MOLECULAR PHYLOGENETICS AND EVOLUTION
Vol. 5, No. 3, June, pp. 495–510, 1996
ARTICLE NO. 0045

Phylogenetics and Evolution of the *Daphnia longispina* Group (Crustacea) Based on 12S rDNA Sequence and Allozyme Variation

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Received January 27, 1995; revised August 28, 1995.

Although members of the crustacean genus *Daphnia* have been the target of much research, there is little understanding of the group's evolutionary history. We addressed this gap by inferring a phylogeny for one of the major species groups (*longispina*) using nucleotide sequence variation of a 525-bp segment of the mitochondrial 12S rDNA and allozyme variation at 21 loci. We identified the major lineages and their relationships, assessed the phylogenetic utility of the few morphological characters in the group, and examined *Daphnia* phylogeography. Nuclear and mtDNA phylogenies were generally concordant in recognizing the same four species complexes. An exception was the position of *Daphnia galeata mendotae*. The allozyme tree paired this species with the *Daphnia rosea* lineage, whereas the mtDNA trees grouped *D. g. mendotae* with *Daphnia galeata galeata*. This discordance was consistent with the reticulate evolution of nuclear genes supporting the hypothesis that *D. g. mendotae* represents a case of homoploid hybrid speciation. Striking morphological stasis in the *longispina* group was evidenced by its very limited morphological divergence over an estimated 100 MY, and by the unusual transitional saturation of the conservative 12S rRNA gene within a species group. Phylogenetic inference also provided evidence that similarities in cephalic crest shape likely resulted from convergent or parallel evolution among species. Endemism at the continental level was indicated for previously cosmopolitan species, but the estimated times of these divisions were inconsistent with vicariance events suggesting recent dispersal among continents. A significant role for divergent selection in new habitats during speciation was suggested by the neighboringly sympatric distributions of four sister species pairs over broad geographic areas.

INTRODUCTION

A lack of phylogenetic knowledge has severely impeded studies of cladoceran comparative biology, biogeography, and evolution. Historical evidence is needed, for example, to evaluate the proposed mechanisms of cladoceran speciation. The much-studied geographic patterns of morphological diversity in cladocerans also have little meaning without historical or genetic knowledge—morphological similarity of taxa (among continents or habitat types) may be due to either convergence, cosmopolitanism, introgression, or shared ancestry.

Where extensive biogeographic information about morphological and genetic variation is available, the long-standing concept of cosmopolitanism has been rejected in favor of endemism and vicariant speciation (Frey, 1987; Glagolev and Alonso, 1990; Sergeev, 1990; Hebert and Wilson, 1994). The generality of endemism in the Cladocera, however, is unclear as the best known groups (chydorids, *Ctenodaphnia*, and *Daphniopsis*) may be unrepresentative with respect to vagility. There is an oft overlooked tendency of these groups either to affix or to have their propagules lodged in the substrate of the waterbody in which they were formed (Frey, 1987; Sergeev, 1990; Fryer, 1991). This impediment to dispersal is certainly not universal in cladocerans. For example, members of the subgenus *Daphnia* often possess buoyant resting eggs, which carpet a waterbody surface increasing exposure to dispersal vectors (e.g., Forbes, 1893; Hobæk and Wolf, 1991).

The genus *Daphnia* has become a model taxon for studies of experimental ecology, life history evolution, toxicology, and evolution of breeding systems (Peters and De Bernardi, 1987), but the genus has remained recalcitrant to the tools of the systematist. The group was known to science as early as 1669 when Swammerdam mistakenly classified it as an aquatic flea (see Edmondson, 1987). Exsanguination of the prey was thought to occur via the pointed rostrum (the frequent blood-red appearance of *Daphnia* due to hemoglobin probably fueled this myth). Unlike some other microcrustaceans (e.g., ostracodes, chydorids, bosminids) there are few useful exoskeletal fossil records for *Daphnia* (Frey, 1987), the chromosomes are too small for detailed cytogenetic investigation, and few if any phylogenetically informative morphological characters have

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1055-7903/96 $18.00

been identified despite 200 years of detailed studies (see Brooks, 1957a; Dodson, 1981; Benzie, 1986). Moreover, individuals of many *Daphnia* species are too small for extensive multilocus allozyme analysis using conventional starch electrophoresis. To compound these technical problems, recent studies have found that introgression, hybridization, and anthropogenic faunal interchanges may have created phylogenetic noise in major *Daphnia* groups (e.g., Taylor and Hebert, 1993 a,b; Hebert and Wilson, 1994).

Biogeographical evidence suggests that the genus has existed for at least 70 MY (Hebert, 1978; Benzie, 1987), and this conclusion may be reinforced by the discovery of fossil *Daphnia*-type ephippia from the early Cretaceous (ca. 130 MYA, Smirnov, 1992). There is, however, little understanding of the age of extant species, although there is a general sense that many species, especially lacustrine forms, have originated since the Pleistocene (Brooks, 1957a). This lack of historical information has prevented assessment of the various processes responsible for speciation.

Three distinct speciation models have been proposed for Cladocera. Vicariant speciation (Frey, 1987; Hebert and Wilson, 1994) occurs when a species distribution is disrupted by a barrier to gene flow (often geological, such as continental drift) and the daughter groups evolve independently, eventually resulting in reproductive isolation. A prediction of this hypothesis is that both the time since divergence and the phylogeography of sister taxa should be concordant with the vicariance event. Lynch (1985) developed a second speciation model for cladocerans that invoked founder effects and divergent selection during habitat shifts. According to this model, lake to pond founder effect speciation should be frequent because (a) lake populations rarely produce sexual propagules, (b) there is strong divergent selection for the pond lifestyle, and (c) ephemeral ponds constrain the founder's effective population size, creating sampling drift. A third mode of speciation that has been proposed for *Daphnia* is hybrid speciation (e.g., Flössner, 1993). In this scenario, hybridization with or without introgression (defined here as interspecific gene flow resulting from backcrossing) leads to the formation of stabilized recombinants that are eventually reproductively isolated from the parent taxa.

In this paper we address the difficult systematics of the *Daphnia longispina*, Müller 1763, species group using a molecular approach. The group is one of two major presumed monophyletic species groups in the subgenus *Daphnia* (*Daphnia pulex* is the other group) and is defined by indistinct combs on the postabdominal claw and reduced male antennules (Hrbácek, 1987). Traditionally, the *D. pulex* group has been defined by the possession of at least one distinct comb on the postabdominal claws and pronounced male antennules. Perhaps the best synapomorphy for the *D. pulex* group is, however, a chromosome count of $2n = 24$ (Beaton and Hebert, 1994). A chromosome count of $2n = 20$ is found in the *longispina* group, other *Daphnia*, and ancestral daphniids. The 12 well-recognized taxa of the *D. longispina* group [i.e., taxa that are recognized both by Brooks (1957a) and by Hrbácek (1987), or have species boundaries genetically confirmed] are listed in Table 1. The group is often further divided into a helmeted complex and the *longispina* complex (species incapable of producing an anterior cephalic crest or helmet). All well-recognized *longispina* species occur in the Holarctic and only one of these species (*Daphnia laevis*) is common beyond this area.

TABLE 1

Well-Recognized Species of the *Daphnia longispina* Group (see Hrbácek 1987; Taylor and Hebert, 1994)

Species	Geographic range	Habitat preferences	Helmet production
D. cristata	Northern Palearctic	Lakes	Yes
D. cucullata	Northern Palearctic	Lakes	Yes
D. dubia	Eastern Nearctic	Lakes	Yes
D. galeata galeata	Palearctic	Lakes	Yes
D. galeata mendotae	Nearctic	Lakes	Yes
D. hyalina	Palearctic	Lakes	Yes
D. laevis	Neotropic, Nearctic, and Ethiopian	Ponds	No
D. longiremis	Holarctic	Lakes	Yes
D. longispina	Palearctic	Ponds	No
D. rosea	Holarctic (and Ethiopian?)	Ponds and small lakes	No
D. thorata	Northwest Nearctic	Lakes	Yes
D. umbra sp. n.	Eastern Nearctic	Lakes and ponds	No

Note. Lakes refer to waterbodies containing fish; ponds are fishless waterbodies. Helmets describe anterior cephalic extensions.

The recent use of sensitive genetic techniques has provided insight into *D. longispina* systematics on a regional scale. Cellulose acetate gel electrophoresis allozyme studies of the *longispina* group have proven useful for establishing species boundaries and affinities in the *longispina* group in Norway (Hobæk and Wolf, 1991) and North America (Taylor and Hebert, 1992, 1994). In addition, RAPD markers (Schierwater *et al.*, 1994) and nucleotide sequences of PCR-amplified cytochrome b genes (Schwenk, 1993) have been used to infer phylogenetic relationships among three European *longispina* species.

The purpose of this study is to use mitochondrial DNA sequence and allozyme variation to (1) identify and compare the major lineages of the *longispina* group, (2) assess the phylogenetic significance of the few morphological characters in the *longispina* group, (3) estimate the time scale of radiation in the group, and (4) gain insights about *Daphnia* phylogeography.

MATERIALS AND METHODS

As the taxonomy of the group is in a state of flux, we included as many historical forms (Flössner, 1972;

TABLE 2

Taxa, Morphological Forms (see Brooks, 1957a; Flössner 1972), and Sampling Sites for *Daphnia* mtDNA Haplotypes

Species/subspecies	Form	Waterbody	Site	Site acronym
cucullata	*cucullata*	Schierensee	Germany	GER
dubia	*nasuta*	Stormy Lake	Wisconsin	WI
dubia	*nasuta*	Wren Lake	Ontario	ON
galeata galeata	*typica*	Derwentwater (lake)	United Kingdom	UK
galeata galeata	*gracilis*	Slapy Reservoir	Czech Republic	CZ
galeata galeata	*gracilis*	Schöhsee	Germany	GER
galeata mendotae	*galeata*	Lost Lake	Oregon	OR
galeata mendotae	*galeata*	Center Lake	Indiana	IN
galeata mendotae	*galeata*	Guelph Lake	Ontario	ON
hyalina	*typica*	Kellersee	Germany	GER
hyalina	*pellucida*	Mondsee	Austria	AUS
laevis	*elongate*	Rondeau pond	Ontario	ON
longiremis	*typica*	Unnamed lake, Melville Peninsula	N.W.T., Canada	NWT
longispina	*caudata*	Toporowy staw (pond)	Poland	POL
rosea	*cavifrons*	Vysne Furkotske, pond	Slovakia	SLV
rosea	*rosea*	Lago Di Campo IV, pond	Piedmont, Italy	ITA
rosea	*dentifera*	Canal Flats pond	British Columbia	BC
rosea	*indianae*	Old Lake	Indiana	IN
umbra (sp. n.)	clear	Igloolik Lake	N.W.T., Canada	IGLK
umbra (sp. n.)	melanic	Pond near Richards Bay	N.W.T. Canada	RB27
thorata	type	Flathead Lake	Montana	MT
magna		Pond	Nebraska	None
lumholtzi	helmeted	Pomme de Terre Lake	Missouri	None

Hrbácek, 1987), geographic variants, and especially allozyme lineages (from Taylor and Hebert, 1994, Taylor, unpublished) as possible. All of the well-recognized taxa in the *D. longispina* group were examined except *D. cristata* (Table 1), which is undoubtedly the sister species of *Daphnia longiremis* (Brooks, 1957a). Table 2 provides the sampling sites and taxonomic designations for the specimens analyzed. North American taxa were identified according to Brooks (1957a) and Taylor and Hebert (1992). A new member of the *longispina* group from the Canadian Arctic, which was defined by allozymes (Taylor and Hebert, 1994), was designated *Daphnia "umbra."* We included geographically distant replicate samples within the following taxa: *Daphnia galeata mandotae* (three sites), *Daphnia galeata galeata* (three sites), *Daphnia dubia, Daphnia rosea,* and *Daphnia hyalina,* and *D. umbra.* Two divergent species of the subgenus *Ctenodaphnia, Daphnia magna* and *Daphnia lumholtzi,* were included as outgroups for the mtDNA parisimony analysis. The 12S rDNA sequence of *D. pulex,* the nominal species of the *D. pulex* group, was obtained from Van Raay and Crease (1994) and added to the analysis.

Allozyme Analyses

In all, 21 putative loci were scored. With the exception of two loci for *D. hyalina,* the minimum sample size for a given locus was 20 individuals per population. The following loci were scored: aconitase hydratase (ACOH-1, EC 4.2.1.3), aldehyde oxidase (AO, EC 1.2.3.1), arginine phosphokinase (APK, EC 2.7.3.3), aspartate aminotransferase (sAAT, mAAT; EC 2.6.1.1), dipeptidase (PEP-A, PEP-A2, EC 3.4.13.11), fumarate hydratase (FUMH, EC 4.2.1.2), glucose-6-phosphate isomerase (GPI, EC 5.3.1.9), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12), lactate dehydrogenase (LDH, EC 1.1.1.27), isocitrate dehydrogenase (IDH-1, IDH-2; EC 1.1.1.42), malate dehydrogenase (MDH-1, MDH-2; EC 1.1.1.37), malate dehydrogenase NADP+ (MDHP-2, EC 1.1.1.40), mannose-6-phosphate isomerase (MPI, EC 5.3.1.8), phosphoglucomutase (PGM-1, PGM-2; EC 5.4.2.2), phosphogluconate dehydrogenase (PGDH, EC 1.1.1.44), and proline dipeptidase (PEP-D, EC 3.4.13.9).

Cellulose acetate gel electrophoresis was conducted according to Hebert and Beaton (1993). Substrates used for PEP-D and PEP-A allozymes were phenylalanyl-proline and leucylglycine, respectively. For pairwise genetic distances we used the chord distance of Cavalli-Sforza and Edwards (1967) because of its low sensitivity to among-group differences in polymorphism (see Swofford and Olsen, 1990). Neighbor Joining (NJ—Saitou and Nei, 1987) was used for tree construction as this method does not assume equal evolutionary rates among lineages. A bootstrap majority-rule consensus tree and the above distance and NJ results were calculated by programs in PHYLIP 3.5c (Felsenstein, 1993).

mtDNA Analysis

Total DNA was extracted from live or previously frozen individuals using 6% Chelex 100 (Bio-Rad, Richmond, CA). Whole animals were placed in 30 μl of Chelex 100 incubated at 60°C for 30 min, vortexed for 30 s, and left overnight at 4°C. Each 50-μl PCR reaction consisted of 10 μl of DNA template (i.e., the supernatant from Chelex 100 extractions), buffer (Boehringer-Mannheim), 1.5 mM $MgCl_2$, 2 mM of each dNTP, 1 μM of each primer, 0.5 to 1 units of DNA polymerase from *Thermus aquaticus* (*Taq*), and 15% w/v trehalose. The primers were designed from conserved regions between *D. pulex* and *Drosophila yakuba* within the 12S rRNA mitochondrial gene (5′-ATGCACTTTCCAGTACATC TAC-3′, 5′-AAATCGTGCCAGCCGTCGC-3′). The PCR consisted of 1 cycle of 1 min at 94°C; 10 cycles of 1 min at 94°C, 2 min at 53°C and 1 min at 72°C; 30 cycles of 1 min at 92°C, 2 min at 53°C, and 1 min at 72°C; followed by 1 cycle of 5 min at 72°C. The product (605 bp in *D. pulex*) was purified using the GeneClean II (Bio-101) kit and sequenced directly using the dideoxy method (Sequenase, United States Biochemical or T7 polymerase, Pharmacia) as modified by Nickrent (1994). The templates were sequenced with each primer resulting in a >20% overlap of information. The DNA sequences were submitted to the GenBank/EMBL database (Accession Nos. U34638–U34652 and U34732–U34739).

Sequences were aligned by Clustal V (Higgins and Sharp, 1988) with a gap penalty of 8, transitions (TS) weighted over transversions (TV) by 2:1, and adjusted by eye using the Seqapp 1.9a sequence editor (Gilbert, 1992). Nucleotide compositions, number of transitions and transversions, TS/TV, and gap statistics were calculated by MEGA 1.02 (Kumar *et al.*, 1993). Phylogenetic analyses of sequences used maximum parsimony (MP) in PAUP 3.1.1 (Swofford, 1993) and NJ of Kimura's two-parameter model distance matrices in MEGA 1.02 (Kimura, 1980; Kumar *et al.*, 1993). The phylogenetic impact of biases in nucleotide composition were assessed using the LogDet transformation methods of Lockhart *et al.* (1994). To assess the phylogenetic signal of the sequences, the g_1 skewness statistic was calculated in PAUP from 10,000 random tree length distributions (Hillis and Huelsenbeck, 1992). One taxon of each strongly supported clade was then removed to examine if the phylogenetic signal was present in deeper branches. We tested the statistical significance of alternate topologies using Wilcoxon's matched-pairs signed-ranks test as implemented by Templeton (1983) for parsimony trees based on DNA sequences.

A mtDNA clock and confidence limits for the 12S rRNA gene was calculated according to Lynch and Jarrell (1993). The estimated asymptotic identity (I_∞) from our data was 0.279. Other parameters, including a substitution rate of 0.489/BY, were from Lynch and Jarrell's (1993) comparison of animal 12S rRNA evolution. For closely related taxa we used the arthropod mtDNA clock proposed by Brower (1994) of 2.3% sequence divergence/MY.

RESULTS

12S rRNA Evolution

A total of 525 continuous bp (approximately 70% of the gene) were compared for the 24 OTUs (Fig. 1). After alignment, there were 229 variable sites, 173 parsimony informative sites, and an average of eight indels per sequence (range = 6–11). Nearly all of the indels (98.5%) were 1 to 2 bp long. The sequences were A-T rich (mean A-T content = 66.5%), with *D. longispina* possessing the lowest value (62.6%) and *D. lumholtzi* the highest value (71%). Pairwise Kimura distances (with complete deletion of gaps and missing sites) ranged from 0 to 28.6% overall and from 0 to 24.8% within the *longispina* group. Within-species sequence divergence was 1% or less in *D. g. galeata, D. g. mendotae, D. dubia, D. hyalina,* and *D. umbra*. Although *D. rosea* divergences were less than 1% within continents, they were 3.6–4.1% between North America and Europe.

The broad range of distances permitted analysis of transition/transversion ratio evolution in the *Daphnia* 12S rRNA gene (Fig. 2). In general, the TS/TV decreased as pairwise sequence divergence increased. The TS/TV ranged from 0.673 to 7 (nine comparisons below 1% sequence divergence lacked transversions). When pairwise comparisons of TS/TV near and beyond saturation (presumably when TS/TV = 1) are excluded, the slope of the regression between TS/TV and the sequence divergences was −20. The point of saturation occurred when pairwise divergence was near 20%. Within the *longispina* group, only comparisons involving *D. laevis, D. dubia,* and *D. longiremis* approach saturation. The nucleotide composition of transversions was biased as 68.7% of all transversions were A ↔ T.

12S rDNA Phylogeny

The g_1 value of −0.783 was well below the critical value ($g_1 = -0.09$, $P = 0.01$) for 25 taxa, indicating that significant phylogenetic signal exists in the data. When one of each closely related taxon pairs was removed (leaving only *D. longispina, D. thorata, D. rosea* SLV, *D. galeata* UK, *D. cucullata, D. laevis, D. longiremis, D. pulex, D. magna,* and *D. lumholtzi*), the g_1 value remained significant ($g_1 = -0.7108$). Eliminating transitions also yielded a significant result ($g_1 = -0.821$). Even when only the most distantly related taxa were included (*D. longispina, D. laevis, D. longiremis, D. pulex, D. magna, D. lumholtzi*) the g_1 test was significant ($g_1 = -1.11$). These results suggest that signal is present among the most divergent taxa in our data.

Distance based trees (not shown) using a Kimura two-parameter distance matrix suggested that *D. pulex, D. magna,* and *D. lumholtzi* are legitimate outgroups for the *longispina* group. In addition, the sequence similarities between these taxa and the ingroups are >75% and within the range recommended for optimizing signal in rDNA phylogenetic studies (Hillis and Dixon, 1991).

Three tree construction methods were used: MP (with gaps coded as an additional character), NJ of Kimura two-parameter distances (with complete and pairwise deletion of gaps and missing sites), and NJ of LogDet transformations. All methods produced a nearly identical topology with respect to *D. longispina* group species (Figs. 3 and 4). The MP bootstrap 50% majority-rule consensus tree differed from the NJ tree in only one important way: the support for a branch that resolves the position of *D. longiremis* was lost (Figs. 3 and 4). Maximum parsimony using branch-and-bound search with multiple populations eliminated for each species found only one shortest tree (Fig. 5). This tree agreed in topology with the multiple populations MP consensus tree (Fig. 4). A NJ tree of LogDet transformed parsimony informative sites (tree not shown) revealed the topology of the *longispina* group was unaltered, indicating that GC content variation creates insignificant phylogenetic noise in the group.

Five major species groups were apparent from analyses of the total data: *thorata*/North American *rosea*, *hyalina*/European *rosea*, *cucullata/galeata/mendotae*, *longispinal/umbra*, and *laevis/dubia*. Within these major groups *D. dubia*, *D. galeata*, *D. hyalina*, and *D. umbra* were monophyletic. The only species that was not monophyletic was *D. rosea*. Using Templeton's (1983) test for comparing parsimony trees, *D. rosea* monophyly required significantly more steps (18 steps longer, $N = 18$, $T = 0$, $P < 0.01$) than the single shortest tree found by the branch-and-bound search (Fig. 5). Monophyly of the *longispina* complex (i.e., unhelmeted taxa), even without including the distantly related *D. laevis*, required significantly more steps than the MP consensus tree (24 extra steps, $N = 48$, $T = 305.5$, $P < 0.01$).

The plot of transition/transversion ratios suggested that phylogenetic noise from transitional saturation may be present in 12S rRNA sequence comparisons involving *D. laevis*, *D. dubia*, *D. longiremis*, and the outgroup. Nevertheless, the relationships among these taxa were unaffected in both the NJ and MP trees (90 MP trees found of 266 steps, CI = 0.735, RI = 0.809) when transitions are eliminated (trees not shown). In addition, the topology of the total data MP tree was not significantly different from the transversion tree topology (2 extra steps, $N = 14$, $T = 45$, $P > 0.05$). Wheeler and Honeycutt (1988) suggested that stem sites in rRNA gene phylogenies be weighted by half or eliminated from the data because of their non-independence dictated by Watson–Crick pairing. We therefore eliminated these sites from our data using the 12S rRNA secondary structure model for *D. pulex* proposed by Van Raay and Crease (1994). NJ of these data (tree not shown) resulted in only one minor topological change from the total data tree as the *longispina/umbra* clade became unsupported. The topology of the total data MP tree was not significantly different from that of the consensus MP tree (based on 27 trees of 239 steps, CI = 0.661, RI = 0.752) with stem sites omitted (3 additional steps $N = 14$, $T = 37.5$, $P > 0.05$).

Nuclear Gene Phylogeny

The NJ consensus tree of allozyme variation (Fig. 6) was generally concordant with the mtDNA trees. The only exception was the grouping of *D. g. mendotae* with the *rosea/thorata/hyalina* clade instead of with *D. g. galeata*. Templeton's test revealed that such a tree is significantly different from the MP tree based on mtDNA sequence (14 steps longer, N = 16, T = 8.5, P < 0.001). Inspection of the allele frequencies in Table A1 revealed that the great nuclear divergence between the subspecies of *D. galeata* was due largely to an increased frequency of alleles that *D. g. mendotae* shares with *D. rosea* (PEP-D, IDH-2, GPI, sAAT) but not with *D. g. galeata*. At least one of these alleles (PEP-D^c) is shared only between the *D. rosea* lineage and *D. g. mendotae*, making shared ancestry unlikely as an explanation for its occurrence in both species. The *D. g. mendotae–D. rosea* sharing of the $sAAT^c$ allele also cannot be explained by shared ancestry if the *longispina/umbra* complex forms a clade with the *rosea/thorata/hyalina* complex. This topology is not significantly different from the MP consensus tree based on 12S rDNA sequence (4 extra steps, $N = 32$, $T = 231$, $P > 0.05$).

Divergence Time Estimates

The 12S rRNA calibration of Lynch and Jarrell (1993) is shown in Table 3. As the calibration is corrected for ancestral intraspecific heterozygosity, the estimated divergence times became negative below a sequence divergence of 9%. The arthropod mtDNA clock of Brower (1994) is expected to be linear until at least 8% divergence and therefore complemented our Lynch and Jarrell (1993) calibrations. The clocks indicated that speciation in the *longispina* group has occurred over the last 100 MY with the greatest radiation occurring in the last 40 MY. The three youngest taxonomic groups (*thorata*–North American *rosea*, *hyalina*–European *rosea*, and *galeata–mendotae*) were estimated to be less than 1 MY. Separation of North American and European *D. rosea* was estimated to be 1.8 MY, whereas the *dubia–laevis* split was substantial at 30 MY. The more ancient *longispina* group lineages (*laevis* and *longiremis*), the *D. pulex–longispina* group separation, and the *Daphnia–Ctenodaphnia* separation were estimated at >100 MY old.

DISCUSSION

The use of independent genetic information and a careful sampling strategy provided clear resolution for the major lineages of the *longispina* group. There is a concordance between our 12S rDNA phylogeny and the phylogeny found using the cytochrome b gene fragment for *D. cucullata*, *D. hyalina*, *D. galeata*, and *D. magna* (Schwenk, 1993). In addition, there is a general agreement between the allozyme and the 12S rDNA phylogenies (see below). Introgression of mtDNA can confound phylogenetic inference, but mtDNA investigations of several *longispina* hybrid complexes agree that mtDNA introgression in the group is negligible (Schwenk, 1993; Taylor and Hebert, 1993a). The low within-species variation for the 12S gene (1% or less) over wide geographic areas suggests that major lineage relationships will be unaffected by increasing the number of individuals per taxon on a continent.

The same is true for allozyme information, as extensive information on this group has been collected on every species indicating very low among-population variation even over wide distances (Korpelainen, 1986; Mort and Wolf, 1986; Wolf, 1988; Hebert *et al.*, 1989; Hobæk and Wolf, 1991; Taylor and Hebert, 1994; Finston and Hebert, unpublished; Taylor, unpublished). It is also unlikely that any new major lineages will be

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                                                                             50
Daphnia longispina POL CTTATCTCCC CTTAAGGAGA ??GTGACGGG CGATATGTAC ACACTTTATT
D. umbra IGLK          ?.......T. .......... GA........ .......... ..........
D. umbra RB27          ???.....T. .......... GA........ .......... ..........
D. thorata MT          ?????????? ?????????? ..??...... ..?....... ..........
D. rosea SLV           ?.......T. .......... GA........ .......... ..........
D. rosea ITA           ........T. .......... GA........ .......... ..........
D. hyalina GER         ????....T. .......... GA........ .......... ..........
D. hyalina AUS         ???.....T. .......... GA........ .......... ..........
D. galeata UK          ????....T. .......... GA........ .......... ..........
D. galeata CZ          ?????????? ?????????? .......... .......... ..........
D. galeata GER         ???.....T. .......... GA........ .......... ..........
D. mendotae IN         ?.......T. .......... GA........ .......... ..........
D. mendotae OR         ?????????? ??????.... GA........ ??........ ..........
D. mendotae ON         ??????..T. .......... GA........ .......... ..........
D. cucullata GER       ????....T. .......... GA........ .......... ..........
D. rosea BC            ??????..T. .......... GA........ .......... ..........
D. rosea IN            ?????????. .......... GA........ .......... ..........
D. laevis ON           ?????????? ???...AG.. GA........ ........G. ..........
D. dubia ON            ?????????. .........? ..?....... ........G. ......C...
D. dubia WI            ??.C...... .......??? ..????.... ........G. ......C...
D. longiremis          .........T ......AG.. G......... ........G. ..........
D. pulex               .........T ...G..A... GA........ ........G. ...TCC....
D. magna               ?????????? ??..GAAG.. GA........ ........G. ...T.C....
D. lumholtzi           ????.....T .....AAG.. GA........ .......... ...T.C...G

                                                                            100
Daphnia longispina POL GCCCCATTCA AGTAGTTTTA CTTAAATTAC TTTACTAATA AATCCACCTT
D. umbra IGLK          A..TT..... .A.GA..... .......C.T .......... ..........
D. umbra RB27          A..TT..... .A..A..... .......C.T .......... ..........
D. thorata MT          ...TT..... ....A..... .......... .......... ..........
D. rosea SLV           ...TT..... .A..A..... .......... .......... ..........
D. rosea ITA           ...TT..... ...GA..... .......... .......... ..........
D. hyalina GER         ...TT..... .A..A..... .......... .......... ..........
D. hyalina AUS         ...TT..... .A..A..... .......... .......... ..........
D. galeata UK          ...TT..... ....A..... .......... .......G.. ..........
D. galeata CZ          ...TT..... ....A..... .......... .......G.. ..........
D. galeata GER         ...TT..... ....A..... .......... .......G.. ..........
D. mendotae IN         ...TT..... ....A..... .......... .......G.. ..........
D. mendotae OR         ...TT..... ....A..... .......... .......G.. ..........
D. mendotae ON         ...TT..... ....A..... .......... .......G.. ..........
D. cucullata GER       ...TT..... ....A..... .......... .......... ..........
D. rosea BC            ...TT..... ....A..... .......... .......... ..........
D. rosea IN            ...TT..... ....A..... .......... .......... ..........
D. laevis ON           A..TT..... GTA.A..... .........A C......... ..........
D. dubia ON            A..TT..... GT..A..... ......C..A C......... ..........
D. dubia WI            A..TT..... GT..A..... ......C..A C......... ..........
D. longiremis          A..A...... .CC.T..... ......A..G .......... ..........
D. pulex               ...TT..... .C.TAC.G.. .....G.A.A .......... ..........
D. magna               ...TTG.... GT.TA....C ..A....A.G C.......C. ..........
D. lumholtzi           ...TT..... .T.TAA...C ..A....A.A ........C. ..........

                                                                            150
Daphnia longispina POL TAGCCAGTTT TAGAG-TTTG ACGTCCGT-C TTTCTAAAGA AAATTGTAGC
D. umbra IGLK          ......-... ..A.AAC.CA ..A.T..... ...T..G.A. ..........
D. umbra RB27          ......-... ..A.AAC.CA ..A.T..... ...T..G.A. ..........
D. thorata MT          C.A....C.. CGAG..C... .AA.....A- .......... ..........
D. rosea SLV           C.A....C.. CGAG..C..A GTA...A.A- ...G...... ..........
D. rosea ITA           C.A....C.. CGAG..C..A GTA...A.A- ...G...... ..........
D. hyalina GER         C.A....C.. CGAG..C..A GTA...A.A- ...G...... ..........
D. hyalina AUS         C.A....C.. CGAG..C..A GTA...A.A- ...G...... ..........
D. galeata UK          C...T..C.. C.A...C..A GAA.....AT .......... ...C......
D. galeata CZ          C...T..C.. C.A...C..A GAA...A.AT .......... ...C......
D. galeata GER         C...T..C.. C.A...C..A GAA...A.AT .......... ...C......
D. mendotae IN         C...T..C.. C.A...C..A GAA.....AT .........G ...C......
D. mendotae OR         C...T..C.. C.A...C..A GAA.....AT .......... ...C......
D. mendotae ON         C...T..C.. C.A...C..A GAA.....AT .......... ...C......
D. cucullata GER       C.A.T.AC.. CGAGA....A GAA.T...AA ..C...GTAG ...C......
D. rosea BC            C.A....C.. C.AG..C... .AA.....A- .......... ..........
D. rosea IN            C.A....C.. CGAG..C... .AA.....A- .......... ..........
D. laevis ON           C.A.T.A... ..A.-...AA GTA.....TT .C.TA.T... .--...?...
D. dubia ON            C.AT..AC.. ATA.A...AA ..A.T...AT .C.TATC... .--....T..
D. dubia WI            C.AT..AC.. ATA.A...AA ..A.T...GT .C.TATC... .--.......
D. longiremis          CGA..T.... -.A.A..AAA G.T.T...AT ...-..G.T. ..........
D. pulex               C.C.T.AG.. .TA...CC.A .AA.....A- ......TTA. ..........
D. magna               C.C...AA.. ATA.-....A .GA.....AT ...T.--TA. ...C......
D. lumholtzi           C..T..AG.. .TA.-..... ..A.....AT ...TA--TT. ..........

                                                                            200
Daphnia longispina POL TCACCTCCAC CCCTACATAA ACTGCACCTT GACCTGAAGT AAAAAAGTT-
D. umbra IGLK          .......... ........G. .......... .......... ..........
D. umbra RB27          .......... ........G. .......... .......... ..........
D. thorata MT          .......... .T...T.... .......... .......... .G......C.
D. rosea SLV           .......... .T...T.... .......... .......... ......A.C.
D. rosea ITA           .......... .T...T.... .......... .......... ........C.
D. hyalina GER         .....T.... .T...T.... .......... .......... ......A.C.
D. hyalina AUS         .....T.... .T...T.... .......... .......... ......A.C.
D. galeata UK          .......... ...C.T.... G......... .......... T.T.....C.
D. galeata CZ          .......... ...C.T..T. .......... .......... T.T.....C.
D. galeata GER         .......... ...C.T..T. .......... .......... T.T.....C.
D. mendotae IN         .......... ...C.T.... .......... .......... T.T.....C.
D. mendotae OR         .......... ...C.T.... .......... .......... T.T.....C.
D. mendotae ON         .......... ...C.T.... .......... .......... T.T.....C.
D. cucullata GER       .......... ..TC...... .......... .......... T.....A.C.
D. rosea BC            .......... .T...T.... .......... .......... .G......C.
D. rosea IN            .......... .T...T.... .......... .......... .G......C.
D. laevis ON           .......... .TTA.....T .......... .......... T.....A...
D. dubia ON            .....G.... ..T.....GC .......... .......... T.G...A...
D. dubia WI            .....G.... .TT......C .......... .......... T.G...A...
D. longiremis          .......... .TTC.T.... .......... .......... C.T...AA..
D. pulex               ...T.A.TG. ..T.TT.... .......... .......... ..G...AA..
D. magna               .....ATTT. .TTC.T.... .......... .......... ......A..A
D. lumholtzi           ...TAATTC. .TT....... .......... .......... ......A.AA
```

FIG. 1. Sequence alignment from a 525-bp sequence of the 12S rDNA gene in 24 *Daphnia* taxa. The nucleotide sequence of *Daphnia longispina* is shown with "." representing identical nucleotides, "?" representing an undetermined nucleotide, and "-" representing a gap. Individuals were from populations identified in Table 2.

found because widely distributed populations and forms of all the well-recognized taxa (both genetically and morphologically) are represented in the data. There are some other morphological forms (e.g., *Daphnia gessneri*) that may be valid species, but they are clearly affiliated with one of the major lineages we have defined. This is also true of Hobæk and Wolf's (1991) *D. umbra*-like melanic "*longispina*" defined by allozymes, and the transparent, Norwegian *D. longispina* from alpine humic ponds. This transparent *D. longispina* of humic ponds and our Polish *D. longispina* match in habitat preference (humic mountain ponds), they share several unique electromorphs (we used the same electrophoretic standard as Hobæk and Wolf, 1991), and their body/head shapes are identical (Fig. 5). Similarly, the nonhumic habitat, allozyme array, and geographic distribution indicates that *D. umbra* and Palearctic pigmented *D.* "*longispina*" (Hobæk and Wolf, 1991; Taylor and Hebert, 1994) are closely related.

An *a posteriori* assessment of the few traditional morphological characters used in *Daphnia* systematics is possible if the concordant relationships inferred from our 12S rDNA and nuclear genes reflect species relationships. Our molecular phylogeny is in agreement with the conventional higher level relations within the genus. The *longispina* group appears monophyletic with respect to *D. pulex* and the subgenus *Ctenodaphnia*. However, a robust test of the monophyly of *longispina* awaits comparison of enigmatic species that possess combinations of *pulex* and *longispina* characters (e.g., *Daphnia curvirostris*). A more certain pattern is the polyphyly of the *longispina* complex. At least three clades possess both helmeted and unhelmeted taxa, suggesting that this character is susceptible to convergent evolution. This similarity transcends mere possession of a helmet, as the shape of these extensions is also remarkably similar among lineages. For example, several populations of the *D. galeata* lineage possess similar helmet shapes to the *D. laevis–dubia* lineage (see Brooks, 1957b). Where these lineages coexist in the same waterbody, Brooks (1957b) reported that the helmet shapes are even more alike. As there is no evidence for introgression in these populations (here and D. Taylor unpublished), the similarity in helmet shapes be-

```
                                                                          250
Daphnia longispina POL CTCTAACTCG AAAACATTCT CTAAAAGTGA TCTGACAACG GCGGTATACA
D. umbra IGLK          ..T.G.T.T. .......... ........A. .......... ..........
D. umbra RB27          ..T.G.T.T. .......... ........A. .......... ..........
D. thorata MT          T.T.....T. .......... .......... .......... ..........
D. rosea SLV           T.T...T.T. .......... ...G....A. .......... ..........
D. rosea ITA           T.T...T.T. .......... ...G....A. .......... ..........
D. hyalina GER         T.T...T.T. .......... ...G....A. .......... ..........
D. hyalina AUS         T.T...T.T. .......... ...G....A. .......... ..........
D. galeata UK          T.T.....T. .......... ........A. .......... ..........
D. galeata CZ          T.T.....T. .......... ........A. .......... ..........
D. galeata GER         T.T.....T. .......... ........A. .......... ..........
D. mendotae IN         T.T.....T. .......... ........A. .......... ..........
D. mendotae OR         T.T.....T. .......... ........A. .......... ..........
D. mendotae ON         T.T.....T. .......... ........A. .......?.. ..........
D. cucullata GER       T.......T. .......... ........A. .......... ..........
D. rosea BC            T.T.....T. .......... .......... .......... ..........
D. rosea IN            T.......T. .......... .......... .......... ..........
D. laevis ON           T.T.TT..T. .......C.. T.T.G...A. .......... ..........
D. dubia ON            A.T.TT..T. .......C.. TAT.G...A. .......... ..........
D. dubia WI            A.T.TT..T. .......C.. TAT.G...A. .......... ..........
D. longiremis          A.A.TTTCT. .......C.. A.G.G...A. .......... ..........
D. pulex               A.T.TT.CT. .....G.... T.T.....A. .......... ..........
D. magna               A.T.T.T-A. .......C.. T.TGG...A. .......... .A........
D. lumholtzi           T.T.T.T.AA .......C.. T.T.G...A. .......... .A........

                                                                          300
Daphnia longispina POL AGCTTTACAA GTAGAGGAAA GATAAAACGG GGACTATCGA TTAAGGGACA
D. umbra IGLK          .......... .A..G..... .......... .......... .......G..
D. umbra RB27          .......... .A..G..... .......... .......... .......G..
D. thorata MT          ....C..... AC........ .......... ..?....... ..........
D. rosea SLV           ....C..... AC........ .......... .......... .......G..
D. rosea ITA           ....C..... AC........ .......... .......... ..........
D. hyalina GER         ....C..... AC........ .......... .......... .......G..
D. hyalina AUS         ....C..... AC........ .......... .......... .......G..
D. galeata UK          .......... A.GA...... .........A .......... ..........
D. galeata CZ          .......... A.GA...... .........A .......... ..........
D. galeata GER         .......... A.GA...... .........A .......... ..........
D. mendotae IN         .......... A.GA...... .........A .......... ..........
D. mendotae OR         .......... A.GA...... .........A .......... ..........
D. mendotae ON         .......... A.GA...... .........A .......... ..........
D. cucullata GER       .....C.... A.GAG..... .........A .......... ...C...G..
D. rosea BC            ....C..... AC........ .......... .......... ..........
D. rosea IN            ....C..... AC........ .......... .......... ..........
D. laevis ON           ....A...T. ..TA...T.. .........A ..T....... ...T...G..
D. dubia ON            ....CA..G. ...A...T.. .........A ..C....... ...TA..G..
D. dubia WI            ....CA..G. ...A...T.. .........A ..C....... ...TA..G..
D. longiremis          ....AA.... AAGA...T.. .........A .......... .......G..
D. pulex               .....A.... AA.AG..T.. .......T.. ........A. ...TA.....
D. magna               ....AA.... A.GA...T.. .........A ..GT...... ...TAA....
D. lumholtzi           .....C.... ...A...T.. .......... .......... ...TAAT...

                                                                          350
Daphnia longispina POL AGCTCCTCTA TTTGGATAAA GTGCCGCCAA AATCTTTGGG TTTGAAGAAC
D. umbra IGLK          .......... .......G.. .......... .......... ..........
D. umbra RB27          .......... .......G.. .......... .......... ..........
D. thorata MT          .......... .........? .......... .......... ..........
D. rosea SLV           .......... .......... .......... .......... ..........
D. rosea ITA           .......... .......... .......... .......... ..........
D. hyalina GER         .......... .......... .......... .......... ..........
D. hyalina AUS         .......... .......... .......... .......... ..........
D. galeata UK          .......... .......... .......... .......... ..........
D. galeata CZ          .......... .......... ..?....... .......... ..........
D. galeata GER         .......... .......... .......... .......... ..........
D. mendotae IN         .......... .......... .......... .......... ..........
D. mendotae OR         .......... .......... .......... .......... ..........
D. mendotae ON         .........C .......... .......... .......... ..........
D. cucullata GER       .......... ..G....... A......... .......... ..........
D. rosea BC            .......... .......... .......... .......... ..........
D. rosea IN            .......... .......... .......... .......... ..........
D. laevis ON           .......... .....T.... .......... .......... ..........
D. dubia ON            .......... .....T.... .......... .......... ..........
D. dubia WI            .......... .....T.... .......... .......... ..........
D. longiremis          .......... .......... A......... .......... .........T
D. pulex               .......... ..A......G A......... .......... ..........
D. magna               .......... .....T..G. A......... ..C....... ..........
D. lumholtzi           .........G .....T..G. A......... .......... ..........

                                                                          400
Daphnia longispina POL AACTTTTACT ACCCTATCT- -AACTATTGC TCTGAATAAC AGGGTATCTA
D. umbra IGLK          .......... .....G.... ........A. .AA....... ..........
D. umbra RB27          .......... .....G.... ........A. .AA....... ..........
D. thorata MT          .......... .....G.... ...A...AA. .TA....... ..........
D. rosea SLV           .......... .....G.... ...A...AA. ..A....... ..........
D. rosea ITA           .......... .....G.... ...A...AA. ..A....... ..........
D. hyalina GER         .......... .....G.... ...A...AA. ..A....... ..........
D. hyalina AUS         .......... .....G.... ...A...AA. ..A....... ..........
D. galeata UK          .......... .....G.T.. ...GA..AA. .GA....... ..........
D. galeata CZ          .......... .....G.T.. ...GA..AA. .GA....... ..........
D. galeata GER         .......... .....G.T.. ...GA..AA. .GA....... ..........
D. mendotae IN         .......... .....G.T.. ...GA..AA. .GA....... ..........
D. mendotae OR         .......... .....G.T.. ...GA..AA. .GA....... ..........
D. mendotae ON         .......... .....G.T.. ...GA..AA. .GA....... ..........
D. cucullata GER       .......... .......... ...GA..AA. .GA....... ..........
D. rosea BC            .......... .....G.... ...A...AA. .TA....... ..........
D. rosea IN            .......... .....G.... ...A...AA. .TA....... ..........
D. laevis ON           .......... .......T.. T..G...GAT .TA....... ..........
D. dubia ON            .......... .......T.A A..AC..AAT .TA.....G. ..........
D. dubia WI            G......... .......T.A A..AC..AAT .TA.....G. ..........
D. longiremis          .......... .......T.. ..TT....AT .TA....... ..........
D. pulex               .......... .......T.. AC......AT .TA....... ..........
D. magna               G......... ........-. ..C......T AGA....... ..........
D. lumholtzi           .......... .......T-. ...TG..GAT AAA....... ..........

                                                                          450
Daphnia longispina POL ATCCTGGTTT TTGACCGAAG TTTTTTA-GC CCCTCTAAAT TTTTAAAGAA
D. umbra IGLK          .......... .AA.T..... .........T .....C.... ..........
D. umbra RB27          .......... .AA.T..... .........T .....C.... ..........
D. thorata MT          .......... .AA.T.A... ........AT T...A.T... ...A......
D. rosea SLV           .......... .AA.T.AG.. ........AT T...A.T... ..........
D. rosea ITA           .......... .AA.T.AG.. .........T T...A.T... ..........
D. hyalina GER         .......... .AA.T.AG.. ........AT T...A.T... ..........
D. hyalina AUS         .......... .AA.T.AG.. ........AT T...A.T... ..........
D. galeata UK          .......... .AA.T.A... .........T T...ACT... ..........
D. galeata CZ          .......... .AA.T.A... .........T T...ACT... ..........
D. galeata GER         .......... .AA.T.A... .........T T...ACT... ..........
D. mendotae IN         .......... .AA.T..... .........T T...ACT... ..........
D. mendotae OR         .......... .AA.T.A... .........T T...ACT... .........G
D. mendotae ON         .......... .AA.T.A... .........T T...ACT... .........G
D. cucullata GER       .......... .AA.T.A... .........T ....AGT... ...A......
D. rosea BC            .......... .AA.T.A... ........AT T...A.T... ...A......
D. rosea IN            .......... .AA.T.A... ........AT T...A.T... ...A......
D. laevis ON           .......... A.CCT.T..A C...C....T T...A..... .A.A......
D. dubia ON            .......... A.T...T..A C...C...AT T..AA..G.. .A.AG.....
D. dubia WI            .......... A.T...T..A C...C...AT T..AA..G.. .A.AG.....
D. longiremis          .......... ..A.T.C..A .........T .....CG... ......GAT.
D. pulex               .......... ..T...T..A .........T .T.AAC.G.. ..C.T.....
D. magna               .......... ...G..CCTT .......TAT ....TC.... AA.......T
D. lumholtzi           .......... ..T.T.TTTA C.......AT T...TA.... A.A.T....T

                                                                          500
Daphnia longispina POL ACAGTGGCCT TTTTAACTGA TTTCACCC-A ACAAAAAGGC TAGATAA-CT
D. umbra IGLK          ....CA..AC ...AT..... ...T...... CT.....A.. ..........
D. umbra RB27          ....CA..AC ...AT..... ...T...... CT.....A.. ..A.......
D. thorata MT          .G..AAA.TC ...AT..... .......... .......A.. ..........
D. rosea SLV           GG.AAAA.TC ...AT..... .......... .......A.. ..A.......
D. rosea ITA           GG.AAAA.TC ...AT..... .......... .......A.. ..A.......
D. hyalina GER         GG.AAAA.TC ...AT..... .......... .......A.. ??????????
D. hyalina AUS         GG.AAAA.TC ...AT..... .......... .......A.. ..A.......
D. galeata UK          GA.AGA..T. C..AT..... .......... .......A.. ..AGA.....
D. galeata CZ          GA.AGA..T. C..AT..... .......... .......A.. ..AGA.....
D. galeata GER         GA.AGA..T. C..AT..... .......... .......A.. ..AGA.....
D. mendotae IN         GA.AGA..TC C..AT..... .......... .......A.. ..AGA.....
D. mendotae OR         GA.AGA..T. C..AT..... .......... .......A.. ....A.....
D. mendotae ON         GA.AGA..T. C..AT..... .......... .......A.. ....A.....
D. cucullata GER       GA.AG.A.T. ...AT.T... .......... C......A.. C.AGA.....
D. rosea BC            .A..AAA.TC ...AT..... .......... .......A.. ..........
D. rosea IN            .G..AAA.TC ...AT..... .......... .......A.. ..........
D. laevis ON           TT.T.A.TA. C..AT.TAA. .......T.. .A...G.TAT A.T.......
D. dubia ON            .T.T.AA.A. ...AT.TAA. .......T.T TG.....TAA A.T.C.....
D. dubia WI            .T.T.AA.A. ...AT.TAA. .......T.T TG.....TAA ..T.C.....
D. longiremis          .TTACAT.TC ...A..TAA. .......T.- -..G...A.A A.T-A....A
D. pulex               TTT--CC.T. AAA.T.AAA. .......TT. T...C..AAT .TC.CC.T..
D. magna               .ATT.ACTTC ...AT.TAA. .......TA. .A...G.AAA ..ATAT....
D. lumholtzi           .TTT.TTTTC C..G...AA. .......TA. .A...G.AAT ACAT.T....

                                                 525
Daphnia longispina POL TAGAA--AAT TCAAC--GTT AACTA
D. umbra IGLK          CTA.G..... .......... ...A.
D. umbra RB27          CTA.G..... .......... .....
D. thorata MT          .?A....... .T........ .....
D. rosea SLV           ..A......? ?????????? ?????
D. rosea ITA           .CAG...... .T........ ...A.
D. hyalina GER         ?????????? ?????????? ?????
D. hyalina AUS         .?A....... .T........ .....
D. galeata UK          .CA....... .G........ .????
D. galeata CZ          .CA....... .G........ .....
D. galeata GER         .CA....... .G........ .????
D. mendotae IN         .CA....... .G.....??? ?????
D. mendotae OR         .CA....... .G???????? ?????
D. mendotae ON         .CA....... .G........ .....
D. cucullata GER       .CA....... .A...TC... ...??
D. rosea BC            .CA....... .T........ .....
D. rosea IN            ..A....... .T........ .....
D. laevis ON           A.TCT..... C.???????? ?????
D. dubia ON            ATATT..... AT...????? ?????
D. dubia WI            ATATT..... CT........ .....
D. longiremis          A.T....... .T..T..... .....
D. pulex               .TT.CGAG.. CG..T..... .....
D. magna               C.A..TC.-. .T.CT..... ..T..
D. lumholtzi           ..A..AT.-. CT.C...... ..T..
```

FIG. 1—*Continued*

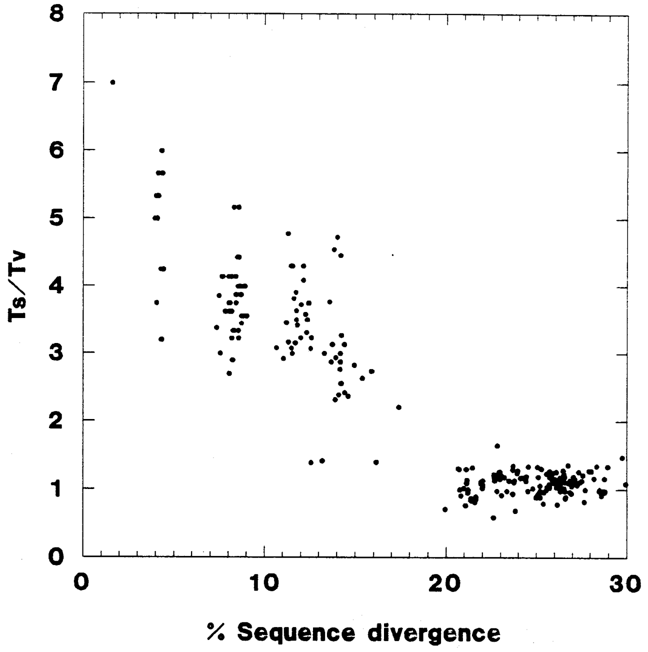

FIG. 2. Plot of TS/TV ratios and all pairwise sequence divergences (Kimura's two-parameter measure with pairwise deletion of gaps and missing data) among 24 taxa of *Daphnia*. Pairwise comparisons of less than 1.5% sequence divergence were excluded because few substitutions (6 cases) or no transversions were observed (11 cases).

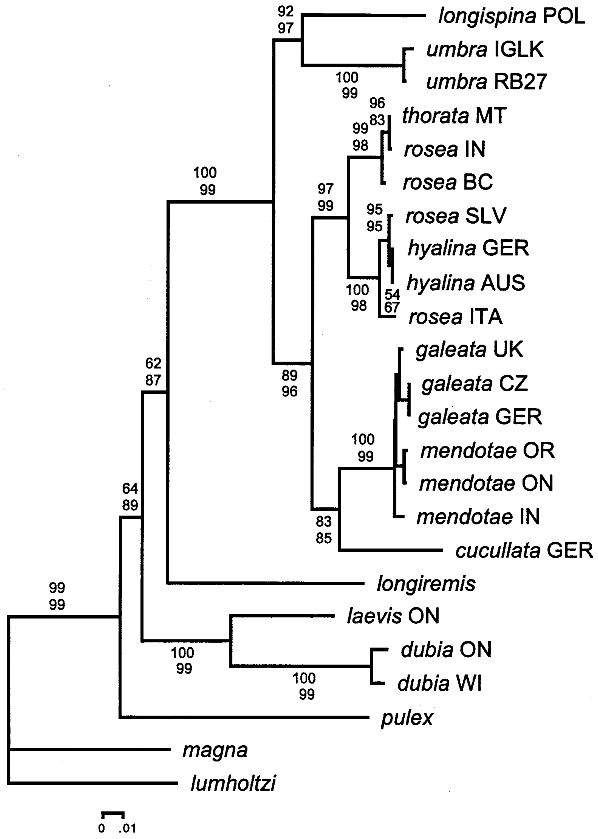

FIG. 3. A phylogenetic tree constructed using the neighbor-joining method (NJ) based on Kimura's two-parameter distances (scale bar) among 24 *Daphnia* taxa. The distances were calculated from mitochondrial 12S rDNA sequences (525 aligned sites) and the NJ was rooted with *Daphnia magna* and *Daphnia lumholtzi*. The upper number of the branches represents the bootstrap confidence limit (1000 replicates with distances based on pairwise deletion of gaps and missing sites) and the lower number represents the confidence probability (based on distances with complete deletion of gaps and missing data). Both values were calculated in MEGA (Kumar *et al.*, 1993).

tween these distantly related lineages is probably due to convergent or parallel evolution of reaction norms.

Another character commonly used to delineate the *longispina* complex, dark bands on the swimming hairs (Hrbácek, 1987), is paraphyletic according to our data. *D. rosea, D. longispina,* and *D. umbra* possess these bands, whereas lacustrine species in this clade lack this character. Indeed, both this character and neckteeth are rendered phylogenetically uninformative in the *longispina* group by their presence in the *D. pulex* group (see Brooks, 1957a).

The lack of morphological evolution in the *longispina* group and the prevalence of interspecific hybrids has been interpreted as indicating a young age (Pleistocene) for much of the group (Flössner and Kraus, 1986). Schwenk (1993) later provided cytochrome b sequence evidence from three species (*D. cucullata, D. g. galeata,* and *D. hyalina*) and the *longispina* radiations are >5 MY. Our 12S rDNA clock calibration of the entire *longispina* group extends the estimated age of the group to over 100 MY. These calibrations possess wide confidence limits but the time scales of the alternate hypotheses we are testing (e.g., vicariance of North America and Europe vs recent dispersal) are also large. Another unusual aspect of the mtDNA evolution is the saturation of transitions in this usually slowly evolving gene (Lynch and Jarrell, 1993) within a subgenus. The saturation of this gene within the subgenus *Daphnia* is likely another indicator of the old age and relative morphological stasis of the group. The two oldest lineages within the group are *D. laevis* and *D. longiremis*. The *laevis* lineage is common in South America, Africa, and the southern half of North America (Brooks, 1957a; Green, 1990). The geographic distribution and estimated time of >100 MYA divergence from other members of the *longispina* group are consistent with a Gondwonaland origin for *D. laevis*.

The age of many other lineages was more unexpected. For example, although the sister group relationship of *dubia* and *laevis* was supported, the 30-MY separation was surprising. *D. dubia*, an endemic of eastern North America glacial lakes was thought to represent a clear example of a postglacial (ca. 10,000 YA) speciation from pond populations near the periphery of the *D. laevis* distribution (Brooks, 1957a). Nevertheless, the mtDNA clock, the numerous allozyme sub-

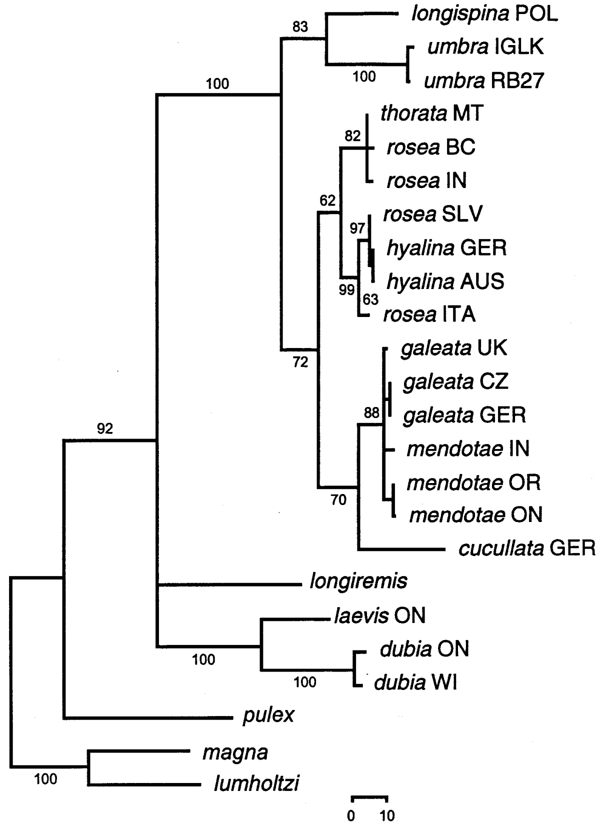

FIG. 4. A boostrap 50% majority-rule consensus tree resolved from an heuristic search with steepest descent option in PAUP of 12S rDNA nucleotide variation in 24 *Daphnia* taxa. Transitions are weighted equal to transversions. The branch lengths are drawn proportional to the number of steps supporting each branch and the scale bar indicates the length of 10 such steps. The bootstrap values (after 200 replications) are shown on the branches. Nine shortest trees were found (length 575, CI = 0.675, RI = 0.756).

stitutions (Appendix), and the near saturation of transitions between these species indicates that the separation occurred much before the last Wisconsinan glaciation. The *dubia–laevis* split therefore may represent a case where present geographic distributions are misleading in recreating speciation scenarios. It is also possible that the *D. dubia* lineage is derived from the sweepstakes dispersal of some unexamined South American *laevis*-group species such as *D. gessneri.*

Another surprising result is the lack of 12S rDNA sequence divergence between North American and European populations of *D. galeata.* It was recently thought that *D. galeata* was either cosmopolitan (Glagolev, 1986) or perhaps separated by vicariance of North America and Eurasia (Frey, 1987). Allozyme studies have now confirmed that *D. galeata* is separated genetically into North American (*mendotae*) and Eurasian groups (*galeata;* Taylor and Hebert, 1993b), but the lack of mtDNA divergence between European and North American *D. galeata* suggests that the present distribution is the result of recent natural dispersal rather than a vicariance event from the continental separation. The results are consistent with Brooks' (1957a) suggestion that *D. g. mendotae* are derived from postglacial dispersal of *D. g. galeata* stock from Siberia or Alaska.

In contrast to *D. galeata,* European and North American populations of *D. rosea* were more divergent than expected. Ever since Brooks (1957a) united North American *longispina* with Eurasian *D. rosea,* this species has often been regarded as cosmopolitan (see Hrbácek, 1987). Yet both allozyme and mtDNA evidence indicate a genetic split between North America and Eurasia. The split is so great that North American and European *D. rosea* are not sister taxa. It should also be noted that ecological differences exist between continents—Eurasian *D. rosea* are often described as arctic-alpine pond dwellers (Flössner, 1972), whereas North American *D. rosea* are strictly temperate and subalpine in distribution (Taylor and Hebert, 1994). Given this broad genealogical concordance between continents, a taxonomic change is warranted (Avise and Ball, 1990). We recommend that the original name of North American *"D. rosea"—Daphnia dentifera* Forbes 1893—be resurrected.

Given the lack of morphological resolution, it is unsurprising that species of the *longispina* group were often united as one before the treatise of Brooks (1957a). Even today the morphological characters (e.g., helmets, neckteeth, spine and seta lengths, homoglobin levels, cuticular pigmentation) used to define species in the group are environmentally induced (e.g., Jacobs, 1961). The minimum forms displayed in lab cultures and at low temperatures in nature exhibit striking morphological uniformity. Morphological evolution, then, in the *longispina* group consists of modification to the norms of reaction because the basic *longispina* morphology has remained intact for over 100 MY (for depictions of phenotypic variability in the group see Fig 4). Fryer (1991) proposed that the evolutionary constancy of the internal body structure in daphniids is maintained by complex functional constraints. But the retention of similar carapace and head shape is surprising in light of the potential for rapid phenotypic evolution of cyclic parthenogens (Lynch and Spitze, 1994) and certainly results from some constraint or cost involving the inducible structures (Jacobs, 1987).

Phylogeographic Patterns

The historical trend of cladoceran biogeography has been from cosmopolitanism to endemism, and from dispersalism to vicariance associated with allopatric speciation (Frey, 1987; Hebert and Wilson, 1994). Our molecular data clearly support endemism in the *longispina* group, but there is little evidence for the primacy of vicariant speciation. Endemism is supported by the genetic division (allozyme and/or mtDNA) of several related groups at geographic boundaries. These include the North America–Eurasia split of *D. galeata*

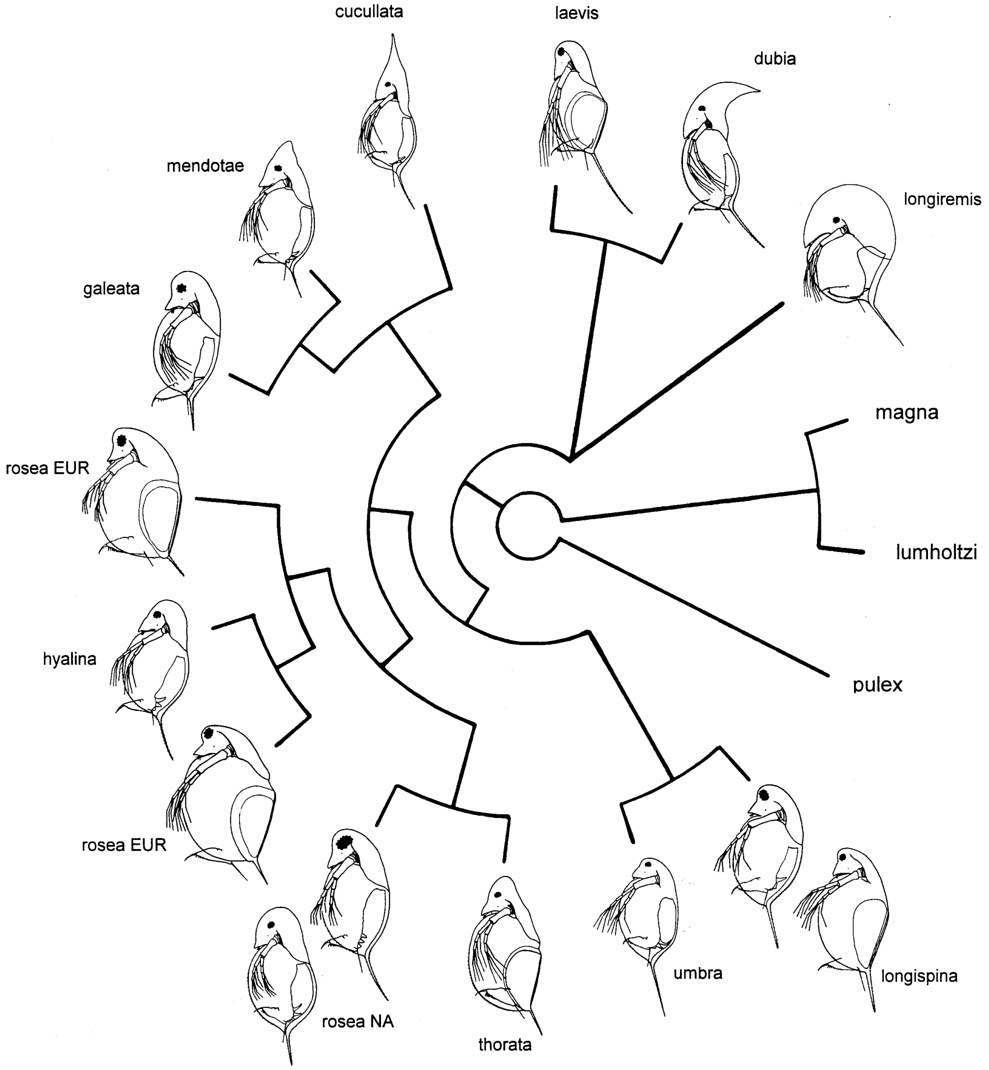

FIG. 5. The single most parsimonious tree found using branch-and-bound search in PAUP (length = 563 steps, CI = 0.65, RI = 0.64). The relationship between *D. longiremis* and *D. laevis/D. dubia* was made polytomous to reflect its lack of resolution upon bootstrapping. *Daphnia* drawings are camera lucida representations of mature females in lateral view. The maximum inducible cephalic extension that is typical of each lineage is shown—uninduced body shapes show striking similarity. The inner North American *D. rosea* is a typical pond form, whereas the outer specimen is found in small lakes. The inner *D. longispina* is from Toporowy Staw Poland and is similar in form to the outer transparent Norwegian *D. longispina* studied by Hobæk and Wolf (1991).

and *D. rosea* as well as the north–south split of *D. laevis* and *D. dubia* within North America. Yet in each case the estimated time of genetic separation of these groups strongly disagrees with the proposed vicariance event. Such discrepancies could indicate that geographic speciation occurred before or after dispersal across geographic barriers or that other evolutionary processes explain speciation patterns in *Daphnia.*

In the subgenus *Daphnia,* a common phylogeographic pattern is the association of habitat shifts with speciation. There are several examples of *Daphnia* sister taxa (relationships based on morphology and allozymes) that live sympatrically in broad habitat mosaics often involving a pond–lake separation (e.g., *Daphnia retrocurva–Daphnia parvula, D. pulex–Daphnia pulicaria,* and *Daphnia catawba–Daphnia minnehaha*). In the *D. longispina* group, the lake/pond contrast is apparent in three sister species associations (*hyalina/rosea, thorata/* N.A. *rosea, dubia/laevis*), while other species (probably related to *D. longispina* and *D. umbra* in this study) are separated by the humic content of the ponds (Hobæk and Wolf, 1991). In each case, the sister species are separated ecologically at the waterbody level and their geographic distributions overlap in broad areas of neighboring sympatry (Brooks, 1957a; Hrbácek, 1987; Edmondson and Litt, 1981; Hobæk and Wolf, 1991). Exceptions to this pattern are the *cucullata/galeata* and *longiremis/cristata* pairs for which

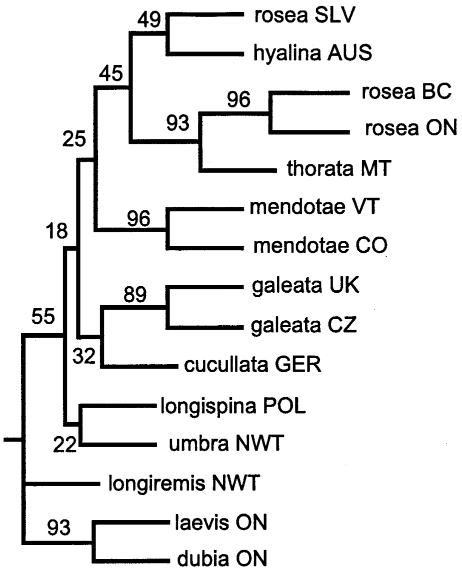

FIG. 6. A bootstrap majority-role consensus tree using neighbor-joining of chord distances (Cavalli-Sforza and Edwards, 1967) calculated from 21 presumptive allozyme loci of the *D. longispina* group. Branch numbers represent the bootstrap values (after 100 replicates). Populations are the same as those used for mtDNA sequences in Table 2 with the following exceptions: *D. rosea* ONT and *D. g. mendotae* data were from Taylor and Hebert (1994) and *D. cucullata* are from Plußsee, Germany.

species often coexist in the same waterbody. Yet, even in these cases, there is strong seasonal or spatial ecological differentiation between the sister species (Hrbácek 1987; Spaak and Hoekstra, 1995).

Divergent selection as a result of such habitat shifts is critical to the founder effect speciation model. However, it remains unclear just how prevalent founder effects are in *Daphnia* populations. Our observation that many sister species live sympatrically in broad habitat mosaics (not in isolated, remote waterbodies) and that many of these taxa produce copious amounts of propagules suggests that opportunities for founder events are rare. Given this information, and the recent lack of experimental and theoretical support for this mode of speciation (Rice and Hostert 1994; Moya *et al.*, 1995), other models might be invoked to account for the phylogeographic patterns in the subgenus *Daphnia*. One candidate is neighboringly sympatric speciation (Grant, 1977). Both models require strong divergent selection, but the neighboringly sympatric speciation model differs in that restricted gene flow is permitted and founder effects are not invoked.

TABLE 3

Estimates of *Daphnia* Clade Ages Based on Two Methods of mtDNA Clock Calibration for the 12S rRNA Gene

	Divergence time estimates (MYA)	
Clade	Brower (1994)	Lynch and Jarrell (1993) with 95% CL
thorata / North American *rosea*	0.3	0
galeata / *mendotae*	0.4	0
hyalina / European *rosea*	0.6	0
European *rosea* / North American *rosea*	1.8	0
cucullata / *hyalina* / *galeata* / *rosea* / *thorata*	4.1	6 (4–24)
laevis / *dubia*	n.a.	30 (17–126)
laevis / *longispina* group	n.a.	140 (79–598)
pulex/ *longispina* group	n.a.	167 (94–713)
Ctenodaphnia / *longispina* group	n.a.	175 (99–751)

Note. The calibration of Brower (1994) was used only with sequence divergences of less than 10%, as this clock may be nonlinear beyond this value.

Hybridization may also lead to speciation as our results suggest that *D. g. mendotae* is a stabilized introgressant. Joint analysis of multiple species-specific allozyme, mtDNA RFLP, and morphological markers have clearly indicated widespread hybridization between North American *D. rosea* and *D. g. mendotae* (Taylor and Hebert, 1992, 1993a). The resulting hybrids are often more abundant than the parent taxa in a lake, providing good conditions for backcrossing. Proposed introgressants are distributed throughout the North American range of *D. g. mendotae*, with nuclear gene flow being unaccompanied by mtDNA gene flow (Taylor and Hebert, 1993a, Taylor, unpublished). Our finding here of a lack of mtDNA sequence divergence between *D. g. mendotae* and *D. g. galeata* together with their great divergence at allozyme loci (where this divergence actually causes *D. g. mendotae* to group with *D. rosea*, Fig. 1) supports the nuclear introgression hypothesis. Moreover, the spatial pattern of North American *D. rosea* being more similar to sympatric *D. g. mendotae* than to allopatric *D. g. galeata* at six loci (IDH-2, GPI, sAAT, mAAT, PGM, and AO, see Taylor and Hebert, 1993a–c) is unpredicted by the alternate hypotheses of symplesiomorphy or convergence. Our phylogenies also show that the widespread presence of the PEP-D^c and mAAT^a alleles in *D. g. mendotae* (Taylor and Hebert, 1993a–c) is consistent only with introgression as they are otherwise restricted to the *D. rosea* clades. Other proposed introgressed alleles (sAAT^c and the high frequency of GPI^i) lose shared ancestry as an explanation if the *D. umbra/D. longispina* clade groups with the *rosea/hyalina/thorata* clade. Such a topology is not significantly different than the shortest MP tree. The adaptive significance of these introgressed genes is unknown, but the observation that *D. g. mendotae* is the most morphologically variable and ecologically successful *Daphnia* species in temperate North American lakes (Brandlova *et al.*, 1972; Carter *et al.*, 1980; Keller and Pitbaldo, 1989) may be partially due to this introgression.

Our evidence indicates that *D. g. mendotae* is a dispersed introgressant, but evidence of reproductive isolation is needed for a speciation scenario. The genetic outcome of recent anthropogenic introductions of Eurasian

D. g. galeata into the Laurentian Great Lakes may provide such evidence as hybrid breakdown is evident between *D. g. galeata* and *D. g. mendotae* (Taylor and Hebert, 1993b). This evidence suggests that postzygotic reproductive isolating mechanisms are acting. The apparent speciation of *D. g. mendotae* from *D. g. galeata* may also be due to allopatric speciation, but the lack of mtDNA divergence between these groups indicates that speciation occurred rapidly, making a quantum speciation mode such as hybrid speciation more likely.

It remains unclear if any of the European *longispina* group species are stabilized introgressants, Schierwater *et al.* (1994) suggested that a discordance they found between a RAPD tree (where *D. galeata* and *D. hyalina* were sister species) and a mtDNA tree might indicate reticulate evolution. Our differing result of nuclear–mtDNA tree concordance may be due to a sampling artifact at some level or to differences in the analysis we used. Another possibility is that there is increased success of introgressants in North America resulting from the differing age of hybrid systems and ecological space available. In North America, the *D. rosea* lineage is restricted to ponds and small lakes throughout much of North America (Brooks, 1957a,), whereas this lineage is very successful in European lakes and often cooccurs with *D. galeata*. It is possible that the success of the introgressant *D. g. mendotae* is due to refugial hybridization with North American *D. rosea* in Beringia followed by dispersal into a mosaic of postglacial North American lakes. In postglacial North America there was likely greater niche space available for recombinants than in Eurasian lakes where habitat differentiation between lineages was weak.

Our data support Lynch and Spitze's (1994) contention that complete reproductive isolation is associated with genetic divergence. All of the naturally hybridizing taxa in the *longispina* group (*D. cucullata, D. g. galeata, D. g. mendotae, D. hyalina,* and *D. rosea*) occur within a clade that we estimate has diverged less than 6 MYA. Apparently complete reproductive isolation requires considerable time in *Daphnia,* but genetic isolation, particularly cytoplasmic isolation, seems to occur much earlier in the speciation process (Schwenk, 1993; Taylor and Hebert, 1993a).

Conclusions

After nearly 30 years of study, the Cold Spring Harbor genetics group led by A. M. Banta (1939) concluded that there are probably only three species of *Daphnia* (*magna, pulex,* and *longispina*) but that a further study "would be of fundamental evolutionary importance" and that future investigators should have long-lived forebears. In this paper we show that, with careful sampling, joint analyses of mitochondrial and nuclear genetic variation can elucidate both the long-standing systematics problems of *Daphnia* and the key evolutionary processes that created this phenotypic phantasmagoria. Our evidence suggests that the *D. longispina* group contains five major species complexes that have retained the *longispina* body plan for an estimated 100 MY. Convergence of helmet shapes in two lineages adds further to the species problem. Speciation probably occurs by several different modes in *Daphnia* (Hebert and Wilson, 1994), but here we provide evidence that neighboringly sympatric speciation and hybrid speciation warrant more attention in this group.

APPENDIX: ALLELE FREQUENCIES AT 21 ALLOZYME LOCI FOR THE *Daphnia longispina* GROUP

	Taxon														
Locus	*mendotae* VT	*mendotae* CO	*galeata* UK	*galeata* CZ	*hyalina* AUS	*rosea* SLV	*rosea* BC	*rosea* ONT	*thorata* MT	*umbra* NWT	*longiremis* NWT	*laevis* ONT	*dubia* ONT	*longispina* POL	*cucul.* GER
mAAT															
A	.000	.000	.000	.000	.000	.000	1.000	1.000	1.000	.000	.000	.000	.000	.000	.000
B	1.000	.962	1.000	1.000	1.000	1.000	.000	.000	.000	1.000	.000	1.000	1.000	.000	1.000
C	.000	.038	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
D	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.000
E	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.000	.000	.000	.000
sAAT															
A	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000
B	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	1.000	.000	.000
C	.920	.500	.000	.000	1.000	.000	1.000	1.000	1.000	.000	.000	.000	.000	1.000	.000
D	.080	.500	1.000	1.000	.000	1.000	.000	.000	.000	1.000	1.000	.000	.000	.000	.000
ACOH-1															
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.000	1.000	1.000	1.000	1.000
B	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.000	.000	.000	.000
AO															
A	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.000	.000	.000	.000
B	.000	.000	.000	.000	.000	.000	.000	.179	.000	.000	.000	.000	.000	.000	.000
C	.000	.000	.000	.000	1.000	1.000	1.000	.821	1.000	.000	.000	.000	.000	.000	.000
D	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.000	.000
E	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.000
F	1.000	1.000	1.000	1.000	.000	.000	.000	.000	.000	1.000	.000	1.000	.000	.000	1.000

APPENDIX—*Continued*

	Taxon														
Locus	*mendotae* VT	*mendotae* CO	*galeata* UK	*galeata* CZ	*hyalina* AUS	*rosea* SLV	*rosea* BC	*rosea* ONT	*thorata* MT	*umbra* NWT	*longiremis* NWT	*laevis* ONT	*dubia* ONT	*longispina* POL	*cucul.* GER
APK															
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.975	1.000	1.000
B	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.025	.000	.000
FUMH															
A	.000	.063	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
B	1.000	.937	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.000	1.000	1.000
C	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.000	.00
GAPDH															
A	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.000	.000	.000	.000
B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.000	1.000	1.000	1.000	1.000
GPI															
A	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.016
B	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.000	.000	.000	.000
C	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.000	.000	.516
D	.000	.000	.000	.000	.000	.159	.000	.000	.000	.000	.000	.000	.000	.000	.000
E	.000	.000	.000	.000	.000	.000	.000	.000	.012	.000	.000	.000	.000	.000	.000
F	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.370	.000	.000
G	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.630	.000	.000
H	.240	.000	1.000	.944	1.000	.841	.000	.000	.000	1.000	.000	.000	.000	.000	.453
I	.760	.915	.000	.000	.000	.000	1.000	1.000	.988	.000	.000	.000	.000	1.000	.016
J	.000	.085	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
K	.000	.000	.000	.056	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
IDH-1															
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.000	1.000	1.000	1.000	1.000
B	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.000	.000	.000	.000
IDH-2															
A	.000	.000	1.000	1.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000
B	1.000	1.000	.000	.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.000
LDH															
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.000	1.000	1.000	.000	1.000
B	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.000	.000	.000	.000
C	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.000
MDH-1															
A	.000	.000	1.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
B	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	1.000	.000
C	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	1.000	.000	.000	.000
D	1.000	1.000	.000	1.000	1.000	1.000	1.000	1.000	.000	1.000	.000	.000	.000	.000	1.000
E	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.000	.000	.000	.000	.000	.000
MDH-2															
A	.000	.000	.000	.114	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
B	1.000	1.000	.000	.841	1.000	1.000	1.000	1.000	1.000	1.000	.000	.000	.000	1.000	1.000
C	.000	.000	1.000	.045	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
D	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.529	.000	.000
E	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.471	.000	.000
F	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.000	.000	.000	.000
MDHP-2															
A	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.000	.000	.000	1.000	.000
B	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.000	.000	.000
C	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.000	1.000	.000	.000	.000	1.000
D	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.000	.000
MPI															
A	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000
B	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.000	.000	.000	.000
C	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.000	.000	.000
D	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.000	.000
E	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.000	1.000	.000	.000	.000	1.000	.000
F	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.000	.000	.000	.000	.000	.000
PEP-A															
A	.000	.000	1.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
B	.000	.000	.000	.000	.000	.325	.000	.000	.000	.000	.000	.000	.000	.000	.000
C	.030	.367	.000	1.000	1.000	.675	1.000	1.000	1.000	1.000	.000	1.000	1.000	.000	1.000
D	.020	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
E	.950	.633	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.000
F	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.000	.000	.000	.000
PEP-A2															
A	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	1.000	.000	.000
B	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000
C	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.000	.000	.000	.000	1.000	.000
D	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.000	.000	.000	.000
E	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.000	.000	.000	.000	.000

APPENDIX—*Continued*

	Taxon														
Locus	*mendotae* VT	*mendotae* CO	*galeata* UK	*galeata* CZ	*hyalina* AUS	*rosea* SLV	*rosea* BC	*rosea* ONT	*thorata* MT	*umbra* NWT	*longiremis* NWT	*laevis* ONT	*dubia* ONT	*longispina* POL	*cucul.* GER
PEP-D															
A	.750	.985	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
B	.000	.000	.000	.188	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
C	.250	.015	.000	.000	1.000	1.000	1.000	1.000	1.000	.000	.000	.000	.000	.000	.000
D	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000
E	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.000	.000	.000
F	.000	.000	1.000	.812	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
G	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.069	.000
H	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.000	.000	.000	.931	.000
I	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.000	.000
J	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.000	.000	.000	.000
PGM-1															
A	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000
B	.000	.000	.000	.000	.192	.000	1.000	1.000	1.000	.000	.000	.000	.000	.000	.000
C	1.000	1.000	1.000	1.000	.808	1.000	.000	.000	.000	.000	.000	1.000	.000	.000	.000
D	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.000	.000	.000	.000	.000
E	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.000	.000	.000	.000
F	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.000	.000
G	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.000
PGM-2															
A	.050	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
B	.000	.054	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
C	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.600
D	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.000	.000	.000	.000
E	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.333	.000	.000
F	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.667	.000	.000
G	.050	.207	.000	.294	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.400
H	.000	.000	.000	.000	.000	.000	.341	.000	.000	.000	.000	.000	.000	.000	.000
I	.900	.739	1.000	.706	.930	.990	.500	.500	1.000	1.000	.000	.000	.000	.000	.000
J	.000	.000	.000	.000	.070	.010	.159	.500	.000	.000	.000	.000	.000	.000	.000
K	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.000
PGDH															
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.000	1.000	1.000	1.000	1.000
B	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.000	.000	.000	.000

ACKNOWLEDGMENTS

We thank the following people for sending *Daphnia* samples: Martin Cerny, Anders Hobæk, and Lawrence Weider, Paul Forde produced the *Daphnia* drawings. We thank Jaroslav Hrbácek for literature and advice on *Daphnia* morphology and Teresa Crease for helpful suggestions and comments. This research was supported by an N.S.E.R.C. postgraduate scholarship to D.J.T. and an N.S.E.R.C. research grant to P.D.N.H.

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