Molecular Ecology (2004) **13**, 97–107 doi: 10.1046/j.1365-294X.2003.02024.x

Mitochondrial DNA variation in North American populations of *Daphnia obtusa*: continentalism or cryptic endemism?

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Abstract

The morphological stasis of many freshwater crustaceans has resulted in the prior delineation of cosmopolitan species and has been explained by their capacity for long-distance dispersal. This study examines the phylogeography of *Daphnia obtusa*, a cladoceran thought to be widespread in North America. However, sequence variation of the mitochondrial cytochrome *c* oxidase subunit I gene indicates that this taxon is composed of two morphologically cryptic species, designated *D. obtusa* NA1 and NA2. NA2 is restricted to the east, whereas NA1 is broadly distributed across the United States, and is subdivided into four phylogroups that show weak genetic differentiation over broad geographical areas, which likely reflects recent long-distance dispersal. The current distributions of the four phylogroups in NA1 can be explained by recent range expansion from different refugia following the last Pleistocene glacial advance. Interestingly, the mitochondrial phylogroups identified in this study do not correspond to lineages detected in a previous allozyme analysis. However, the latter groups are associated with a habitat shift suggesting that natural selection may have played a role in their divergence. The results of this and previous studies illustrate the complicated biogeographical history of freshwater cladocerans.

Keywords: Cladocera, COI, Crustacea, dispersal, mtDNA, phylogeography, speciation

Received 22 May 2003; revision received 22 September 2003; accepted 22 September 2003

Introduction

The evolutionary history of the cladoceran crustaceans, specifically species in the genus *Daphnia*, has been studied extensively. This genus was initially believed to consist of a small number of taxa exhibiting morphological similarity across their broad distributions (Brooks 1957; Hrbácek 1987). Limited diversification of regional gene pools was expected as a result of their dispersal capabilities (Mayr 1963; Schwenk *et al*. 2000). However, it is now accepted that these 'cosmopolitan' species are often assemblages of genetically divergent taxa (Frey 1987; Hebert & Wilson 1994) whose distributions are endemic to a single continent (Adamowicz 2002). Moreover, studies have shown that many taxa with 'continental' distributions are species complexes composed of morphologically cryptic lineages whose individual distributions are narrow (Hebert & Wilson 1994; Taylor *et al*. 1998; Hebert *et al*. 2003).

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Initial studies of DNA sequence divergence in cladoceran populations, which were restricted to a microgeographical scale, found marked local diversity in gene frequencies among conspecific populations (Crease *et al*. 1990). It was thought that this pattern of regional genetic divergence would be amplified at larger distances (Ebert 1994; Lynch & Spitze 1994), but subsequent investigations into the genetic diversity of broadly distributed taxa have revealed very limited macrogeographical differentiation (Crease *et al*. 1990; Hebert *et al*. 1993; Taylor *et al*. 1996; Weider *et al*. 1999). However, these studies focused on North American species that primarily occur in areas glaciated during the Pleistocene. Thus, the limited genetic variation in these taxa may reflect the recent recolonization of these areas (Hebert *et al*. 1993). In contrast, studies on Australian daphniids, living in areas not impacted by glaciations, revealed extensive regional gene pool differentiation (Hebert & Wilson 1994). Similarly, recent continental-scale surveys of daphniids from the warm temperate regions of North America, such as *Daphnia laevis* (Taylor *et al*. 1998), have discovered enough divergence to suggest that these

supposed 'cosmopolitan' species actually consist of complexes composed of several largely allopatric phylogroups. Indeed, phylogroups from the *D. laevis* study correspond to recognized species, namely *D. laevis*, *D. dubia* and *D. magniceps*. Certainly, these studies are demonstrating that environments with relatively stable landscapes can invoke marked regional differentiation in daphniids.

Historically, speciation in *Daphnia* has been associated with both hybridization and ecological differentiation. Specifically, hybridization, although frequently resulting in low-fitness hybrids (Crease *et al*. 1989; Taylor & Hebert 1992; Schwenk 1993; Colbourne & Hebert 1996; Schwenk *et al*. 2000), has been linked to the origin of polyploid polar taxa (Dufresne & Hebert 1997) and to a case of rapid speciation following introgression (Taylor & Hebert 1993; Schwenk & Spaak 1995). Speciation via disruptive selection due to habitat shifts, especially lake-to-pond transitions (Taylor *et al*. 1996; Pfrender *et al*. 2000; Schwenk *et al*. 2000), has also been proposed. However, recent studies support a dominant role for allopatric divergence in the speciation of daphniids occupying stable landscapes (Hebert & Wilson 1994; Taylor *et al*. 1998; Schwenk *et al*. 2000; Adamowicz 2002; Hebert *et al*. 2003). Thus, it is apparent that further examination of the evolutionary histories of daphniids is necessary to establish the preponderance of any one speciation mechanism.

Because most prior studies have examined *Daphnia* population divergence on a small scale, it has been difficult to confirm larger patterns of intraspecific genetic diversity. However, Hebert & Finston (1996) examined allozyme diversity in populations of the cyclically parthenogenetic, pond-dwelling *D. obtusa* Kurz across its entire North American range. As most of its current range remained unglaciated during the Pleistocene, this study provided an opportunity to examine a species that may have occupied this area for a long time (Schwartz *et al*. 1985; Hebert 1995). This allozyme study showed that *D. obtusa* is divided into three largely allopatric groups with eastern, central and southwestern distributions (Fig. 1). Populations belonging to a group exhibit low variability over large distances, whereas large genetic divergences were detected over relatively small distances at contact zones between groups. These results indicate that a simple monotonic relationship between genetic divergence and geographical distance does not exist in North American *D. obtusa*, at least at the allozyme level (Hebert & Finston 1996).

In this study, mitochondrial DNA (mtDNA) diversity in *D. obtusa* was surveyed in North America to determine if three allopatric groups are also evident at the mtDNA level. Mitochondrial DNA variation should show even greater subdivision due to the haploid condition and uniparental transmission of mtDNA (Avise 1994). Viewed from a broader perspective, this study sought to provide further insight into the patterning of genetic diversity as well as the evolutionary history of this particular group of daphniids.

Materials and methods

Sampling

Populations of *Daphnia obtusa* examined in this study are listed in Table 1 and derive from most of its known range in North America. In total, 33 sites comprising 44 populations were included with representatives of all three allozyme lineages (Fig. 1). Total genomic DNA was extracted using the Isoquick kit (Orca Research) from single animals that were flash frozen in liquid nitrogen in the field, or multiple individuals that were propagated from a single female in the laboratory.

DNA amplification and sequencing

A polymerase chain reaction (PCR) was used to amplify a 709-bp fragment of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene using universal primers, LCOI490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2918 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer *et al*. 1994). Amplification of 675- and 731-bp fragments for troublesome samples was achieved using an alternate set of primers, COIFDP (5′-TTCTCAACTAATCAYAARGAYATTGG-3′) and COIFDP2 (5′-CAATTTAATGCCTACTCCTCGGCC-3′), respectively, with COIRDP (5′-TGGTATAAGATCGGA-TCTCCCCCTCCTGC-3′). The PCR was of 50 μL total volume and contained 2.0 mM $MgCl_2$, 5 pmol of each primer, 40 μM dNTPs, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 10–50 ng of genomic DNA and 1 unit of *Taq* DNA polymerase (Roche). The amplification reaction was performed in an MJ PTC-100 thermal cycler and the thermocycling profile consisted of one cycle of 1 min at 94 °C, 40 cycles of 30 s at 94 °C, 30 s at 50 °C and 1 min at 72 °C, with a final incubation of 5 min at 72 °C.

The PCR products were electrophoresed in 0.8% TAE agarose gels, stained with ethidium bromide and visualized under UV light. DNA fragments were excised from the gel and purified using either the Microcon PCR Centrifugal Filter kit (Millipore) or a freeze / thaw method. The freeze / thaw protocol was as follows: after excision, the agarose band was frozen in the top of a filter-plugged pipette tip (50 μL), thawed and then spun at maximum speed for 10 min in a 1.5-mL microfuge tube. The resulting eluant was precipitated in ethanol. It was not possible to amplify sufficient DNA to provide quality sequence data from a few samples due to the small amount of DNA template available. These samples were cloned using the TOPO TA Cloning kit for Sequencing (Invitrogen), and either multiple clones were sequenced or high fidelity JumpStart™ *Taq* DNA polymerase (Sigma-Genosys) was

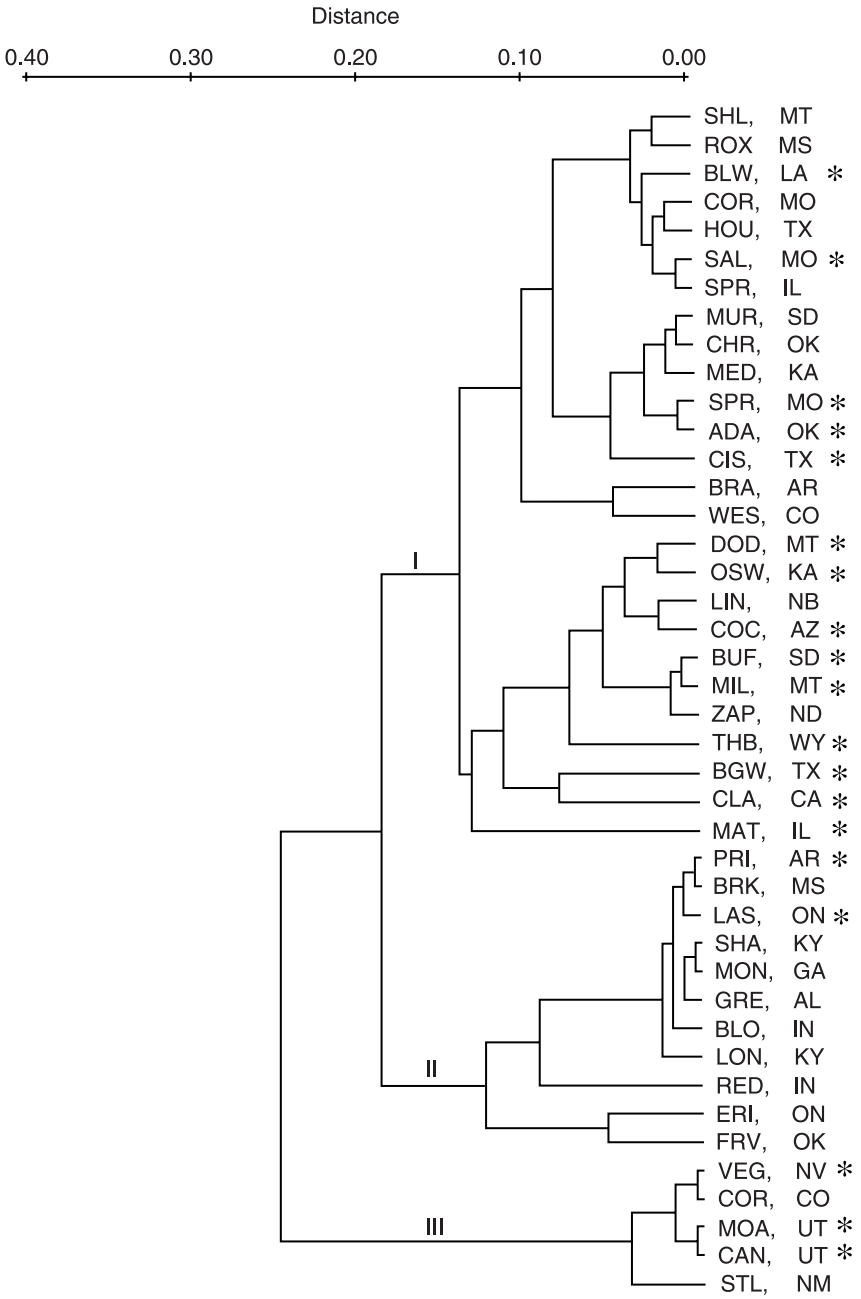

Fig. 1 UPGMA tree of genetic distances among North American populations of *Daphnia obtusa* based upon allele frequencies at polymorphic allozyme loci (reproduced from Hebert & Finston 1996). The three allozyme lineages are indicated with Roman numerals. Asterisks mark populations used in this study.

used to generate the cloning template to avoid errors associated with the misincorporation of nucleotides by *Taq* DNA polymerase. All purified samples were sequenced using 20–50 ng of template with 5 pmol of the primers LCOI490, COIFDP and COIFDP2 as appropriate, using the ABI Prism TaqFS dye terminator kit (Perkin–Elmer). The sequences were resolved on an ABI 377 automated sequencer. Whenever possible, two individuals from each population were analysed. Whenever a new variant was detected within a sequence that was not well resolved on the electropherogram, the sequence was rerun to confirm it, or was sequenced in the opposite direction.

Alignment and phylogenetic analysis

A 645-bp region of the COI gene was aligned by eye using BIOEDIT (http://jwbrown.mbio.ncsu.edu/Bioedit/bioedit.html). Identical DNA sequences were grouped together and a representative was chosen for phylogenetic analyses. Bayesian inference of phylogeny was achieved through the use of Mr Bayes (http://morphbank.ebc.uu.sc/mrbayes) (Huelsenbeck 2000). The model of DNA substitution was first chosen by characterizing the sequences using MODELTEST v3 (http://inbio.byu.edu/Faculty/kac/crandall_lab/modeltest.htm) (Posada & Crandall 1998). The results

Table 1 List of 33 collection sites for *Daphnia obtusa* together with the number of populations examined at each location. The allozyme group identified by Hebert & Finston (1996) is also given. 'Clones' represent situations where DNA was extracted from a pool of 10 animals. The COI gene was subsequently PCR amplified from this DNA and cloned into a plasmid. The number of plasmid clones that was sequenced is indicated

Site code	Number of populations surveyed	Number of individuals per population	Collection site	State or Province	Allozyme lineage
AD	1	1	Ada	Oklahoma	I
AN	1	3 clones	Golf Course Pond, St. Andrews	New Brunswick	—
BG	2	2	Big Wells	Texas	I
		2			
BL	2	1	Bellwood	Louisiana	I
		1			
BS	1	1	Busey, Champaign County	Illinois	—
BU	3	1	Buffalo	South Dakota	I
		2			
		2			
BW	1	1	Buffalo Wallow Pond, Urbana	Illinois	—
CA	1	2	Canyonlands	Utah	III
CI	2	2	Cisco	Texas	I
		2			
CL	1	3 clones	Clayton	California	I
CO	1	2	Coconino	Arizona	I
CS	1	2	Congaree Swamp	South Carolina	—
CT	1	3	Coal Tipple Pond, Portsmouth	Ohio	—
DO	1	2	Dodson	Montana	I
DV	1	2 clones	Davisville	Missouri	—
FH	1	2 clones	Fountain Hill	Arkansas	—
HE	1	1	Hey-A-Pond, St Joseph	Illinois	—
JP	1	5 clones	Jepson Prairie	California	—
LA	1	1 clone	Ojibway Park, LaSalle	Ontario	II
		1			
LT	1	1	Lake Texoma	Oklahoma	—
MA	1	1 clone	Matoon	Illinois	I
MI	3	1	Miles City	Montana	I
		1			
		2			
MO	1	2	Monticello-Moab	Utah	III
NA	1	2	Natchez	Tennessee	—
NP	1	4	Nobody's Pond, Morton	Illinois	—
OS	1	3 clones	Oswego, Southwest of Pittsburgh	Kansas	I
PR	2	3	Princeton	Arkansas	II
		2			
SA	1	2 clones	Salisbury	Missouri	I
SL	1	2 clones	Salem	Missouri	—
SP	3	2	Springfield	Missouri	I
		2			
		2			
SR	1	2	Shiprock	Colorado	—
TH	1	1	Thunder Basin	Wyoming	I
VE	2	2	Red Rock, Las Vegas	Nevada	III

of this analysis were then incorporated into the Bayesian phylogenetic analysis. The number of generations run was 600 000 and all trees constructed before confluence (20 000 generations) were discarded as 'burn in' (Huelsenbeck 2000). Cladistic analysis was performed using the maximum parsimony (MP) criterion in PAUP v10 (Swofford 2002) under the default parameters with a heuristic search algorithm and tree-bisection and reconnection (TBR). The results of the MODELTEST analysis were also incorporated into this analysis. Sequences were added randomly in 50 replicate trials, with one tree held at each step. Bootstrap values for MP trees were based upon 200

pseudo-replicates. The Kimura 2-parameter model (K2P) (Kimura 1980) was used to estimate sequence divergence in MEGA v2.1 (Kumar *et al*. 1993). Phenetic analysis of the resulting distance matrix was performed using the neighbour-joining (NJ) algorithm in MEGA (Saitou & Nei 1987) with pairwise deletion of missing sites. The bootstrap percentages from 1000 pseudo-replicates were calculated in MEGA. Sequences of the COI gene for *D. neo-obtusa*, *D. cheraphila*, *D. prolata*, *D. pileata* and *D. catawba* were obtained from Adamowicz (2002), while a sequence of *D. pulex* was obtained from GenBank (Accession no. AF117817). The arthropod mtDNA sequence divergence rate of 2.3%/Myr (Brower 1994) was used to estimate the time of divergence among closely related taxa (< 8% sequence divergence).

Matrix correlation analysis

The Mantel matrix correlation test available in ARLEQUIN (Schneider *et al*. 2000) was used to determine if there is a correlation between geographical distance among populations and sequence divergence among their COI haplotypes. Each unique COI haplotype in each population was included as an element in the matrix. The geographical distance between different COI haplotypes from the same population was considered to be 0. The number of permutations was set to 1000.

Results

COI gene diversity

Thirty-nine unique sequences were found among the 86 isolates examined. More than one sequence was found in 18 of the 31 populations in which more than one individual was analysed. Among these sequences, 222 bp of 645 bp positions were polymorphic, 168 of which were phylogenetically informative. There were no indels among the sequences. Twenty-eight variable amino acid positions were detected, with only two amino acids occurring at most variable sites, but three amino acids were detected at two positions. Six of the 28 variable amino acid positions were phylogenetically informative. The pairwise divergences between unique DNA sequences originally identified as *Daphnia obtusa* ranged from 0.2 to 25.4%. All sequences are available in GenBank under Accession nos AY380411–AY380454.

Phylogenetic analysis of sequence divergence in the COI gene

Most of the COI gene sequences from individuals originally identified as *D. obtusa* are very similar. However, a few highly divergent sequences were detected and these were compared with those obtained in a comprehensive survey of COI diversity in North American daphniids (Adamowicz 2002). One sequence from Louisiana (BL) was very similar to that of a typical *D. pulex*, whereas three sequences from CL and four from JP (both in California) were closest to the COI sequence of *D. neo-obtusa*. The BL and CL populations were originally identified as *D. obtusa* by Hebert & Finston (1996) based on allozyme analysis, but it is possible that these were mixed populations.

The Bayesian tree shows that North American populations of *D. obtusa* are divided into two major clades (Fig. 2), denoted as NA1 and NA2 (NA = North America). NA1 is composed of four distinct mitochondrial lineages (1A, 1B, 1C and 1D, Fig. 3) with differing geographical distributions, whereas NA2 is restricted to the southeastern United States (Fig. 4). Lineages 1B and 1C overlap substantially throughout the Mississippi watershed (Fig. 4), although lineage 1A has a western distribution and ranges into the Rocky Mountains. The east–west transition between 1A and 1B/1C occurs within the Great Plains Region and roughly coincides with a shift in elevation from high in the west to low in the east. Lineage 1D is represented by only two isolates from Illinois so the extent of its distribution is unclear. A contact zone occurs for all lineages just west of the Mississippi river. For example, sites in both Missouri (SP) and Arkansas (PR) contained three of the NA1 lineages (Figs 3 and 4), whereas both 1B and 1C haplotypes, and an NA2 haplotype were found in the NP population from Illinois.

Trees produced using different phylogenetic analyses are generally consistent. Support is strong for the monophyly of the two *D. obtusa* clades (NA1 and NA2; Figs 2 and 3) and for the monophyly of the four lineages (1A–1D) in NA1 (Fig. 3). However, support for the relationships among these lineages and for the relationships among haplotypes within each lineage is very low. There is support for the sister group relationship between NA1 and NA2, and strong support for the clustering of the CL and JP isolates with *D. neo-obtusa*, and the BL isolate with *D. pulex*. All known species in the *D. obtusa* group in North America (Colbourne & Hebert 1996) are included on this tree (Fig. 2), and it is clear that *D. obtusa* NA2 does not cluster closely with any of them. The only significant difference among trees constructed using different methods concerns the relationship between *D. neo-obtusa* and *D. pulex*. They are sister taxa in the Bayesian and MP trees, but *D. neo-obtusa* clusters with the other members of the *D. obtusa* complex in the NJ tree, as expected from previous studies (Colbourne & Hebert 1996; Adamowicz 2002). In addition, *D. pileata* is the sister group to *D. cheraphila* + *D. prolata* in the NJ tree. Support for all these topologies, as well as those from previous studies, is relatively low (Fig. 2), so further analysis of the phylogenetic relationships among these taxa is required.

The average sequence divergence among haplotypes within NA1 lineages ranges from 0.2% (1D) to 0.9% (1C),

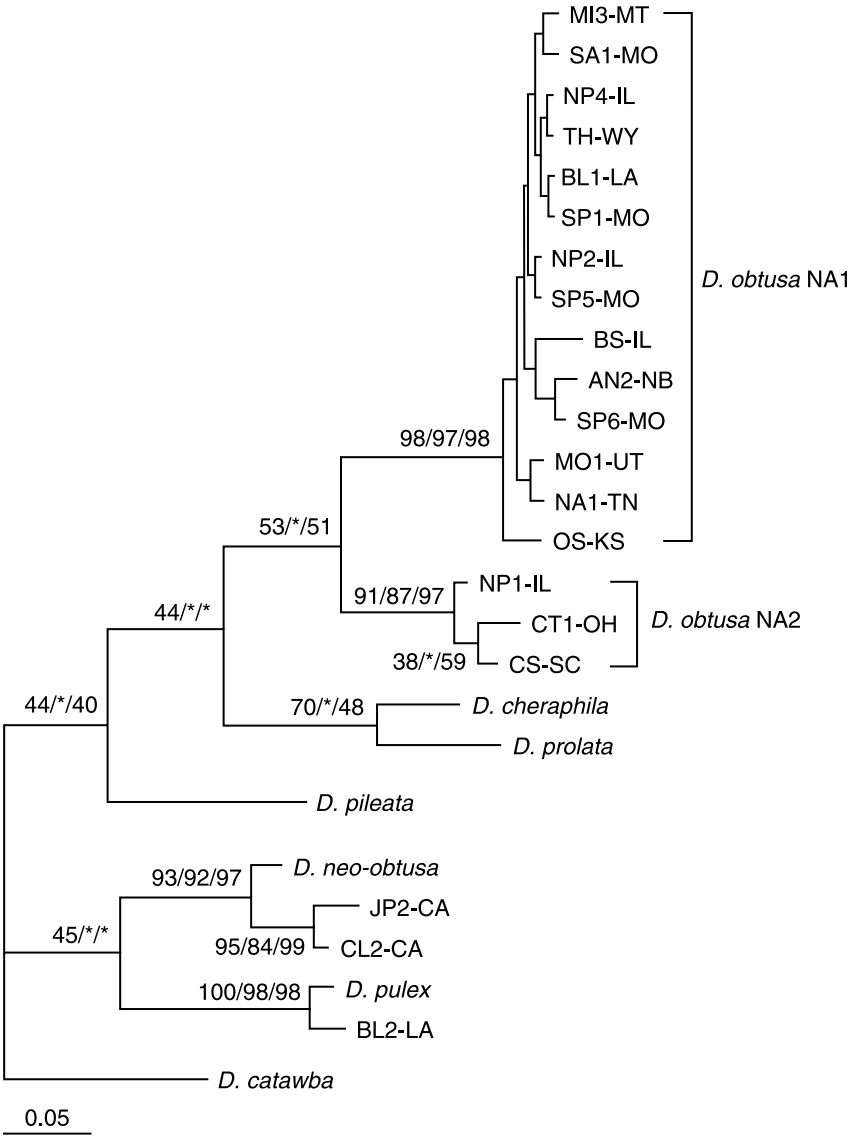

Fig. 2 Bayesian phylogram of mitochondrial COI gene variation in North American *Daphnia obtusa*. Only the first and second codon positions of each sequence were included in the analysis. Numbers beside major nodes represent clade credibility values from Bayesian analysis, and bootstrap support from maximum parsimony and neighbour-joining analyses (BA/MP/NJ). Asterisks indicate that there was no support in the MP or NJ analysis for the topology shown. The scale bar indicates sequence divergence. The isolates are identified as follows: the first two letters specify the collection site (Table 1), the number (if present) denotes the haplotype within the collection area, and the last two letters specify the state or province of origin. The trees were rooted with *D. catawba*. A NJ tree including all haplotypes was constructed first. Owing to the large number of haplotypes in *D. obtusa* NA1, a few representatives of each subgroup were chosen for subsequent Bayesian and MP analyses. There is significant rate variation across sites based on the TrN model of DNA substitution (number of substitution types = 6) and the gamma model ($P < 0.000001$). The gamma distribution shape parameter is 0.0681 and the substitution rate matrix is R[C-G] = 1.0000, R[A-G] = 2.5245, R[A-T] = 1.0000, R[C-] = 1.0000, R[C-T] = 28.2845, R[G-T] = 1.0000.

whereas that among *D. neo-obtusa* haplotypes is 2.4%. Interestingly, one haplotype (SP5-MO) from a Missouri (SP) population has the plesiomorphic state at all sites that define the four lineages of NA1, and the position of this isolate within this clade (Fig. 3) varies among the different tree building methods, all with low support.

Mean sequence divergence between haplotypes from different lineages within NA1 ranges from 1.2 to 2.0% (average 1.5%) (Table 2). Using Brower's (1994) estimate of arthropod mtDNA sequence divergence of 2.3%/Myr, the divergence of the four NA1 lineages occurred 520 000–870 000 years ago, during the Pleistocene. In contrast, sequence divergence between *D. obtusa* NA1 and NA2 is much higher (15.3%) suggesting that they are separate species with long histories of isolation. As Brower's clock becomes nonlinear over 8% sequence divergence, it cannot be used to estimate their divergence time. However, Taylor *et al*. (1998) estimated that *D. magniceps* and *D. laevis* diverged from one another on the order of 12 Ma based on data from the mitochondrial 12*S* ribosomal RNA (rRNA) gene. Adamowicz (2002) subsequently sequenced the COI gene from these two taxa and found 13.6% sequence divergence between them. If the COI gene is evolving at approximately the same rate in this lineage as it is in the *D. obtusa* lineage, then the two *D. obtusa* clades are likely to have diverged at least 12 Ma.

Correlation between genetic distance and geographical distance

The matrix of geographical and genetic distance between COI gene sequences was significant ($r = 0.158$, $P = 0.014$) suggesting that the closer two populations are geographically,

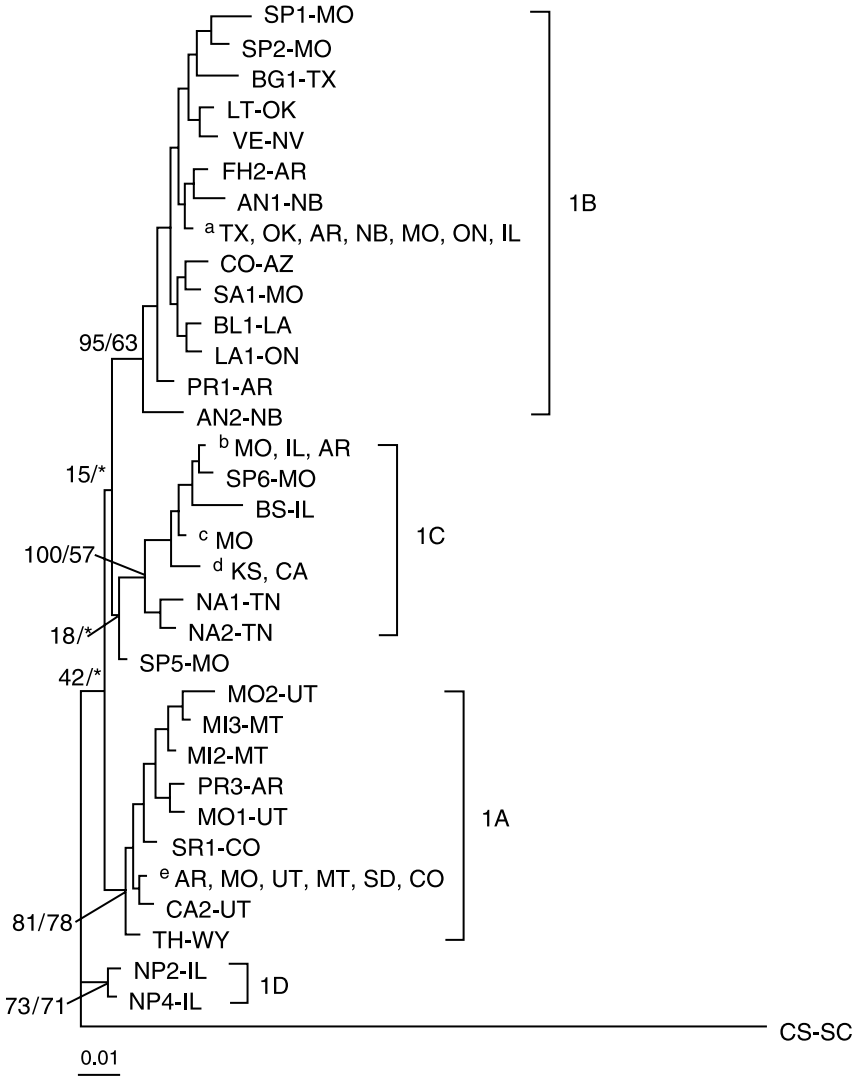

Fig. 3 Bayesian phylogram showing the topology of the four mitochondrial COI gene lineages within *Daphnia obtusa* NA1. All three codon positions of each sequence were included in the analysis. The trees were rooted with a haplotype from *D. obtusa* NA2. Only the state is provided for haplotypes found in multiple populations. Complete designations are as follows: a = AD-OK, AN3-NB, BG2-TX, CI-TX, FH1-AR, HE-IL, LA2-ON, MA-IL, SA2-MO; b = BW-IL, NP3-IL, PR4-AR, SP3-MO; c = DV-MO, SL-MO; d = JP1-CA, OS-KS; e = BU-SD, CA1-UT, DO-MT, MI1-MT, PR2-AR, SP4-MO, SR2-CO. Numbers beside major nodes represent clade credibility values from Bayesian analysis and bootstrap support from neighbour-joining analysis (BA/NJ). Asterisks indicate that there was no support in the NJ analysis for the topology shown. The scale bar indicates sequence divergence. There is significant rate variation across sites based on the HKY85 model of DNA substitution (number of substitution types = 2) and the gamma model ($P < 0.00001$). The gamma distribution shape parameter is 0.463.

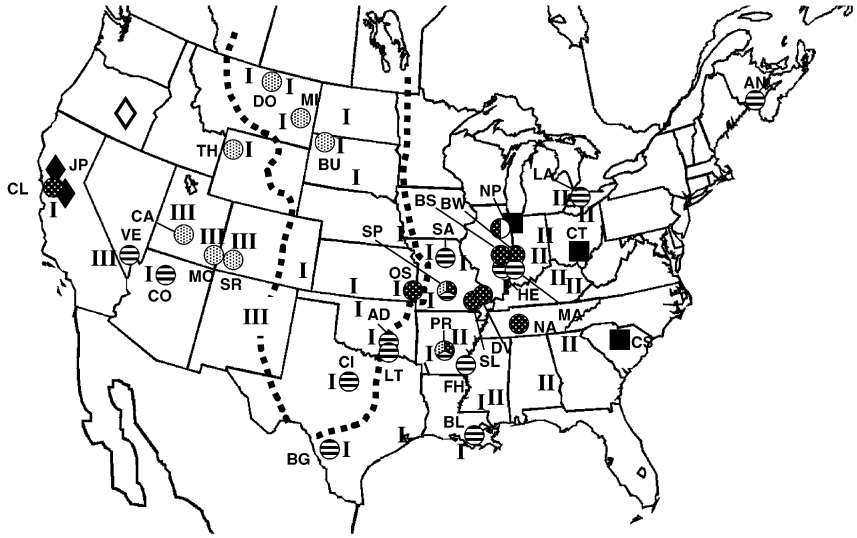

Fig. 4 Geographical distribution of mitochondrial COI haplotypes in three species of the *Daphnia obtusa* complex. Populations of *D. obtusa* NA1 are denoted by circles, populations of *D. obtusa* NA2 are denoted by squares and populations of *D. neo-obtusa* are denoted by diamonds. The two-letter population codes correspond to those given in Table 1. The open diamond denotes the only previously documented population of *D. neo-obtusa* (Colbourne & Hebert 1996). Roman numerals refer to the allozyme groups of Hebert & Finston (1996). The shading of the *D obtusa* NA1 populations denotes the mitochondrial COI phylogroup: 1A = ◍; 1B = ⊜; 1C = ●; 1D = ○. The heavy dotted line encircles the Great Plains Region.

Table 2 Mean sequence divergence of the mitochondrial COI gene between phylogroups of *Daphnia obtusa* NA1, the other species in the North American *D. obtusa* complex, *D. pulex* and *D. catawba*. The mean sequence divergence between major groups is shown above the diagonal. Numbers below the diagonal are standard errors from 1000 bootstrap replicates, estimated in MEGA

	D. obtusa 1A	*D. obtusa* 1B	*D. obtusa* IC	*D. obtusa* ID	*D. obtusa* NA2	*D. neoobtusa*	*D. pulex*	*D. cheraphila*	*D. prolata*	*D. pileata*	*D. catawba*
D. obtusa 1A		0.015	0.016	0.012	0.154	0.225	0.237	0.195	0.214	0.214	0.239
D. obtusa 1B	0.004		0.020	0.014	0.154	0.230	0.240	0.198	0.220	0.218	0.249
D. obtusa 1C	0.004	0.004		0.014	0.155	0.225	0.244	0.200	0.219	0.217	0.249
D. obtusa 1D	0.004	0.004	0.004		0.148	0.227	0.240	0.192	0.216	0.212	0.247
D. obtusa NA2	0.017	0.017	0.017	0.017		0.245	0.234	0.209	0.224	0.211	0.245
D. neo-obtusa	0.020	0.020	0.019	0.020	0.022		0.228	0.223	0.245	0.219	0.237
D. pulex	0.020	0.020	0.021	0.021	0.021	0.019		0.230	0.260	0.207	0.253
D. cheraphila	0.018	0.019	0.018	0.018	0.019	0.020	0.020		0.139	0.199	0.254
D. prolata	0.019	0.019	0.019	0.019	0.020	0.021	0.021	0.015		0.197	0.264
D. pileata	0.020	0.020	0.020	0.020	0.019	0.022	0.019	0.018	0.019		0.238
D. catawba	0.021	0.021	0.021	0.021	0.021	0.020	0.021	0.021	0.020	0.021	

the more similar their COI gene sequences tend to be. Even so, the geographical proximity of populations with haplotypes from different lineages along the 'transition zone' between phylogroups 1A in the west and 1B/1C in the east likely contributes to the relatively low value of the correlation coefficient.

Discussion

Following Darwin's (1859) observation of the morphological similarity and cosmopolitan distributions of freshwater crustaceans, considerable attention has focused on their evolutionary history and biogeographical patterns. The results of recent genetic studies have found significant regional gene pool differentiation, enough to warrant the recognition of multiple species (Hebert & Wilson 1994; Taylor *et al*. 1998). Consequently, the historical view of cosmopolitan species has now often been replaced by cryptic endemism (Frey 1987; Hebert & Wilson 1994; Taylor *et al*. 1998). Our study examined the patterning of mtDNA diversity in *Daphnia obtusa*, a species thought to possess a broad continental distribution in North America.

Although just one mtDNA gene (COI) was examined, this study provides new information on the geographical patterning of genetic diversity within this taxon. Populations identified as *D. 'obtusa'* (*sensu* Schwartz *et al*. 1985) were shown to consist of three species in the *obtusa* complex, one of which, *D. neo-obtusa*, has been recognized previously (Hebert 1995). Previous work (Colbourne & Hebert 1996) has shown that the *obtusa* complex consists of five taxa in North America; *D. obtusa*, *D. neo-obtusa*, *D. pileata*, *D. cheraphila* and *D. prolata*. COI sequence divergence among these taxa ranges from 19 to 23% (Adamowicz 2002) suggesting that they all diverged from one another at least 15 Ma, and possibly much earlier. Previously, *D. neo-obtusa* had only been detected in eastern Oregon but the populations identified in this study, JP and CL, were both from California (Fig. 4) suggesting that this species has a broader range along the Pacific coast than previously thought. Moreover, the sequence divergence between the COI genes of isolates from the two states is 5.2%, suggesting that there are geographically separated phylogroups of this species. Although the allozyme profile of population CL was similar to that of the other *D. 'obtusa'* populations analysed by Hebert & Finston (1996), the results of this study indicate that this population is *D. neo-obtusa*. Alternatively, it may contain both species but further studies are required to determine if this is the case.

The second species identified in this study, *D. obtusa* NA2, is an undescribed, morphologically cryptic taxon. The separation of *D. obtusa* NA1 and this newly recognized taxon has strong support (Fig. 2) and the COI sequence divergence between them is 15.3%. Moreover, it is clear that *D. obtusa* NA2 is not closely related to any of the other taxa in the *D. obtusa* complex. Unfortunately, none of the populations of *D. obtusa* NA2 were included in the allozyme survey of Hebert & Finston (1996), so it is not known whether it can be distinguished from *D. obtusa* NA1 with these markers. Individuals of the two species were found to coexist in a single pond (NP) from Illinois in our study, but is seems unlikely that they would interbreed given the depth of sequence divergence between their COI genes. Even so, future sampling of habitats in the region, followed by subsequent allozyme and mtDNA analyses, should be performed to confirm that they are reproductively isolated (Witt & Hebert 2000). Overall, the results presented here strongly suggest the presence of yet another member of the North American *obtusa* complex.

D. obtusa NA1 is broadly distributed across the central USA and is itself composed of four phylogroups (1A–1D;

Fig. 3) with strong support for the monophyly of each. Divergences among these phylogroups, which range from 1 to 2% (Table 2), are too shallow to determine their phylogenetic relationships. The use of Brower's (1994) arthropod mtDNA clock suggests that these lineages diverged within the Pleistocene, < 1 Ma. Thus, they were likely restricted to different refugia during the glacial advances. Currently, the ranges of groups 1B and 1C overlap substantially throughout the Mississippi watershed, but group 1B has spread further north and south.

Within each of the NA1 phylogroups, there is significant, but modest, genetic differentiation over broad geographical areas except at contact points between lineages, a pattern that is common to species impacted by glaciations (Bernatchez & Wilson 1998). Thus, it seems that the glacial advances influenced this species more than might be expected given its occurrence in the unglaciated regions of the continent. Moreover, sharing of gene pools by populations separated by large distances (Fig. 4) is likely the result of recent long-distance dispersal (Hebert & Finston 1996; Taylor *et al*. 1998), which is possible through the passive dispersal of resting eggs by either biotic or abiotic factors (Taylor *et al*. 1998). Indeed, the Western, Mississippi and Atlantic avian flyways may be an important dispersal mechanism that maintains cohesion within phylogroups along north–south axes (Taylor *et al*. 1998).

Comparison of the earlier allozyme results (Hebert & Finston 1996) and those in this study reveals a lack of concordance between mtDNA and allozyme groups. Allozyme group I generally occurs in the west, throughout the Great Plains Region (Fig. 4), whereas group II occurs in the east; a transition that roughly coincides with the prairie–forest ecotone. Indeed, Hebert & Finston (1996) found ecological differentiation among populations as those from eastern habitats occupy clear water ponds, whereas their midwestern counterparts inhabit ponds with high levels of colloidal clay. Conversely, the east–west transition between mtDNA groups 1A and 1B/1C occurs within the Great Plains Region and roughly coincides with a substantial shift in elevation from high in the west to low in the east; a transition that likely reflects dispersal from different glacial refugia. That long-distance dispersal along an east–west axis does sometimes occur is evident from population CO in Arizona, which has mtDNA and allozyme genotypes more typical of populations in Texas or Oklahoma, and population CL in California, which is more similar to populations from Kansas or Missouri (Fig. 4). In addition, the western phylogroup, 1A, was detected in Arkansas and Missouri.

Surveys of mtDNA variation have been conducted for two other species of *Daphnia*, *D. laevis* (Taylor *et al*. 1998) and *D. ambigua* (Hebert *et al*. 2003), with broad geographical distributions across unglaciated North America. *D. laevis* consists of two major clades showing ~ 14% sequence divergence at COI. One clade, *D. laevis* s.s, occurs east of the Appalachian Mountains, whereas the other, *D. magniceps*, occurs from the central United States to the Pacific coast. Taylor *et al*. (1998) estimated that they diverged from one another at least 12 Ma. This situation resembles that in *D. obtusa*, where the sequence divergence between the eastern and central species is 15%. The congruence of this phylogeographical pattern and its timing suggests that both the *laevis* and *obtusa* species groups were influenced by the same vicariant event. The last major orogenesis in eastern North America involved a series of isostatic uplift pulses during the Miocene (23.8–5.3 Ma) that produced the modern Appalachian peaks (Levin 2002), and which likely acted as the barrier that prompted divergence in both of these *Daphnia* taxa.

Phylogeographical structure within *D. magniceps* and *D. obtusa* NA1 based on mtDNA variation is also similar with sequence divergence between groups of 1–2% within each species. Groups 1B and 1C in *D. obtusa* NA1 have a similar distribution to the central group in *D. magniceps*. In both cases there is very little sequence divergence and little structure across broad geographical areas within phylogroups. However, the western phylogroups of each species have quite different distributions. Group 1A in *D. obtusa* NA1 occurs in the western Great Plains Region and into the Rocky Mountains, whereas *D. magniceps* occurs along the Pacific coast, west of the Sierra Nevada-Cascades range. The closest relative to *D. obtusa* in this region is *D. neo-obtusa*, from which it is likely to have diverged prior to the formation of these mountains between 10 and 15 Ma (Ruddiman *et al*. 1989). In addition, the divergence between the western and central groups of *D. magniceps* is also evident from analysis of allozyme variation (Taylor *et al*. 1998), whereas the geographical distribution of allozyme and mtDNA groups in *D. obtusa* is not concordant. The distribution of groups identified with both types of markers suggests that there has been gene flow between them. However, the possibility that selection also plays an important role in maintaining differentiation at nuclear loci in *D. obtusa*, despite the gene flow, deserves further consideration.

D. ambigua (Hebert *et al*. 2003) also shows divergence into phylogroups with eastern, central and Pacific distributions. However, in this case, sequence divergence between all three phylogroups (2–5%) is of the same order of magnitude. Furthermore, it is higher than the divergence observed between phylogroups within *D. magniceps* and *D. obtusa* NA1. Sequence divergence between North American populations of *D. ambigua* and those in South America is ~ 4% (Hebert *et al*. 2003) suggesting that this species may have invaded all three regions of North America (eastern, central, Pacific) from the south in the early Pleistocene and diverged during the subsequent glacial periods with little contact between regions. Although *D. ambigua* has occupied North America for a much shorter period than the *laevis*

and *obtusa* species complexes, it shows a similar pattern of phylogeographical structure suggesting that persistent topographic features produce predictable patterns of population divergence and phylogeographical structure, regardless of the timing of the colonization events.

Conclusions

This study provides new evidence suggesting that North American populations formerly assigned to *Daphnia 'obtusa'* actually consist of two distinct species showing largely allopatric distributions. This pattern has been documented in other North American daphniids. The two North American species of *D. obtusa* (NA1 and NA2) are morphologically similar and inhabit comparable ecological niches (clear water ponds) in the east. However, the high degree of their mtDNA divergence suggests that they are cryptic species. NA2 has a narrow distribution restricted to the southeastern United States, whereas NA1 has a broad distribution spanning most of the central United States, west of the Appalachian Mountains. However, this 'continental' species is divided into four mitochondrial phylogroups with somewhat allopatric distributions. The current distribution of these groups likely reflects the recent range expansion of fragmented populations previously restricted to different glacial refugia during the Pleistocene. In addition, our study has revealed a lack of correlation between mtDNA lineages and nuclear allozyme groups. Together, these results illustrate the complicated biogeographical history of freshwater crustaceans whose evolutionary analysis is confounded by such factors as cryptic endemism and habitat shifts. Further studies on this and other taxa are necessary to test the generality of these results with respect to other freshwater crustaceans.

Acknowledgements

Financial support for this work was provided by a research grants from the Natural Sciences and Engineering Research Council to TJC and PDNH. We thank A. Holliss for sequencing, C. Bros for assistance with sample collection and PCR analysis, and A. Robertson, A. Gozdzik and J. deWaard for assistance with phylogenetic reconstruction. We thank S. Adamowicz and J. Colbourne for the use of COI sequences described in her MSc thesis, and D. Taylor, L. Weider and D. Innes for providing some of the *Daphnia* samples analysed in this study.

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