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# Phylogeographical patterning in *Daphnia ambigua*: Regional divergence and intercontinental cohesion

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*Abstract*

*Daphnia ambigua*, one of the most broadly distributed members of its genus, occurs in Europe as well as in North and South America. This investigation examines diversity in two mitochondrial genes, COI and 12S rDNA, to ascertain the geographical distribution of variation in this species. The results establish that North American *D. ambigua* are separable into four phylogroups with an average of 4% mtDNA sequence divergence. One of these groups is dominant, occupying Mexico and the central United States, whereas the other groups are restricted to the Atlantic or Pacific coasts. Their distributional centroids coincide with refugial areas identified in prior studies, reinforcing evidence that the Appalachian and West Coast ranges have been important in the isolation of zooplankton lineages. Gene exchange, mediated by migratory birds whose flyways are structured by these mountains, is likely instrumental in maintaining north–south cohesion within each phylogroup. European populations of *D. ambigua* are closely allied to those from the eastern United States, a result concordant with their presumed recent introduction from North America. By contrast, South American populations last shared an ancestor with North American lineages approximately 2 million yr ago. The phylogeographic information now available suggests that topographic barriers generate predictable patterns of population divergence in zooplankton species and that allopatric speciation has played an important role in their diversification.

Genetic studies are providing new insights into both the taxonomic diversity of zooplankton and the extent of regional diversification in single species. Cladocerans have been the target of particularly intensive studies, which have revealed that many ''species'' are actually a complex of reproductively isolated lineages (e.g., Hebert and Finston 1996; Taylor et al. 1998). This result has been especially true in comparisons involving ''conspecific'' populations from different continents, which often show more than 20% sequence divergence at mitochondrial genes (Rowe 2000; Cox and Hebert 2001). However, studies have also revealed that variation occurs on single continents. Particularly strong regional divergence (5–10% sequence divergence at mitochondrial genes) has been noted in taxa such as *Sida* and *Holopedium*, whose resting eggs are poorly adapted for transfers among habitats (Rowe 2000; Cox and Hebert 2001). By contrast, members of the genus *Daphnia*, whose diapausing eggs are packaged in structures that aid dispersal, show less regional divergence (Weider et al. 1999). However, one factor complicates this conclusion. Most prior work on daphniids has focused on species from recently deglaciated areas, whereas the distributional centroids of the other cladocerans lie in areas that were ice-free throughout the Pleistocene. Moreover, a study of the *Daphnia laevis* complex, a group restricted to temperate regions, revealed both an overlooked species and evidence for genetically divergent phylogroups (Taylor et al. 1998). This result suggests that marked regional divergence is a general feature of cladoceran species from areas where environmental conditions have been stable enough to permit sustained occupancy of the landscape. However, further work is needed both to establish this fact and to probe the factors responsible for such regionalization.

The present study investigates the phylogeographic structure of *Daphnia ambigua* Scourfield, a species native to the temperate regions of the New World. Although first described from Kew Gardens (Scourfield 1947), it was later recognized as an introduced species in Europe (Johnson 1952), where it is now broadly distributed (Brooks 1957; Dumont 1974; Maier 1996). The species has an immense natural distribution, occurring from southernmost Canada through the United States and Mexico into South America as far as mid-Argentina (Brooks 1957; Paggi 1973; Hebert 1995). *D. ambigua* shows conspicuous phenotypic variation, both seasonally and among populations, on local and regional scales. Much of this diversity is a response to variation in temperatures, food levels, and abundance of the predatory dipteran *Chaoborus* (Hebert and Grewe 1985; Hanazato 1991). However, there has never been any genetic verification that populations assigned to *D. ambigua* are conspecific.

The present investigation had two goals. It aimed firstly to establish the extent and patterning of genetic diversity among North American populations of *D. ambigua* as a test of their taxonomic status. If this taxon represents an aggregate of reproductively isolated forms, this analysis would be expected to reveal deeply divergent lineages, some of which might occur sympatrically (Witt and Hebert 2000). Alter-

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natively, if *D. ambigua* is a single species, the analysis should reveal little or no divergence. The second goal of this study was to evaluate the genetic similarity between North American populations of *D. ambigua* and those from Europe and South America.

## Methods

*Specimen collections*—Populations of *D. ambigua* were collected from 34 sites across its range in North America, from four sites in southern South America, and from one site in Europe (Table 1). Samples were either flash-frozen in liquid nitrogen or preserved in 90% ethanol for subsequent molecular analysis.

*Mitochondrial DNA analysis*—Total DNA was isolated from two to five individuals from each population using modified proteinase K methods (Schwenk et al. 1998). Ethanol-preserved specimens were first soaked in water for at least 30 min. Each individual was then homogenized in 50 $\mu$l of H3 extraction buffer and 20 $\mu$g of proteinase K and incubated for 24 h in a 50°C oven. Following denaturation of the proteinase K with a 10-min incubation in a 96°C water bath, DNA extracts were stored at −20°C. Polymerase chain reaction (PCR) was used to amplify two mitochondrial gene fragments. A 710–base pair (bp) fragment of the cytochrome *c* oxidase subunit I (COI) gene was amplified using the primer pair LCOI490 and HCO2918 (Folmer et al. 1994), and a 550-bp fragment of the 12S rRNA gene was amplified using primers designed for *Daphnia* (Taylor et al. 1996).

Each 50-$\mu$l PCR reaction consisted of 2–5 $\mu$l of DNA template, 5 $\mu$l of 10× PCR buffer (10 mmol $L^{-1}$ Tris-HCl pH 8.3, 50 mmol $L^{-1}$ KCl), 0.2 $\mu$mol $L^{-1}$ of each primer, 2.2 mmol $L^{-1}$ $MgCl_2$, 0.2 mmol $L^{-1}$ of each dNTP, and 1 unit of *Taq* DNA polymerase. The thermal regime for amplification of COI was as follows: one cycle of 1 min at 94°C; 5 cycles of 1 min at 94°C, 1.5 min at 45°C, and 1.5 min at 72°C; 30 cycles of 1 min at 94°C, 1.5 min at 50°C, and 1.5 min at 72°C; and a final extension of 5 min at 72°C. The thermal regime for 12S consisted of one cycle of 1 min at 94°C; 10 cycles with declining annealing temperatures: 1 min at 94°C, 1.5 min at 60/58/56/54/52°C (2 cycles each), and 1.5 min at 72°C; 25 cycles of 1 min at 94°C, 1.5 min at 50°C, and 1.5 min at 72°C; and a final extension of 5 min at 72°C. PCR products were gel-purified using Qiaex II (Qiagen) and sequenced using the ABI prism BigDye terminator 3 sequencing kit (25 cycles, 55°C annealing for COI, 50°C for 12S). Products were sequenced in one direction using primers LCOI490 and 12S-A. Electrophoresis was performed on an ABI 377 automated sequencer (Applied Biosystems). Sequence electropherograms were aligned using the SeqApp 1.9 sequence editor (Gilbert 1992). The 12S sequences were aligned using the Clustal algorithm (Higgins and Sharp 1988) with default conditions and adjusted by eye. Each unique COI and 12S haplotype is available from GenBank (AF523684–AF523738), with one exception (COI from site MI), which contained too many unknown nucleotides.

Phenetic analysis of the sequence data was performed in MEGA 2 (Kumar et al. 2001) for each gene separately and then for a combined data set, which included individuals that were sequenced for both genes. Matrices of genetic distances based on Kimura's (1980) two-parameter model (K2P) were used to construct phenograms by the neighbor-joining (NJ) algorithm (Saitou and Nei 1987). In all cases, pairwise deletion of missing sites was employed, gaps were treated as missing data, and bootstrap values were based on 1,000 pseudoreplicates. Sequences from two other species of the subgenus *Daphnia, D. pulex* and *D. parvula*, were included as outgroups. *D. pulex* sequences were from Crease (1999) (GenBank AF117817). The *D. parvula* 12S sequence was from Colbourne and Hebert (1996), whereas COI was sequenced from a *D. parvula* population collected near Amarillo, Texas, obtained from the archived collections held by one of us (P.D.N.H.).

Cladistic analysis was performed according to the maximum parsimony (MP) criterion in PAUP* 4.0 (Swofford 1998). Two divergent representatives from each major cluster, as identified by the phenetic analysis, were included in the MP analysis using a combined COI and 12S data set, for a total of 10 *D. ambigua* individuals plus the two outgroup taxa. Because this data set was small, an exhaustive search was performed. PAUP default settings were employed, except that taxa were added randomly in 100 replicate trials, with 10 trees held at each step. Sites containing gaps were omitted from this analysis, and bootstrap values were based on 1,000 pseudoreplicates.

Because estimated rates of sequence divergence for COI range from 1.4 to 2.6% per million years (Knowlton et al. 1993; Knowlton and Weight 1998; Schubert et al. 1998), we chose an intermediate clock rate of 2.0% per million years to estimate divergence times of lineages from the COI data.

## Results

*mtDNA sequence variation*—The alignment of COI sequences was 640 bp in length and was unambiguous; no gaps were present. However, an initial phase of data analysis revealed an anomalous haplotype in two individuals from a riverine population in Argentina (Coronda River, province of Santa Fe). These sequences were divergent (23–26%) from all other *D. ambigua*, but only 12% divergent from *D. retrocurva.* This population likely represents an undescribed species within the *retrocurva* complex and was excluded from further consideration in the present study. Among the remaining populations, 29 COI haplotypes were detected among 62 individuals from 34 North American populations, and three COI haplotypes were detected from the four South American populations, but COI was not successfully amplified from the French population. These 32 COI haplotypes had 91 variable sites, of which 65 were parsimony-informative. The average nucleotide composition of the sequences was moderately A/T biased, but similar, among isolates ($\chi^2$ homogeneity test, $p > 0.99$): 33.7% T, 20.6% C, 23.8% A, and 21.9% G.

The final 12S alignment was 571 bp long and was also unambiguous; only five gaps of one to two nucleotides were required for alignment, even including the outgroup taxa. Twenty-two North American and two South American se-

Table 1. Collection sites for *Daphnia ambigua* from North and South America and Europe. N/A, not available.

| Site code | Sampling location | Latitude | Longitude | GenBank accession Nos. COI (haplotype) | GenBank accession Nos. 12S (haplotype) |
|---|---|---|---|---|---|
| North America | | | | | |
| AR | Holly Springs, Arkansas | 33°50′N | 92°40′W | AF523713(Central B) | AF523721(AR-a)<br>AF523722(AR-b) |
| CA1 | Campo, California | 32°39′N | 116°23′W | AF523685 (CA1-a)<br>AF523688 (CA1-b) | AF523714(CA1-a)<br>AF523724(CA1-b) |
| CA2 | Anza, California | 33°34′N | 116°36′W | AF523706(CA2-a)<br>AF523713(CA2-b=AR) | —<br>— |
| CA3 | Sacramento, California | 38°31′N | 112°22′W | AF523689 | AF523730 |
| FL1 | Lake Okeechobee, Florida | 26°56′N | 80°50′W | AF523695 | AF523718 |
| FL2 | Lake Hatchinella, Florida | 28°01′N | 81°25′W | AF523695 (FL2-a=FL1)<br>AF523698(FL2-b) | —<br>AF523726(FL2-b) |
| FL3 | Butler Lake, Florida | 30°03′N | 82°20′W | AF523694(FL3-a)<br>AF523704(FL3-b) | —<br>— |
| FL4 | Eustis Lake, Florida | 28°51′N | 81°43′W | AF523701(FL4-a)<br>AF523703(FL4-b) | —<br>— |
| FL5 | Montgomery Lake, Florida | 30°11′N | 82°37′W | AF523705(FL5-a)<br>AF523702(FL5-b) | —<br>— |
| FL6 | Dowling Lake, Florida | 30°03′N | 82°20′W | AF523694(FL6=FL3-a) | AF523736 |
| FL7 | Lake Trafford, Florida | 26°25′N | 86°30′W | AF523695(FL7-a=FL1)<br>AF523699(FL7-b) | —<br>AF523731(FL7-b) |
| ID1 | Rexburg, Idaho | 43°51′N | 111°46′W | AF523713(ID1=AR) | AF523723(Central C) |
| ID2 | Sand Dune Lake, Idaho | 42°26′N | 116°51′W | AF523713(ID2=AR) | AF523723(ID2=ID1) |
| LA1 | Bellwood 2, Louisiana | 31°33′N | 93°16′W | LA1 AF523709 | AF523723(LA1=ID1) |
| LA2 | Bellwood 8, Louisiana | 31°33′N | 93°16′W | AF523712(Central A) | AF523727 |
| MA1 | Cape Cod 1, Massachusetts | 41°40′N | 70°20′W | AF523697 | AF523720 |
| MA2 | Cape Cod 4, Massachusetts | 41°40′N | 70°21′W | AF523700 | AF523735 |
| MEX1 | Little Presa, Mexico | 20°45′N | 98°41′W | AF523687 | AF523716 |
| MEX2 | Presa Vincente Aguire, Mexico | 20°26′N | 99°29′W | AF523686 | AF523723(MEX2=ID1) |
| MI | Pleasant Lake, Michigan | 42°23′N | 84°17′W | — | AF523725 |
| MO1 | Springfield, Missouri | 37°13′N | 93°16′W | AF523713(MO1=AR) | AF523723(MO1=ID1) |
| MO2 | Thomas Hill, Missouri | 39°32′N | 92°38′W | AF523712(MO2=LA2) | AF523723(MO2=ID1) |
| NC | Santeetlah, North Carolina | 35°47′N | 81°25′W | — | AF523729 |
| NE | Ainesworth, Nebraska | 42°32′N | 99°53′W | AF523710 | — |
| NY | New York | 41°23′N | 74°18′W | AF523696 | AF523737 |
| OH | Flushing, Ohio | 40°09′N | 81°02′W | AF523713(OH=AR) | AF523719 |
| OK1 | Ada, Oklahoma | 34°47′N | 96°41′W | AF523712(OK1=LA2) | AF523723(OK1=ID1) |
| OK2 | Cherokee Oklahoma | 36°45′N | 98°21′W | AF523712(OK2=LA2) | AF523728 |
| OR | Carter Lake, Oregon | 43°50′N | 124°08′W | AF523684(OR-a)<br>AF523707(OR-b) | AF523715(OR-a)<br>AF523723(OR-b=ID1) |
| RI | Tucker Pond, Rhode Island | 41°36′N | 71°10′W | AF523693 | AF523717(RI-a)<br>AF523734(RI-b) |
| TX1 | Cisco, Texas | 32°23′N | 98°59′W | AF523708(TX1-a)<br>AF523711(TX1-b) | —<br>— |
| TX2 | Austin, Texas | 30°16′N | 97°45′W | AF523713(TX2=AR) | AF523723(TX2=ID1) |
| TX3 | Big Wells, Texas | 28°34′N | 99°33′W | AF523712(TX3-a=LA2)<br>AF523713(TX3-b=AR) | —<br>— |
| South America | | | | | |
| ARG1 | Lago Rosario, province of Chubut, Argentina | N/A | | AF523692 | AF523733 |
| ARG2 | Embalse (reservoir) Paso de las Carretas, province of San Luis, Argentina | 33°20′S | 65°52′W | AF523691 | AF523732 |
| ARG3 | Embalse La Florida, San Luis, Argentina | 33°11′S | 66°01′W | AF523691(=ARG2) | AF523732(=ARG2) |
| CHILE | Natri Lake, Chiloé Island, Chile | N/A | | AF523690 | — |
| Europe | | | | | |
| FRANCE | Etong de Bellebouche | 46°72′N | 1°11′E | — | AF523738 |

quences were detected. The sequence obtained from the sole European population was identical to one in a population from New York State. The 12S sequences contained 42 variable sites, of which 26 were parsimony-informative. The nucleotide compositions of the 12S sequences were more A-T biased than for COI but were also similar among haplotypes ($\chi^2$ homogeneity test, $p > 0.99$): 34.5% T, 13.5% C, 32.7% A, and 19.3% G.

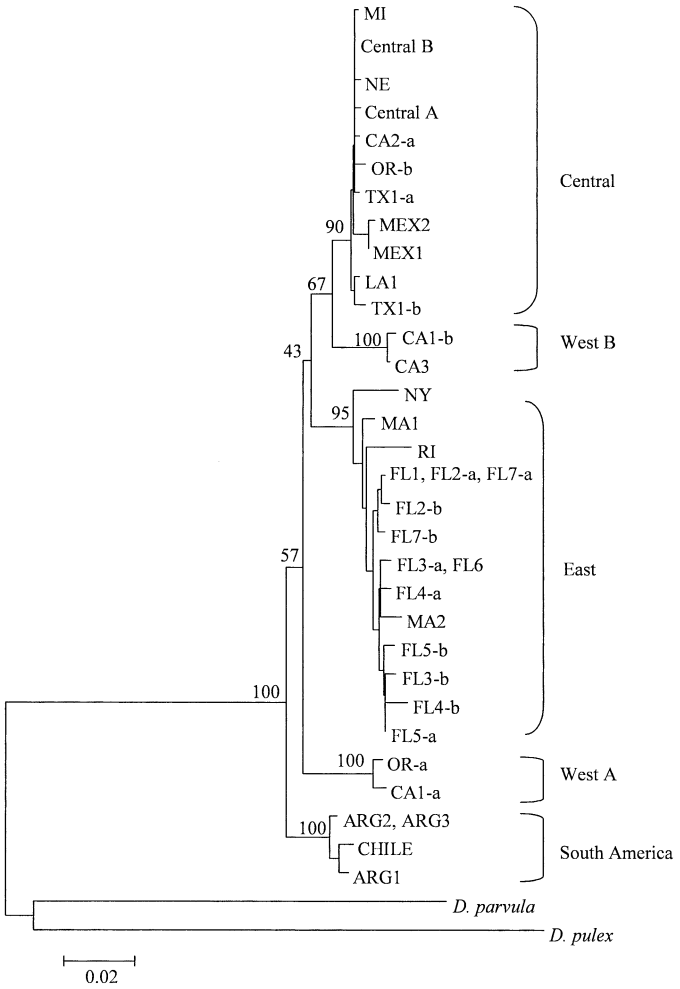


Fig. 1. Neighbor-joining tree constructed using all unique COI sequences from *Daphnia ambigua.* Bootstrap values for the deep nodes and each major cluster are presented. Site codes follow those in Table 1. The Central A haplotype was detected at five sites (LA2, MO2, OK1, OK2, and TX3). The Central B haplotype was found at eight sites (AR, CA2, ID1, ID2, MO1, OH, TX2, and TX3). Different haplotypes detected at the same site are designated by lowercase letters. The scale bar represents the K2P distance.

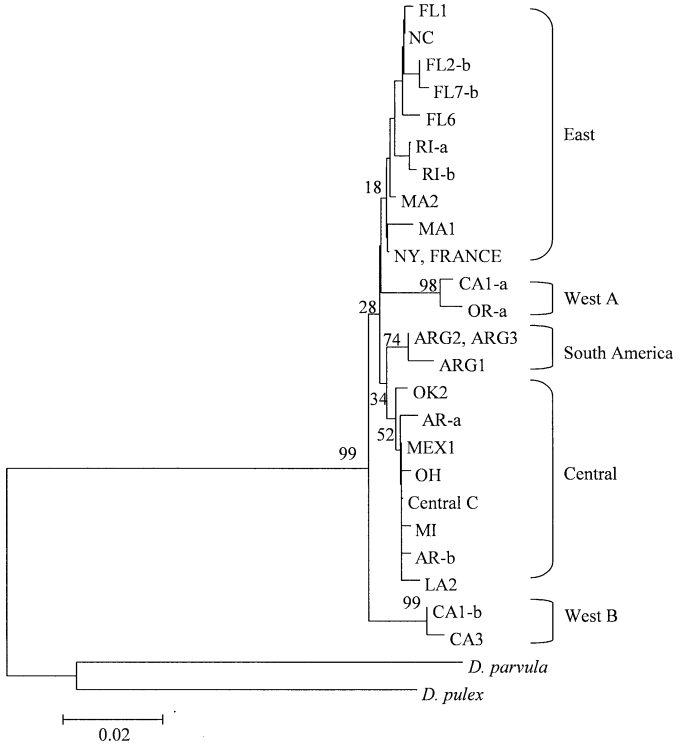


Fig. 2. Neighbor-joining tree based on all unique 12S sequences, with selected bootstrap values presented. Haplotype C was found at nine sites (ID1, ID2, LA1, MEX2, MO1, MO2, OK1, OR, and TX2). The scale bar represents the K2P genetic distance.

*Phylogeographic groups in North America*—A NJ phenogram indicated that the 32 COI haplotypes were separated into five clusters supported by bootstrap values >90 (Fig. 1). Because both genes were not amplified for all individuals, the 12S phenogram is based on a slightly different set of individuals. Even so, the 12S phenogram revealed the same five clusters, albeit with weaker bootstrap support (Fig. 2). Mean sequence divergence among the COI clusters ranged from 2.6 to 5.7% (Table 2), whereas divergences between 12S clusters ranged from 1.2 to 3.1%. Membership in the clusters was stable, indicating that North American populations of *D. ambigua* are separable into four groups, whereas the South American populations comprise a fifth cluster.

When the locations of individuals assigned to each of the four North American groups were mapped, it was apparent that the members of each cluster possessed largely allopatric distributions (Fig. 3). The Central cluster dominated the core of North America, occurring in the north from Ohio west to Idaho and then south to the Gulf coast and into Mexico. The remaining clusters were restricted to the coastal margins of the continent. Members of the Eastern cluster occurred from Florida northward on the eastern side of the Appalachians and also contained the single population from France. By contrast, members of the other two clusters occurred west of the Sierra Nevada and Coast Ranges. There was more mixture of phylogroups in this area. Two of the Californian populations (CA1 and CA3) contained members of the West B cluster, but individuals from CA2 belonged to the Central cluster. The sole population from Oregon was dominated (80%) by individuals belonging to the West A cluster, but one isolate from this population was a member of the Central cluster. As well, one individual from CA1 also belonged to the West A cluster.

Table 2. Average K2P sequence divergence in the COI gene among the five phylogroups of *Daphnia ambigua* identified by phenetic analysis.

| | Average K2P distance (%) | | | |
|---|---|---|---|---|
| Phylogroup | East | Central | West A | West B |
| Central | 3.8 | — | | |
| West A | 4.8 | 4.1 | — | |
| West B | 5.0 | 2.6 | 4.5 | — |
| South American | 5.7 | 3.2 | 4.9 | 4.1 |

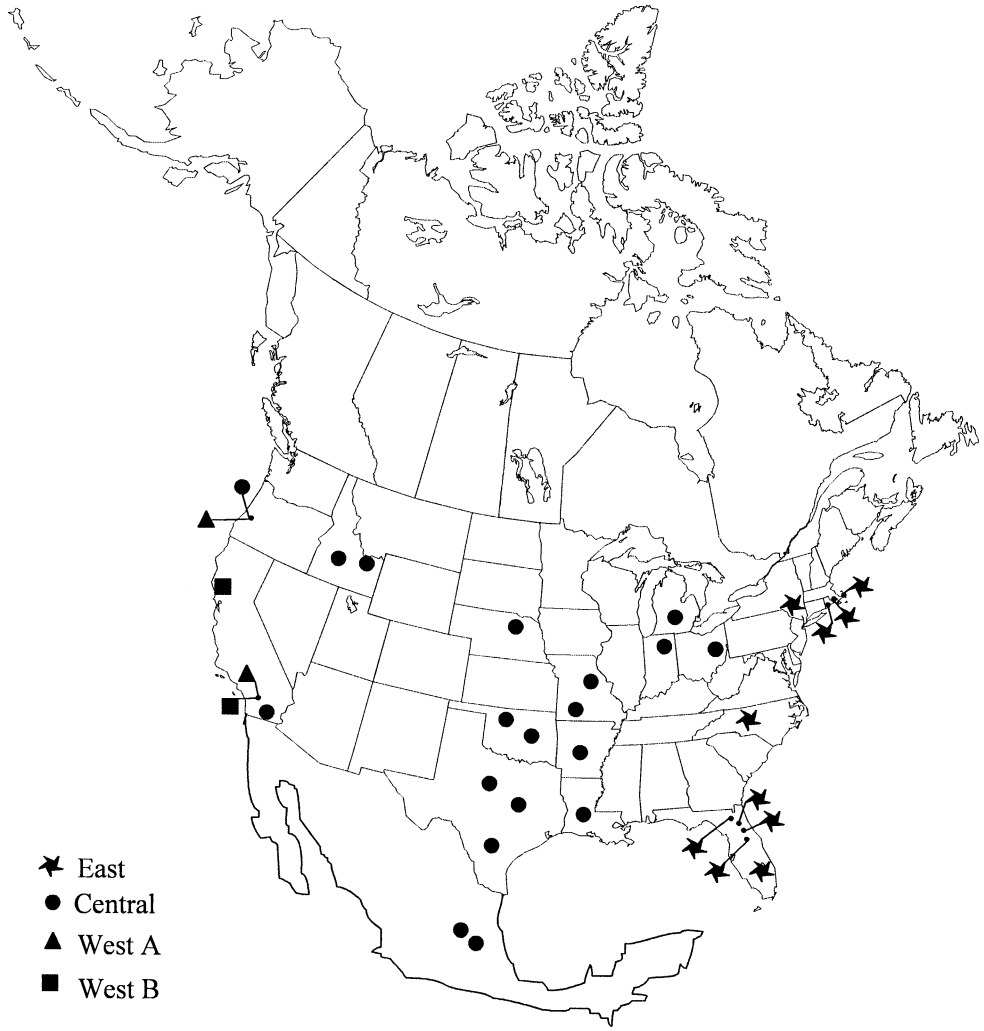


Fig. 3. Geographical distribution of the four mtDNA clusters of *Daphnia ambigua* within North America.

*Age and relationships of the phylogeographic groups*—Although the existence of five phylogroups was clear, the phylogenetic relationships among them were ambiguous. The COI tree suggested that South American populations were a monophyletic sister group to all North American populations, but the 12S data suggested that they were most closely related to the Central cluster. Combined analysis, based on both COI and 12S sequences, produced a third topology, placing the South America cluster as sister group to the Central and West B groups (Fig. 4). Bootstrap support for the 12S tree was weakest, whereas the COI tree had marginally higher bootstrap values for the deeper nodes than the combined tree, with a bootstrap value of 57 supporting the monophyly of the North American populations. Maximum parsimony analysis using 150 parsimony-informative characters of the COI + 12S dataset further supported the sister relationship between North and South American populations (Fig. 5). The exhaustive search resulted in a single best tree with moderate bootstrap support that placed North and South American populations as sister groups.

The average percent sequence divergence between pairs of the four clusters of North American COI haplotypes varied from 2.6 to 5.0% (Table 2). Assuming 2% sequence divergence per million years, the four North American phylogroups of *D. ambigua* last shared common ancestors from 1.3 to 2.5 million yr ago. The South American phylogroup showed just 3.2% sequence divergence from members of the Central cluster, suggesting their separation at about 1.6 million yr.

## Discussion

Past genetic studies have revealed that morphologically based taxonomies have seriously underestimated species diversity in many microcrustacean groups (Hebert and Wilson 1994; Hebert and Finston 1996; Witt and Hebert 2000). By contrast, the present study suggests that the taxonomy of *D. ambigua* is relatively simple, with one species occurring on three continents. However, the genetically divergent *D. ambigua*-like species detected in a riverine habitat in Argentina requires further attention. This taxon, which is a member of the *D. retrocurva* complex, apparently represents a case of morphological convergence.

There has been enough work done on freshwater life to

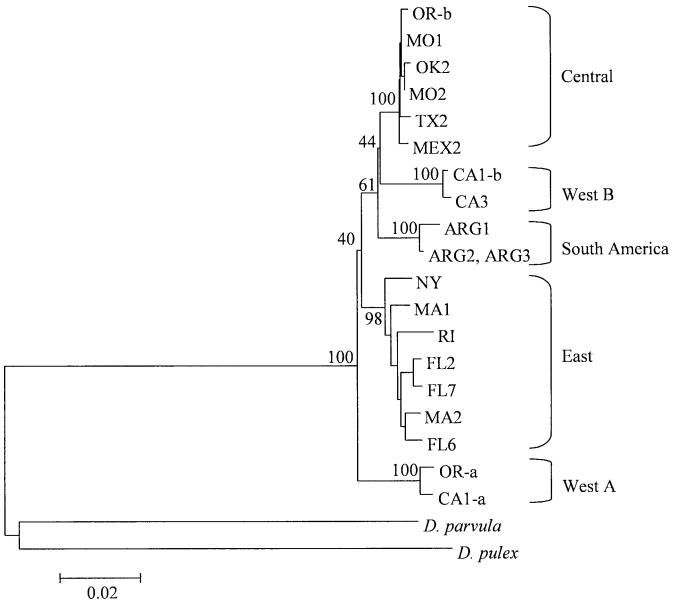


Fig. 4. Neighbor-joining tree of sequence variation in the CO1 and 12S genes. The scale bar represents the K2P distance.

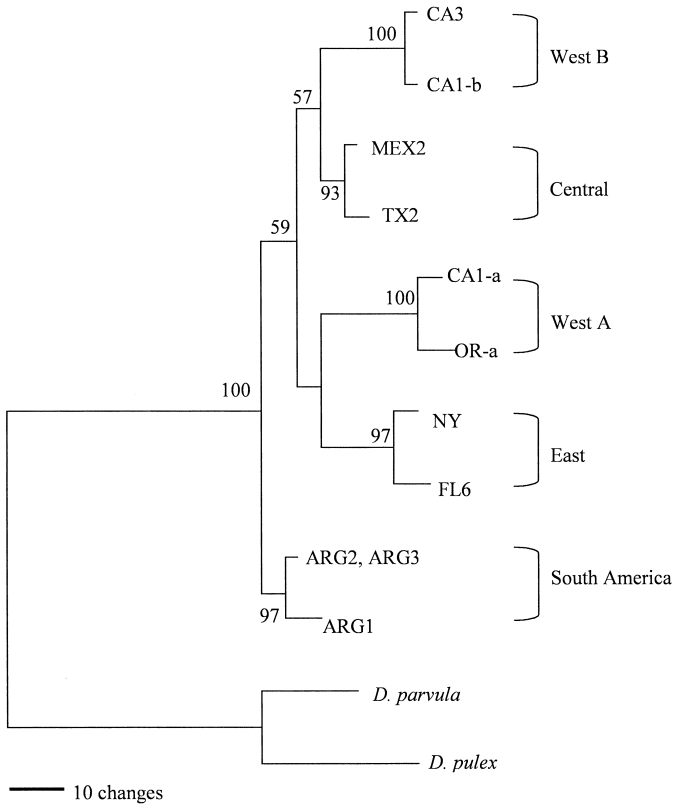


Fig. 5. The single best maximum parsimony tree obtained in an exhaustive search based on sequence variation for the CO1 and 12S genes. Bootstrap values were based on a heuristic search with taxa added randomly in 100 replicate trials, 10 trees held at each step, and 1,000 bootstrap pseudoreplicates. Tree statistics are as follows: $g_1 = -2.83$ (based on 250,000 randomly generated trees), $g_{1Crit} = -0.16$, tree length = 239, consistency index = 0.78, homoplasy index = 0.22, and retention index = 0.77.

establish that populations of most species are fractured into several phylogeographic groups (Avise 2000). The most detailed information derives from work on fishes where phylogroups often coincide with major watershed barriers (Bernatchez and Wilson 1998). Although there is less information on aquatic invertebrates, they also regularly show regional divergence. Surprisingly, the phylogroups in these organisms often also coincide with watershed boundaries, even in taxa that produce diapausing eggs enabling penetration of these barriers. The most comprehensive study to date showed, for example, that North American populations of the cladoceran *Sida crystallina* are separated into six phylogroups showing deep genetic divergence and remarkably sharp boundaries (Cox and Hebert 2001). A similar phylogeographic pattern was observed among members of the *D. laevis* complex (Taylor et al. 1998). Work on these taxa suggested the isolation of populations east of the Appalachians from those in the central portion of the continent and from those in the west. The present study reveals a congruent phylogeographic pattern. North American populations of *D. ambigua* are separated into eastern, central, and western lineages. However, there were two significant differences from earlier studies. First, the western regions of North America are occupied by two phylogeographic groups of *D. ambigua*: one in the south and the other in the north. Admittedly, there was evidence of limited exchange between these regions, but more work is needed to clarify its extent. As well, the levels of sequence divergence among phylogroups of *D. ambigua* are lower than those in *S. crystallina* or in the *D. laevis* complex. In fact, the present results suggest that its North American lineages have diversified within the last 1–2 million yr. Their recent origins prevent any firm decision concerning relationships among the phylogroups, but a lineage from the Pacific (West B) seems most closely allied to that from the Atlantic region, despite the large geographic distance separating these areas.

This study is one of the few investigations that have examined the levels of divergence among populations of a microcrustacean species on different continents. Prior work on *D. ambigua* suggested that European populations invaded recently from the New World, and the present results confirm this hypothesis, suggesting that they derive from the northeastern United States. By contrast, the clear genetic divergence between *D. ambigua* from South America and all North American phylogroups indicates that populations from these two continents last shared a common ancestor some 2 million yr ago. Although this is a substantial interval, it is a briefer history of isolation than might have been expected, given that these continents possess a number of ancient endemics, indicating that both regions have provided suitable habitats for daphniids for a long interval.

The present study has provided information that extends our understanding of the role of long-distance dispersal in structuring cladoceran populations. The rapid colonization of Europe by *D. ambigua* and the similar occupancy of large blocks of territory by other invading cladocerans (Havel et al. 2000) suggest that their populations ordinarily show little genetic diversity in the early phases after colonization of a new continent. However, the regular presence of phylogeo-

graphic structure in cladoceran populations across a continent suggests that regionalization and genetic diversification follow the initial occupancy. It is significant that different North American species show similar phylogeographic structure, but varying time frames for their divergence. This pattern suggests that persistent topographic features produce predictable patterns of population divergence and phylogeographic structure, regardless of the timing of the colonization event. The phylogroup boundaries detected in North American cladocerans coincide with major migratory flyways, suggesting that dispersal mediated by birds can curb the balkanization of cladoceran populations over high-traffic corridors (Crease et al. 1997). South American populations of *D. ambigua* were likely also founded as a consequence of bird-mediated dispersal, perhaps during a redirection of migration routes in the early Pleistocene. However, the genetic divergence among North American populations and between South and North America supports the conclusion that such gene flow is insufficient to prevent regional gene pool differentiation. In fact, if its regional populations persist for another few million years, *D. ambigua* will likely fragment into an assemblage of species. However, it is perhaps more likely that changing conditions will lead to the extinction of populations in all but one or two regions, curbing genetic divergence and speciation. Although the long-term fate of these populations cannot be predicted, the occurrence of regional differences in modern populations is a fact that should be considered in ecological studies. It is, for example, likely that modern populations of *D. ambigua* also show regional variation in life history traits and morphology that have arisen as a coincidental by-product of their long histories of isolation.

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