of origin.

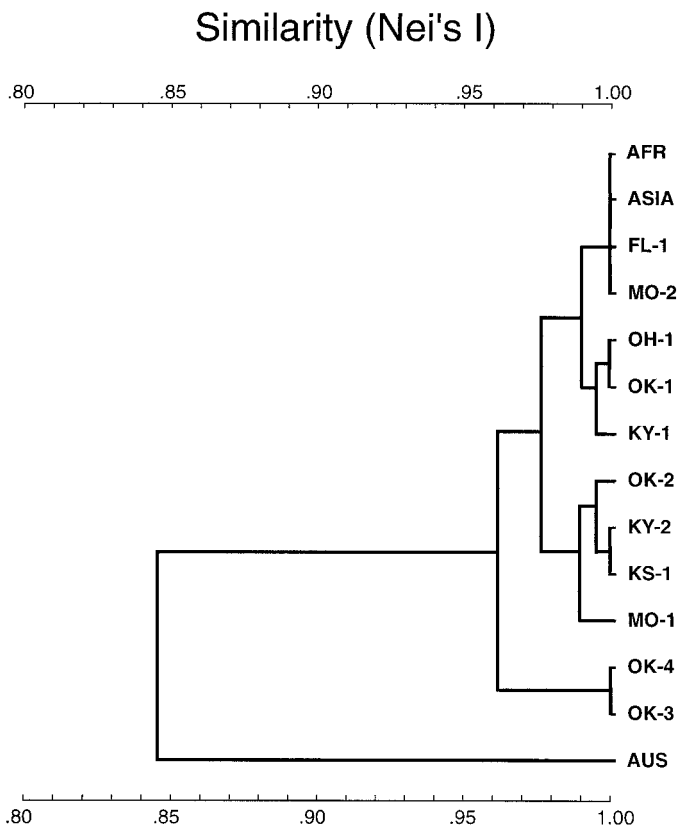


Fig. 1. Results from an unweighted pair group method with arithmetic averaging (UPGMA) cluster analysis of Nei's (1978) unbiased identities between 14 populations of *D. lumholtzi* at six allozyme loci. Population codes are listed in Table 1. Cophenetic correlation = 0.951.

*D. lumholtzi* was collected by plankton tows from 26 sites over a 6-yr period (Table 1). Twenty-one of these sites were located in 11 states of the United States, whereas five other sites were located on the three continents within the natural range of this species (Africa, Asia, and Australia; Benzie 1988). Samples from 18 populations were preserved for allozyme analysis and 14 for DNA analysis (Table 1). Most samples were flash-frozen within 8 h of collection and stored in liquid nitrogen until analysis. The others were either preserved in trehalose for allozyme analysis (Taylor et al. 1994), or in 90% ethanol for DNA analysis.

*Genetic variation at allozyme loci*—Allozyme phenotypes were determined using cellulose acetate gels following standard methods (Hebert and Beaton 1993). Cultures of nine isofemale lines from three U.S. populations (KS-1, OK-3, and MO-1) served as allozyme markers on gel plates. Both live and frozen animals yielded equally good enzymatic activity. Although some trehalose-preserved samples had a number of active enzymes, many did not and were useful only for allelic comparisons among populations.

Most allozyme gel plates were run for 15 min in a Tris-glycine buffer pH 8.5 at 1.5 mA per plate. The exception was aspartate aminotransferase, which was examined after running gel plates for 20 min in Tris-citrate buffer (0.75 M Tris, 0.25 M citric acid, pH 7.0). Individual *Daphnia* could usually be stained for six enzymes, although juveniles and those showing low enzyme activity were only stained for three. For polymorphic loci, alleles were numbered in order of increasing anodal mobility (i.e., 1 slowest).

All populations were examined for variation at six enzymes representing seven loci: aldehyde oxidase (*Ao*, EC 1.2.3.1), arginine kinase (*Ark*, EC 2.7.3.3), mitochondrial and cytoplasmic aspartate aminotransferase (*Aat-F* and *Aat-S*, EC 2.6.1.1), proline dipeptidase (*Pep-D*, EC 3.4.13.9 [sub-

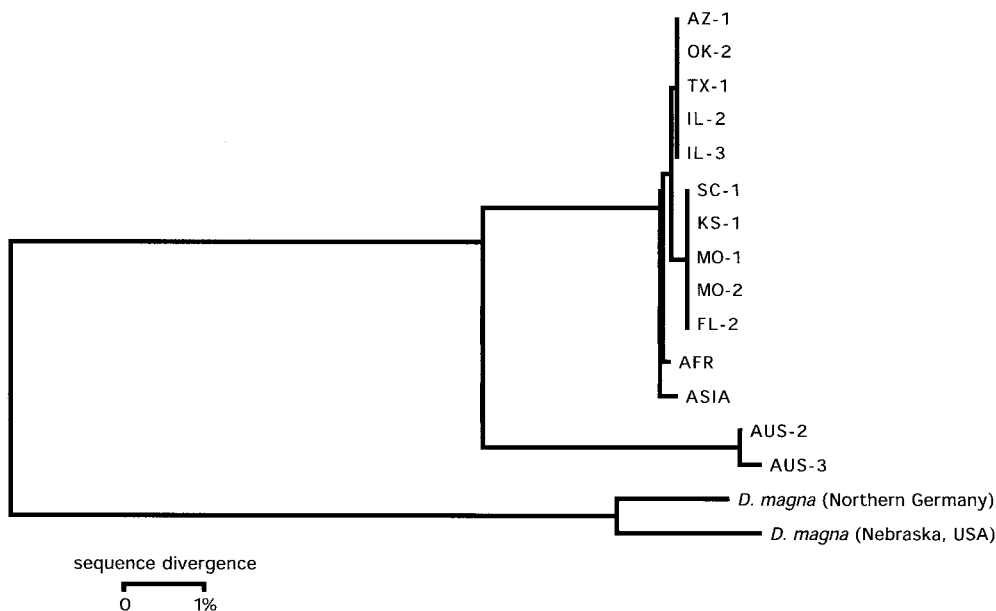


Fig. 2. Sequence divergence among populations of *D. lumholtzi* at the 12S rDNA (540 nt) and cytochrome *c* oxidase subunit-1 (646 nt) genes of the mitochondrial genome. Populations are listed in Table 1.



Table 3. Nucleotide variation in 12S rDNA and cytochrome c oxidase subunit 1 mitochondrial genes from sequenced haplotypes of *Daphnia lumholtzi*. Haplotype A belongs to North American isolates AZ-1, OK-2, TX-1, IL-2, and IL-3. Haplotype B belongs to North American isolates SC-1, KS-1, MO-1, MO-2, and FL-2. Only variable characters are shown. Numbers indicate nucleotide positions along the sequenced fragments of each gene sequence. Transitions leading to amino acid changes are indicated by (\*) and appear in positions 57, 84, and 131 of the COI gene.

	12S rDNA	COI
	11122234455	111111112222223333333333444555555566666666666
	2801411876601	1244556789233466791123468011123448811302455789900011233344
	2785303681633	9209272644119809501705450703651062928956509705814736514736
		* * *
Haplotype A	AACGAAGAGTAAC	CCGTCCCGTCCATGTACCTATCCCTGGCCCTAATCCGACTCACTTCCCTGGATGCAAT
Haplotype B	AACGAAAAGCAAC	GCGTCCCGTCCATGTACCTATCCCTGGCCCTAATCCGACTCACTTCCCTGGATGCAAT
AFR	AACGAAGAGTAAC	GCGTCCCGTTCGTGTACCTATCCCTGGCCCTAATCCGACTCACTTCCCTGGATGCAAT
ASIA	AACGAAGGTAAC	GCGTCTCGCCGTGTACCTATCCCTGGCCCTAATCCGACTCACTTCCCTGGATGCAAT
AUS-2	GGAATAGAATGGT	CTAATTTTTCTGCACGTTAGTTAGATTGTCGGCTTATGCTGTCGTTCCAGAATGGC
AUS-3	GGAATTGAATGGT	CTAATTTTTCTGCACGTTAGCTTAGATTGTCGGCTTATGCTGTCGTTCCAGAATGGC

(dTTP), 10 mM Tris-HCl (pH 8.3) buffer, 50 mM KCl, 2.2 mM MgCl<sub>2</sub>, and 1 unit of Taq polymerase (Perkin-Elmer). Amplification involved a 1-min initial denaturation at 94°C, followed by four cycles of 1 min at 94°C, 1.5 min at 45°C and 1 min at 72°C, and subsequently followed by 36 cycles of 1 min at 92°C, 1.5 min at 50°C and 1 min at 72°C. PCR products from both genes were electrophoresed within 2% agarose gels, and then purified using a QIAEX II kit (Qiagen). Fifty to 100 ng of the purified DNA fragments were sequenced using 3 pmol of one of the amplification primers and the ABI Prism TaqFS dye termination kit (Perkin-Elmer). The sequencing reactions were analyzed on an ABI 377 automated sequencer.

A 540-nucleotide (nt) sequence of the 12S rDNA and a 646-nt sequence of the COI gene from all populations were aligned by eye using SeqEd version 1.0.3 (ABI). Estimates of sequence divergence were calculated for the combined data and for individual genes using the Kimura two-parameter model of molecular evolution in MEGA, version 1.02 (Kumar et al. 1993). Phenetic analysis of the divergence matrix calculated from the combined data was performed by the neighbor-joining method, also in MEGA.

Although the accuracy of molecular clocks is uncertain when substitution rates are based on calibrations for distantly related taxa, these clocks are helpful in testing biogeographic hypotheses. In the current study, a calibration on the basis of the arthropod mtDNA clock of Brower (1994) and another on the basis of the rate of COI divergence in a crustacean (Knowlton et al. 1993) were used to verify if vicariance events could explain the wide geographic distribution of *D. lumholtzi*.

Of the 1,186 sequenced nt from both mitochondrial genes, 232 nt were variable between *D. lumholtzi* and *D. magna*, but only 71 nt were polymorphic among the isolates of *D. lumholtzi*. The analysis of sequence divergence for the combined data showed that all isolates from the United States clustered with the isolates from Uganda and Nepal. (Fig. 2) Only 3 nt varied between the two mitochondrial haplotypes found in North America, but an additional 5 nt varied with the inclusion of the African and Asian samples (Table 3). By contrast, the two Australian isolates showed 5.4% sequence divergence from the other cluster, with 9 and 51 nt differences distinguishing Australian haplotypes at the 12S

gene and COI genes respectively (Table 3). By comparison, the European and North American isolates of *D. magna* showed just 3.1% sequence divergence from each other.

**Discussion**—Both the allozyme and mtDNA data indicate a high similarity between the Asian and African populations of *D. lumholtzi* and their marked divergence from Australian populations. The application of molecular clocks to the mtDNA data suggests that the Australian members of *D. lumholtzi* diverged from the lineage found on the other continents between 2.3 and 4 mya, depending on the clock calibration that is used. Brower's (1994) general estimate of the nt substitution rate for arthropod mitochondrial genomes is 2.3% per million years. On the basis of this calibration, the Australian lineage of *D. lumholtzi* has been evolving independently for 2.35 million years. It is known that molecular evolutionary rates differ among genes (Li 1997), with most protein coding regions of mtDNA accumulating nt substitutions more rapidly than ribosomal DNA (Lynch and Jarrell 1993), a pattern apparent in the sixfold greater incidence of nt substitutions in the COI versus 12S rDNA sequences noted in this study. However, because the COI calibration currently available for crustaceans (2.2–2.6%/million years; Knowlton et al 1993) is similar to that for the mt genome at large, its application shifts the estimated divergence time of the two lineages of *D. lumholtzi* to 3.3–4 mya. Despite this uncertainty in dates, our results rule out the possibility that a broadly distributed ancestral population was split during the fragmentation of Gondwanaland some 65 mya. Instead, the distribution of *D. lumholtzi* reflects its subsequent dispersal among continents.

*D. lumholtzi* is fairly closely allied to the *D. carinata* complex (Hebert, unpublished data), a group that dominates the Australian fauna; this relationship suggests that *D. lumholtzi* may have originated on that continent, although further analysis of the Afro-Asian fauna is needed to test this suspicion. Presuming that *D. lumholtzi* originated in Australia, a subsequent dispersal event led to the colonization of either Africa or Asia some 2–4 mya. The later spread of the species from one of these areas to the second occurred much more recently, likely within the last 100,000 years, as indicated by the very limited divergence between Asian and African populations. All North American populations of *D. lumholtzi*

showed a high genetic similarity to these Afro-Asian populations. Although our genetic data could not discriminate between these alternate sources, Africa is the likely source as fish were imported from Lake Victoria to a Texan reservoir (Sorensen and Sterner 1992). Viewed from a larger perspective the colonization of North America is simply the most recent in a series that have led to the broad geographic distribution of *D. lumholtzi*.

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