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A Hierarchical Molecular Phylogeny within the Genus *Daphnia*

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The nucleotide sequences of two mitochondrial DNA regions were used to determine phylogenetic relationships in the genus *Daphnia* (water fleas), a group for which systematics are historically unstable. A portion of the small ribosomal RNA was used to reconstruct higher-level relationships among species, while a portion of the more rapidly evolving control region was used to reconstruct lower-level relationships among populations. Two unexpected results were obtained. First, the subgeneric status of *Ctenodaphnia* falls into uncertainty on the basis of the failure of the 12S rRNA sequences to support these species as comprising an outgroup to the remainder of *Daphnia* species. Second, the high similarity of 12S rRNA sequences of *Daphnia pulex* and *Daphnia pulicaria* samples, coupled with the dual paraphyly of these samples as reconstructed from control-region analysis, suggests that they are two clonotype constellations within the same species complex. A combination of a variety of ecological influences has apparently resulted in the evolution of sets of *Daphnia* genotypes that are genetically cohesive despite their phenotypic divergence. © 1995 Academic Press, Inc.

INTRODUCTION

Phylogenetic delineations within the genus *Daphnia* Muller, 1785 (Class Crustacea, Order Cladocera) are uncertain and are often described as being in a state of flux (Hebert, 1977; Edwards, 1980; Hebert and Loaring, 1986; Benzie, 1986; Korpelainen, 1986; Hebert, 1987). Since the taxonomy of the genus was last revised by Brooks (1957), several factors have contributed to taxonomic confusion, including morphological variation within populations (Hrbacek and Hrbackova-Esslova, 1960; Edwards, 1980; Dodson, 1981), breeding system variation within species (Benzie, 1987; Hebert *et al.*, 1993; Cerny and Hebert, 1993), and interspecific hybridization (Brooks, 1957; Hebert, 1985; Taylor and Hebert, 1992; Hebert and Wilson, 1994). In particular, recurrent evolution of otherwise distinctive traits such as obligate parthenogenesis (Hebert *et al.*, 1989a; Crease *et al.*, 1989) and cuticle pigmentation (Hebert and McWalter, 1983) has obscured true phylogenetic relationships. The genus is fraught with examples of cyclomorphosis or environmentally induced morphological adaptations (reviewed in Hrbacek, 1987), which transcend species boundaries. In fact, the original concept of a reaction norm (Woltereck, 1909) was based on observations of variation in *Daphnia* clones in the laboratory.

Hrbacek (1987) and Benzie (1986) have provided recent discussions of *Daphnia* systematics, and both support the division of the genus into two subgenera, *Ctenodaphnia* and *Daphnia sensu strictu*. This split is maintained by at least five diagnostic morphological traits (Schwartz and Hebert, 1984). The subgenus *Daphnia s. str.* has been further subdivided into the *D. pulex* group and the *D. longispina* group. *Ctenodaphnia* species are typically larger (1.6 mm or longer in adult length) than *Daphnia s. str.* species. Exceptions exist however, and Benzie (1986) noted that the number of sound morphological synapomorphies that support the subgeneric split are few. An additional complication is the existence of species that have been included as members of an additional genus *Daphniopsis* (Hann, 1986), but that have been aligned *within* the subgenus *Ctenodaphnia*. Some of the more widespread *Daphnia* species and their current taxonomic affiliations are listed in Table 1.

In the northern hemisphere, species determinations in the subgenus *Daphnia s. str.* are particularly obfuscated by the presence of at least two species complexes in which interspecific hybridization is common. In the longispina group, hybridization is known to occur among *D. galeata, D. hyalina,* and *D. cucullata* (Wolf and Mort, 1986; Schwenk, 1993) and between *D. galeata* and *D. rosea* (Taylor and Hebert, 1992). In the pulex group, the named forms *D. pulex, D. pulicaria, D. schoedleri,* and *D. middendorffiana* comprise a collection that will be referred to here as the PPSM species complex. These are species prevalent in North America that have been the subject of numerous ecological–genetic studies (e.g., Lynch, 1983; Weider and Hebert,

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TABLE 1

Traditional Systematics of Selected Species in the Genus *Daphnia* and Their Geographic Distributions (Hrbacek, 1987)

Species	Distribution
Subgenus *Daphnia sensu strictu*	
Daphnia pulex group	
D. pulex	Holarctic
D. pulicaria	North America and Europe
D. schoedleri	Holarctic
D. middendorffiana	Circumpolar Arctic and N. temperate alpine
D. obtusa	Palearctic, Europe, New Zealand, and U.S.A.
D. curvirostris	Europe, Africa, and N. American Arctic
D. catawba	E. North America, Greenland, and Arctic Canada
D. parvula	S.E. U.S.A., South America, Central Europe
D. retrocurva	Glaciated continental N. America
D. ambigua	Holarctic
Daphnia longispina group	
D. longispina	Europe and Asia
D. frigidolimnetica	Lapland
D. rosea	Holarctic
D. galeata	Holarctic
D. hyalina	Europe
D. cucullata	Old World Holarctic and northern Palearctic
D. zschokkei	Central Europe
D. thorata	Western and midwestern U.S.A.
D. longiremis	Glaciated Europe and North America
Subgenus *Ctenodaphnia*	
D. magna	Temperate northern hemisphere
D. similis	Northern hemisphere
D. lumholtzi	Southern Europe, Asia, and Australia
D. carinata	Orient and Australia
D. cephalata	Orient and Australia
D. barbata	Africa
D. brooksi	Eastern Utah
Daphniopsis ephemeralis	Laurentian Great Lakes
Daphniopsis chilensis	Chile

1987; Lynch *et al.,* 1989; Hobaek *et al.,* 1993). Such studies were performed with an acknowledgment of the uncertainty of the genetic distinction of the clones used. In disagreement with Brooks (1957), *D. pulicaria* has been elevated to species status by some (Hrbacek, 1959; Brandlova *et al.,* 1972), and despite several reports of polyphyly among pulex and pulicaria clones, the species designation of pulicaria has remained popular. Traditionally, limnologists describe lake-dwelling populations as *D. pulicaria* and temporary pond-dwelling populations as *D. pulex* (Brandlova *et al.,* 1972). Recent surveys, however, relying either on subtle morphological characters such as an isometric reticulation on the head of *D. pulex,* but not *D. pulicaria,* females or on diagnostic alleles at a few allozyme loci, have provided almost as many exceptions to this rule as confirmations (c.f. Cerny and Hebert, 1993).

The purpose of this study is not to revise definitively the taxonomy of *Daphnia* species. It is instead aimed at constructing a molecular–genetic framework into which morphological and ecological surveys can be placed and at providing a foundation for hypothesis testing involving phylogenetic components. We have used an analysis of nucleotide-sequence variation among selected *Daphnia* species in two mitochondrial DNA regions that evolve at different rates in order to examine phylogenetic relationships at the subgeneric, species, and population levels.

MATERIALS AND METHODS

Species Sampling

Five *Daphnia* species, *D. magna, D. similis, D. galeata, D. curvirostris,* and *D. obtusa,* all from North American lakes or ponds (Table 2), were chosen to provide an indication of higher-order relationships. The species identity of these samples was based on traditional morphological discrimination as per Brooks (1957). Two clones of *D. middendorffiana* (one pigmented and one unpigmented) from the Canadian low Arctic and two pulex-like clones from the Canadian high Arctic (one pigmented and one unpigmented) were included and identified primarily on the basis of morphology (including pigmentation) and sampling location.

The remaining samples were from the midwestern United States and from Oregon and were chosen in order to investigate lower-order relationships (Table 2). All of these samples were assumed to belong to the PPSM species complex on the basis of gross morphology and allozyme analysis (see below). These samples included permanent lake populations, semipermanent small lake populations, and ephemeral pond populations. The midwestern U.S. samples were given species names based on the consensus from many recent ecological surveys of their source populations. With the exception of *D. similis,* the Oregon samples were not assigned species names and include melanic dune-pond forms (Florence and Siltcoos ponds, Table 2), the only known pigmented *Daphnia* outside of Arctic or alpine habitats (Hebert and Emery, 1990).

Allozyme Analysis

The placement of the Oregon samples in the PPSM species complex and the *D. pulex*/*D. pulicaria* distinction based on allozyme allele frequencies were possible through the previous analysis of Lynch and Spitze (1994). These authors performed starch-gel and cellulose-acetate protein electrophoresis on several Oregon and Midwest populations with the following 11 loci: *Pgm, Pgi, Aat* (formerly *Got*), *Pep, Fum, Mpi, Ldh, Me, Mdh, Hex,* and *Apk* (Table 3). Recently, the Dunes and Desert populations have been added to this survey (K.

TABLE 2

***Daphnia* Samples Included in This Study**

Species/population name	Sampling location	Habitat type
D. pulex-reference	Amana, Iowa	Temporary pond
D. pulex-unpigmented	Tuktoyaktuk, NWT, Canada	Temporary pond
D. pulex-pigmented	Tuktoyaktuk, NWT, Canada	Temporary pond
D. pulex	Portland Arch, Indiana	Permanent lake
D. pulicaria	Mendota, Wisconsin	Permanent lake
D. middendorffiana-unpigmented	Churchill, Manitoba	Temporary pond
D. middendorffiana-pigmented	Churchill, Manitoba	Temporary pond
D. obtusa	Urbana, Illinois	Temporary pond
D. curvirostris	Tuktoyaktuk, NWT, Canada	Temporary pond
D. galeata	Mendota, Wisconsin	Permanent lake
D. magna	Tuktoyaktuk, NWT, Canada	Temporary pond
D. similis	Buckaroo Lake, OR	Temporary pond
Lava-6	Lava Lake, OR	Permanent lake
Dorena-109	Dorena Reservoir, OR	Permanent lake
Desert-KCO	Burns, OR	Temporary pond
Desert-DWA	Burns, OR	Temporary pond
Desert-RCK	Burns, OR	Temporary pond
Valley-MCV	Springfield, OR	Temporary pond
Valley-CCC	Florence, OR	Temporary pond
Valley-AMA	Eugene, OR	Temporary pond
Elbow-7	Elbow Lake, OR	Permanent lake
Dunes-S13	Dunes City, OR	Semipermanent dune pond
Dunes-S81	Dunes City, OR	Semipermanent dune pond
Dunes-S82	Dunes City, OR	Semipermanent dune pond
Dunes-S89	Dunes City, OR	Semipermanent dune pond
Dunes-F10	Florence, OR	Temporary dune pond
Dunes-K8	Florence, OR	Temporary dune pond
Dunes-X20	Florence, OR	Temporary dune pond

Note. Numbers refer to clone numbers from laboratory cultures. One- or three-letter code (e.g., AMA, F) refers to separate ponds. All Dunes populations contain pigmented individuals. Dunes pond S refers to Siltcoos Pond, a semipermanent large pond located in the dunes 3 km from Siltcoos Lake. Valley-CCC is a population of unpigmented individuals found near, but not in, the Oregon dunes; this population had previously been typed as a "Valley" population based on allozyme data.

Spitze, unpublished data). Electrophoresis and staining protocols were performed as described by Lynch (1983) and Hebert and Beaton (1989). Hebert *et al.* (1993) utilized a diagnostic *Ldh* allele to help distinguish between Canadian *D. pulex* and *D. pulicaria* individuals. They report a slow allele as indicative of *D. pulex* and a fast *Ldh* allele as indicative of *D. pulicaria*, with *D. pulex* / *D. pulicaria* hybrids as slow/fast heterozygotyes. In addition to *Ldh*, apparent fixed allelic differences at the *Pep* and *Hex* loci have been used for discrimination (Hebert *et al.*, 1993; K. Spitze, pers. comm.).

DNA Extraction, Amplification, and Sequencing

DNA was either extracted from individual clones grown in culture and then batch processed for genomic DNA purification or isolated from single individuals using the Chelex method (Walsh *et al.*, 1991; Morin and Woodruff, 1992). For batch processing, animals were grown in 250 ml of pond water (Amazon pond, Eugene, OR) until the density of animals was sufficient to harvest 250 μl of wet, lightly compacted individuals. In the laboratory, most *Daphnia* species will produce iteroparous clutches by parthenogenesis such that all individuals will have the same genotype, barring mutation. After gentle homogenization by hand, DNA isolation was performed by the standard phenol extraction/ethanol precipitation method (Maniatis *et al.*, 1982) and resuspended in 50 μl H_2O. For individual processing, a single animal was placed in 250 μl 10% Chelex (Bio-Rad Laboratories), autoclaved 15 min, and placed on ice. The supernatant from this procedure can be directly used in the polymerase chain reaction (adapted from Morin and Woodruff, 1992). This procedure was also used to prepare outgroup samples, *Moina brachycephala* (representative of a separate cladoceran family) and *Cyzicus mexicanus* (representative of a separate branchiopod crustacean order, Conchostraca).

For phylogenetic analysis, the sequence of a 3667-nucleotide-pair region of the Amana, Iowa *D. pulex* mitochondrial DNA was used for reference (GenBank Accession No. Z15015, Van Raay and Crease, 1994). For all other samples, DNA amplification was performed via the polymerase chain reaction (Mullis *et al.*, 1986) using oligodeoxynucleotide primers specific for two regions of the mtDNA genome (Fig. 1). The 12S rRNA region was amplified with the use of the following primers in a single-step PCR reaction: 1862 (5′-AAGAGCGA CGGGCGATGTGT-3′) and 1863 (5′-AAACTAGGATTA GATACCCTATTAT-3′). These primers produce a 385-nucleotide-pair fragment and were designed from sequence regions conserved between *D. pulex* and *Drosophila yakuba* (Clary and Wolstenholme, 1985). The control region was amplified in a two-step nested reaction. Initial amplification was performed using the primers 12S (5′-TAACCGCGACGGCTGGCAC) and fMet (5′-GGGCATGAACCCACTAGCTT-3′), which produced fragments averaging 1000 nucleotide pairs in length. Internal amplification was then performed on a 1000-fold dilution of this reaction with the primers DPUDL-1 (5′-CAATCTAGAGCCAAAGCCAGATTCA-3′) and DPUDL-2 (5′-CCTCTGCAGGTAGCCCTTTAA TCAGGCATC-3′), which produced fragments averag-

TABLE 3

Summary of Allozyme Analysis

Species/population	*Pgm*	*Pgi*	*Aat*	*Pep*	*Fum*	*Mpi*	*Ldh*	*Me*	*Mdh*	*Hex*	*Apk*
D. pulex (traditional)	F/M/S	M	M	M/**S**	M/S	S	S	M	M	M	M
D. pulicaria (traditional)	F/M/S	F/M/S	F/M	M	S	S	**F**	M	M	S	M
D. obtusa	M	S/S	**S**	M	M	**F**/M	S	M	M	M	M
Desert-DWA, -KCO	**F′/M$^+$**	**F′/S′**	M	**F$^+$**	**F**/M	M	S	M	M	M	M
Lava, Dorena, Elbow, Desert-RCK	F/M/S	F/M/S	F/M	M	S	S	F	M	M	S	M
Valley	F/M/S	M/S/S	M	nd	S	M	S	M	M	M	M
Dunes-K8, -X20	M/S′	S	F	M	S	S	S	M	M	**F**	**F**
Dunes-S13, -S81, -S82, -S89	M/S′	M/**F$^+$**	nd	nd	nd	nd	F/S	nd	nd	nd	M

Note. Alleles are indicated if they are present in a population at a frequency of >10%. Allelic designations F$^+$, F′, F, M$^+$, M, S′, and S indicate relative mobilities from fastest (cathodally) to slowest; nd indicates undetermined. Traditional *D. pulex, D. pulicaria,* and *D. obtusa* allelic patterns are as per Cerny and Hebert (1993) and Lynch and Spitze (1994). The *Ldh, Hex,* and *Pep* loci have been traditionally used to distinguish between *D. pulex* and *D. pulicaria* individuals. The *Aat* locus has previously been designated *Got.* Alleles indicated by boldface type are potentially diagnostic, being present in only one of these groupings.

ing 750 nucleotide pairs in length. Both control-region primer pairs were designed from the Amana *D. pulex* sequence. Amplifications from purified genomic DNA preparations utilized 0.1 µl genomic DNA; amplifications from the Chelex process utilized 10 µl Chelex supernatant. All amplifications were performed in 50-µl reaction volumes containing 8.75 nmol each primer and 1.25 U *Taq* DNA polymerase (Perkin–Elmer). Thermal-cycling parameters were 94°C 5 min + (92°C 1 min, 53°C 1 min, 72°C 1 min) × 30 (×40 for Chelexed samples) + 72°C 10 min in a Perkin–Elmer Cetus thermal cycler.

The DNA sequence of the amplified fragments was determined by the direct sequencing method for double-stranded DNA (c.f., Garza and Woodruff, 1992), following fragment purification by GeneClean (Bio 101). Sequencing was carried out using [^{35}S]dATP, the primers used for PCR amplification, the modified T7 DNA polymerase Sequenase (ver. 2.0, U.S. Biochemical Corp.), dideoxynucleotide triphosphates, and the accompanying reagents. Template-primer annealing in these reactions was accomplished by denaturing approximately one-fourth of the amplification products from a single PCR reaction with 100 nmol of the appropriate primer at 100°C for 5 min and then flash cooling in an ethanol/dry-ice slurry. Reaction products were separated on 6% polyacrylamide 0.4-mm gels containing 7 M urea and 0.5× TBE buffer and visualized by autoradiography. Spot sequencing of duplicate PCR products from a single template revealed no amplification-dependent sequence variation within samples.

Data Analysis

Alignment of all sequences, for both mtDNA regions, was performed by eye. In the 12S rRNA region, no gaps were observed and thus gap weighting was not an issue. In the control region, gaps and insertions were observed (see Results). As variable gap/substitution relative weightings less than 10 : 1 did not affect the topology of the resultant phylogenies, this ratio was set to 1 : 1, with all insertions/deletions of adjacent nucleotides, no matter how numerous, being coded as a single event. Parsimony analysis was performed using the program PAUP version 3.1.1 (Swofford, 1993). Exhaustive searches were performed on subsets of each data set containing 11 or fewer taxa. Phylogenetic signal for each tree reconstruction was assessed with the g_1 statistic of tree-distribution kurtosis (Hillis and Huelsenbeck, 1992). Character-state changes and specific phylogenetic hypotheses were investigated using the program MacClade (Maddison and Maddison, 1992). For such analyses, insertions and deletions were considered additional presence/absence characters, not missing data. Initially, all character-step changes were

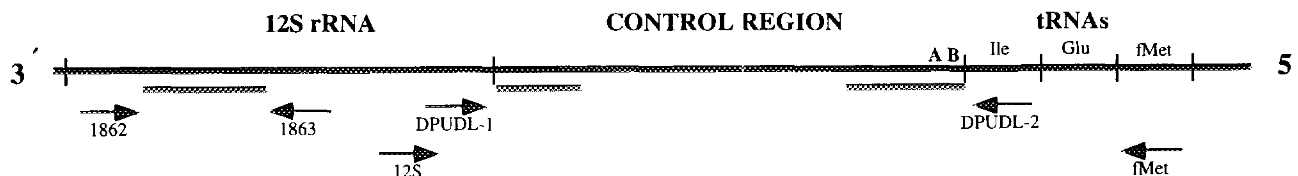

FIG. 1. *Daphnia* mitochondrial DNA regions sequenced in this study and locations of PCR primer annealing sites. Shaded strips indicate regions used for phylogenetic reconstruction. "A" indicates the Valley insertion (9–12 nt); "B" indicates the Dunes deletion (17 nt) (see Fig. 2).

weighted equally, including transitions vs transversions. Maximum-likelihood analysis was performed assuming a Poisson model of mutation and a molecular clock and analyzed using the "mlk.dna" program contained within the PHYLIP software package (Felsenstein, 1993). For the 12S rRNA data set, this analysis was pseudoreplicated 30 times using variable taxon addition order and a transition/transversion ratio of either 1 : 1 or 2 : 1. Neighbor-joining and UPGMA reconstructions were performed on nucleotide-distance matrices from data from all sites, corrected for multiple hits with the Jukes–Cantor method, using the "neighbor" program contained within the PHYLIP package. The UPGMA reconstruction was pseudoreplicated 100 times.

Using the 12S rRNA distance matrix, estimation of absolute divergence times of internal branch nodes in the UPGMA reconstruction was accomplished by the mtDNA molecular clock calibration of Lynch and Jarrell (1993). Pairwise divergence times were estimated using the formula $\hat{T}_{ij} = \hat{D}_{ij}/2\delta$, where $\hat{D}_{ij}$ is the fitted divergence between two species and δ is the estimated substitution rate in the gene region of interest. The fitted divergence $\hat{D}_{ij}$ is obtained by correcting the observed sequence identity (I_{ij}, from the distance matrix) for sequence heterogeneity in the ancestral species (baseline heterozygosity $\neq 0$). This correction is done with the formula

$$\hat{D}_{ij} = [-\ln\{(I_{ij} - I_\infty)/(1 - H_0 - I_\infty)\}](1 - I_\infty),$$

where I_∞ is the baseline sequence identity estimated from empirical nucleotide frequencies ($I_\infty = \Sigma_{A,G,C,T}\, q^2$, where $q = f(A, G, T, \text{or } C) \neq 0.25$) and H_0 is the baseline heterozygosity (Lynch and Jarrell, 1993). The parameters δ, I_∞, and H_0 are all estimated from regression of mitochondrial 12S rRNA sequence divergences on fossil divergence times of animal species from whale to *Caenorhabditis elegans* (Lynch and Jarrell, 1993). Only the portion of the 12S rRNA gene that was actually sequenced was employed in the present calibration.

RESULTS

The published allozyme results of Lynch and Spitze (1994) describe monophyletic pulex and pulicaria clades, well separated from *D. obtusa.* Extension of this study to include additional Oregon populations revealed distinct Desert and Dunes clades outside the pulex/pulicaria group. All of the putative PPSM populations included in this study fell within a monophyletic assemblage distinct from *D. obtusa,* indicating that they were indeed constituents of the species complex (Table 3). The Lava, Dorena, Elbow, and Desert-RCK samples possessed genotypes, including *Ldh* and *Hex* alleles, that would classify them as *D. pulicaria* under the current convention (see Discussion). The Dunes and Valley populations possess heterozygosity at these loci that precludes assigning them to either the traditional pulex or pulicaria. The Desert-DWA and Desert-KCO samples possessed multilocus genotypes that would place them as distinct from either pulex or pulicaria.

For the 12S rRNA region, complete sequences were obtained over a 288-nucleotide-pair portion of the gene for all 27 ingroup taxa and both outgroup taxa. Of these sites, 143 were variable and 94 (33%) were potentially phylogenetically informative, i.e., had variations shared in 2 or more taxa. The majority of this variation was contributed by ingroup taxa; removal of the 2 outgroup taxa eliminated only 17 of the 94 informative sites. Only one small (3 nucleotide) insertion in 1 taxon (*D. galeata*) was observed in the 12S region, and this autapomorphic character was not considered in the subsequent analyses.

For the control region, amplification and sequence readability were reliable only for samples in the PPSM species complex, although some information was obtained from the *D. galeata* sequence. When the reference Amana pulex sequence was compared to the Mendota pulicaria sequence over the entire 715-nucleotide-pair span between the DPUDL-1 and DPUDL-2 primers, 43 variable sites were observed, including gaps (94% similarity). Variation was distributed throughout the region, with the ends being slightly more variable. This study utilized a comparison of variation of 116 nucleotide sites from the 3′ end, of 212 nucleotide sites from the 5′ end, and of two large (9–17) nucleotide insertion/deletions from the 5′ end (Figs. 1 and 2). In total, across all samples, 79 variable sites from the control region could be defined, 46 of which were phylogenetically informative.

Most of the 12S rRNA variation was a consequence of differentiation among the named species outside of the PPSM species complex. Within the PPSM complex, several of the samples possessed identical sequences, and many samples were very similar; the Amana pulex and Mendota pulicaria samples differed at one of 288 positions. Only the two *D. middendorffiana* samples, (which differed from each other at 4 of 288 positions) and two of the Oregon Desert samples (DWA and KCO, which differed at one position) stood out as being distinct. No more than 7 nucleotide changes separate any two of the other PPSM taxa. Consequently, 11 taxa were chosen for an exhaustive search of parsimonious tree topologies using only Amana pulex, Mendota pulicaria, Desert-DWA, pigmented *D. middendorffiana,* and the non-PPSM species (Fig. 3). In this case, a single most parsimonious tree was obtained, with a length of 302 unambiguous character-state changes. Based on a g_1 statistic of -0.60, this tree has significant phylogenetic signal ($P < 0.01$) when compared to a random distribution of tree lengths (Hillis and Huelsenbeck,

1992). Altering the choice of Amana pulex and Mendota pulicaria for PPSM taxa has no effect on the topology of the most parsimonious tree. In all cases, both the *D. similis / D. magna* and the *D. curvirostris / D. galeata* pairs form monophyletic assemblages, with the latter being an outgroup to the former. This is of interest because *D. similis* and *D. magna* are members of *Ctenodaphnia,* while *D. curvirostris* and *D. galeata* are members of *Daphnia s. str.* With different sets of taxa, the ambiguity in parsimonious reconstruction concerns only the relative placement of the Desert-DWA and *D. obtusa* branches. This ambiguity is not resolved by other methods of reconstruction (see below).

Maximum-likelihood analysis supported the overall topology resulting from the parsimony analysis. A 10-taxa tree from the 12S rRNA sequence clusters the PPSM samples as a monophyletic group and strengthens the placement of the *Ctenodaphnia* species as not being an outgroup to the other *Daphnia* species. Pseudoreplication of 288 characters with replacement from the complete data matrix (bootstrapping) allowed a test of the robustness of this topology. Only 1 of 30 pseudoreplicates ($P < 0.05$) placed the *Ctenodaphnia* species as an outgroup. However, 8 replicates constructed topologies similar to that in Fig. 3 but with the similis/magna and curvirostris/galeata clades together as monophyletic with respect to the other *Daphnia* samples. These replicates placed *D. obtusa* inside, outside, and monophyletic with the Desert-DWA sample in a 3 : 2 : 1 ratio.

Selection of a transition/transversion ratio (i/v) is an important consideration in maximum-likelihood analyses because this ratio can reach as high as 10/1 in comparisons involving most animal mtDNA genes. However, for reconstructions at the family level and below, mtDNA ribosomal RNA genes often exhibit i/v ratios that are less skewed. In the *Daphnia* sequences presented here, the i/v ratio was 1.2/1 for observed changes reconstructed from either the entire 11-taxa parsimony tree or the terminal branches leading to the obtusa, similis, magna, curvirostris, and galeata taxa. However, this ratio increased to 6.2/1 when only changes in the more closely related Desert and PPSM taxa were considered. Varying this ratio from 1/1 to 10/1 did not affect the topology of the resulting tree in the optimal maximum-likelihood analysis, and setting this parameter at 1/1 or 2/1 in the replicate maximum-likelihood analyses gave indistinguishable results.

The two distance-based methods of phylogenetic reconstruction, UPGMA and neighbor-joining, generated identical topologies at the higher-order level (Fig. 4). Notably, these methods provide evidence that the *Ctenodaphnia* may indeed have an outgroup relationship to the other species included in this report, although this node is not significant upon distance-matrix bootstrapping (Fig. 4). Both methods cluster all PPSM samples as monophyletic with little internal resolution and also support a monophyletic arrangement of the Desert-DWA/KCO and *D. obtusa* samples. With a recalibration of the 12S rRNA molecular clock using the technique of Lynch and Jarrell (1993) applied only to the relevant 288-nucleotide-pair region, estimates of δ = 0.55 nucleotide changes per position per billion years, I_∞ = 0.283, and H_0 = 0.0834 were obtained. These values allowed placement of a divergence-time scale on the distance-based phenogram (Fig. 4). The *Cyzicus / Moina* divergence time was estimated at 292 million years ago (95% C.I.: 164 million–1.32 billion), while the *Daphnia* interspecific radiation was estimated at approximately 120 Mya from Fig. 4. All divergence times in the PPSM complex were negative, indicating that among-species variation is obscured by within-species variation (H_0) for these samples.

Clearly, the 12S rRNA gene is unable to resolve branching orders within the PPSM complex, except for the distinctiveness of both *D. middendorffiana* clones. The control region has been demonstrated to evolve at a rate 1.4–5.0 times faster than the rest of the mtDNA genome, at least in vertebrates (Brown, 1985), and to be useful for comparisons of closely related taxa (Hoelzel *et al.,* 1991; Morin *et al.,* 1993). These sequences were therefore analyzed with the same techniques as above. In this region many insertions and deletions, of one or more nucleotides, were present and possessed phylogenetic information, i.e., they appeared in more than one sample. Most apparent were two sites at the very 5′ end of the control region that exhibited insertions or deletions of more than 9 nucleotides (Fig. 2). In certain melanic Dunes samples that were analyzed, a 14-nucleotide fragment is deleted, with respect to the Amana *D. pulex.* Two of the seven Dunes samples included in this study (K8 and X20) have this deletion. Although a complete sequence for non-PPSM samples could not be obtained using the PCR primers described above, it could be determined that this ≈14-nucleotide portion was present in *D. galeata,* as well as in all other

FIG. 2. Sequences and alignments from the mitochondrial control region of 18 *Daphnia* PPSM clones. Sequences read from 3′ to 5′ left to right as in Fig. 1 and in Van Raay and Crease (1994). The first 116 sites are from the 3′ end of the control region, while the remaining 212 sites and the two large (9–17 nt) insertions/deletions are from the 5′ end of the control region; the two segments are separated by exclamation marks. Position 1 in this figure lies at position 8 from the 3′ end of the control region; position 117 lies at position 218 from the 5′ end of the control region; the 5′ position of the Dunes deletion lies at the 5′ end of the entire control region (Fig. 1 and Van Raay and Crease, 1994). The Dunes deletion is present in the K8 and X20 clones and in >90% of all melanic *Daphnia* from the Florence, Oregon dune area (N.L., unpublished data). The abbreviations Dpx and Dpc refer to "*D. pulex*" and "*D. pulicaria*" clones, respectively (see Discussion). The letter Y refers to an uncertain base identity of C or T. A black dot by the taxon name indicates a melaninized clone.

```
                     10         20         30         40         50         60         70         80         90        100
Dpx-Amana      TCCAGAAGGA CCTTAAAAGT ATGGATAAAA ACTCGGAACC ACTTTAGCGC AAGTATTTAA CGACCG-CTT TGAAA-TTTG CCCGCCAAAA TTCAATCTTT
Dpx-Arctic     C......... .......... .......... .......... .......... .......... ......-... .A..GG.... .......... ..........
Dpx-Arctic•    C..T...... .....G.... .C........ .......... .......... .........G A.....A... .....-...A .......... ..T.......
Dpc-Dorena     C......... .......... .......NNN N......... .......... ........NN N.....-... .....-...A .......... ..........
Dpc-Lava       C......... .......... .C........ .......... .......... .......... ......-... .....-...A -......... ..........
Dpc-Elbow      C......... .......... .......... .......... .......... .......... ......-... .....-...A TY........ ..........
Dpc-Mendota    C......... .......... .......... .......... .....GC... ........GG ......A... .....-.... .......... ......T...
Valley-CCC     C......... .......... .......... .......... ......A... .........N NN....-... .....-.... ......NNNN NNNNN.....
Valley-MCV     C......... .......... .......... .......... ......A... .........G ......-... .....-.... .....T.... ..........
Valley-AMA     C......... .......... .....N.... .......... ......A... .........G ......-... .....-.... .....T.... ..........
Dunes-F10•     C......... .......... .......... .......... .......... .......... ......-... .....-...A T......... ..........
Dunes-K8•      C......... .......... ...A...... .......... .......... .....C...G ......A... .....-...A .....T.... .........-
Dunes-S13•     C......... .......... .......... .......... .......... .......... ......-... .....-...A .......... ..........
Dunes-S81•     C......... .......... .......... .......... .......... .......... ......-... .....-...A T......... ....T.....
Dunes-S82•     C......... .......... .......... .......... .......... .......... ......A... .....-...A ...T...... ..........
Dunes-S89•     C......... .......... .C........ .......... .......... .......... ......-... .....-...A -......... ..........
Dunes-X20•     C......... .......... ...A...... .......... .......... .....C...G ......A... .....-.... ...-.T.... .........-
Desert-RCK     C......... .......... .......... .......... .......... .......... ......-... .....-...A .......... ..........

                    110     <*>120        130        140        150        160        170        180        190        200
Dpx-Amana      CGAGGGG-AT CCCCCC!TTAA TGTTTGGTCC AAAAATTAGA AAAGGGGTTT AAACTGAATA GATACTTATT AGGAACCACT TTCGTGAGGA ACTTTTCAAC
Dpx-Arctic     .......-.. ......!.... ..N....... NNNNN..... .......... .......... .......... .......... .......... ..........
Dpx-Arctic•    .......-.. ......!.... .......C.. .......... .........A .......... .C.GT..... .......... ...CCA.... ..........
Dpc-Dorena     .......-.. ......!.... .......... .......... .......... .......... .......G.. .......... .......... ..........
Dpc-Lava       .......-.. ......!.... ..N....... .......... .......... .......... .......... .......... .......... ..........
Dpc-Elbow      .N-.....-. NN....!.... .......... .......... .......... .......... .......... .......... .......... ..........
Dpc-Mendota    T.....-.-. ......!.... .......C.. .......... .......... .......... .C..T..... .......... .......... ..........
Valley-CCC     TN-.....-. ......!.... .......G.. .......... .......... .......... ....TC.... .......... .......... ..........
Valley-MCV     T.C.....-. ......!.... .......A.. C......... .......... ...T...... .......... .......... ...A...... .TA.......
Valley-AMA     T.......-. ......!.... .......C.. .......... .......... ...T...... .......... .......... .......... .TA.......
Dunes-F10•     .....-A.-C ......!NNNN .......... N......... .......... .......... .......... .......... .......... ..........
Dunes-K8•      .T-.....-A ......!.... .......... .......... .......... .....AG... .C.GT..... ...G...... ....C..... ..-.......
Dunes-S13•     ........G. ......!NNNN NNNNNNNNNN NNNNNNNNN. .......... .......... ......C... ..A....... .......... ..........
Dunes-S81•     .....--.-A ......!..NN .......... NN........ .......... .......... .......... .......... .......... ..........
Dunes-S82•     T.......-. ......!.... .......C.. .......... .......... .......... ....T..... .......... .......... ..........
Dunes-S89•     .......-.. ......!.... .......... .......... .......... .......... .......... .......... .......... ..........
Dunes-X20•     .T-.....-A ......!NNNN NN........ .......... .......... .....AG... .C.GT..... ...G...... ....C..... ..-.......
Desert-RCK     .......-.. ......!.... .......... .......... .......... .......... .......... .......... .......... ..........

                    210        220        230        240        250        260        270        280        290        300
Dpx-Amana      -TTTTTTGAA GAAG-AAAGG TCTTCGCCCC CCAAAAAAGT TGAAAAGTT- CCTCCGAATA ATATTGAAAA GTGGTTCCGA GTTTTCATTG ATATTTTTAT
Dpx-Arctic     --...NNN.. ....A..... ...G...-.. .......-.. .........- ....A..... .......... .......... .......... ..........
Dpx-Arctic•    --.....--- ....A..... ...G...... .......-.. .........- ..C.A..... .......... .......... ......G... ..........
Dpc-Dorena     -......--- ....A..... ...G...... .......-.. .........- ....A..... .......... .......... .......... ..........
Dpc-Lava       NN.....--- ....A..... ...G...... .......-.. .........- ....A..... .......... .......... .......... ..........
Dpc-Elbow      -......--- ....A..... ...G...... .......-.. .........- ....A..... .......... .......... .......... ..........
Dpc-Mendota    --.....--- ....A..... ...G...-.. .......-.. .........- ....A..... .......... .......... ......G... ..........
Valley-CCC     -......N-- ....-...A. ...G...... .......-.. .........- ....A..... .......... ......T... ......G... ..........
Valley-MCV     -.....---- ....-...A. ...G...-.. .......... .........- ....A..... .......... .......T.. ......G... ..........
Valley-AMA     -......--- ....A..... ...G...... .......-.. .........- ....A..... .......... .......T.. ......G... ..........
Dunes-F10•     --......-- ....A..... ...G...... .......-.. ......-..- ....A..... .......... .......... ......G... ..........
Dunes-K8•      T.......-. A..T-..... ...G...-.. .......... ......-..C ....A..... .......... .......... ......G... ..........
Dunes-S13•     -.......-- ....A..... ...G...-.. .......-.G .........- ....A..... .......... .......... .......... ..........
Dunes-S81•     C-......-- ....A..... ...G...-.. .......-.. .........- ....A..... .......... .......... .......... ..........
Dunes-S82•     -......--- ....A..... ...G...-.. .......-.. .........- ....A..... .......... .......... ......G... ..........
Dunes-S89•     -......--- ....A..... ...G...-.. .......-.. .........- ....A..... .......... .......... .......... ..........
Dunes-X20•     T......... -..T-..... ...G...-.. .......... ......-..C ....A..... .......... .......... .......... ..........
Desert-RCK     -......--- ....A..... ...G...... .......-.. .........- ....A..... .......... .......... .......... ..........

                    310        320            VALLEY INSERT  DUNES DELETION
Dpx-Amana      GCAAGCAAGT GAGTAATTTT TTTAAA-G   ------------   tttttt---aaaaacaa
Dpx-Arctic     .......... .......... ........   tttttttttaag   tttttt-aaaaaaccaa
Dpx-Arctic•    .......... .G........ ........   ------------   ttttttttttaaaa----
Dpc-Dorena     .......... .......... ........   ------------   ttttttt--aaaaccaa
Dpc-Lava       .......... .......... ......A.   ------------   ttttt---aaaaaccaa
Dpc-Elbow      .......... .......... ........   ------------   ttttttt-aaaaaccaa
Dpc-Mendota    ...G.....A .G........ ........   ------------   tttttt--aaaag--aa
Valley-CCC     .......... .....G.... ......A.   tttttt-aaaag   ttt-----aaaaaccaa
Valley-MCV     .......... .......... ........   ttttt---aaag   ttttt---aaaaaccaa
Valley-AMA     .......... .......... ........   tttttt--aaag   tttt----aaaaaccaa
Dunes-F10•     .......... .......... ........   ------------   tttttt-aaaaacccaa
Dunes-K8•      ...GA..... .G........ ........   ------------   -----------------
Dunes-S13•     .......... .......... ........   ------------   tttttttaaaaaancaa
Dunes-S81•     .......... .......... ........   ------------   tttttt-aaaaacccaa
Dunes-S82•     G......... .......... ........   ------------   tttttttanaaaannnn
Dunes-S89•     .......... .......... ......A.   ------------   ttttt---aaaaaccaa
Dunes-X20•     ...GA..... .G........ ........   ------------   -----------------
Desert-RCK     .......... .......... ........   ------------   ttttttt aaaaaccaa
```

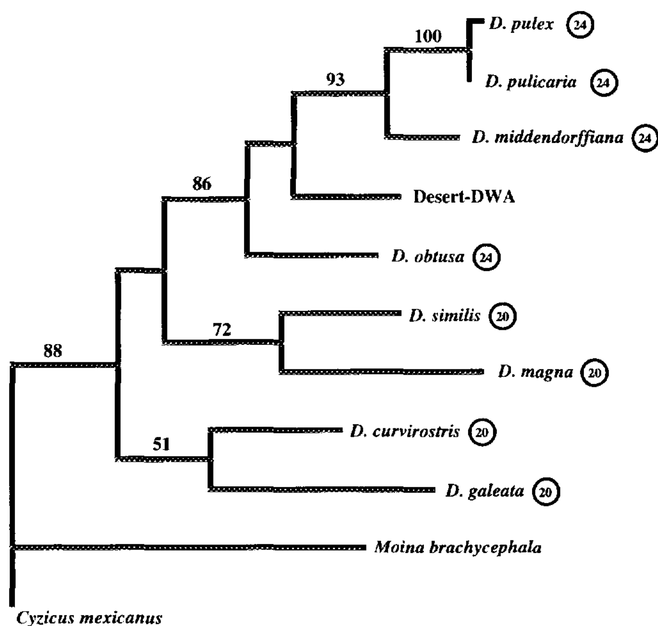

FIG. 3. Maximum-parsimony tree for 11 *Daphnia* taxa. Sequences and alignments are from a 288-nucleotide-pair segment of the mitochondrial 12S ribosomal DNA gene of the ingroup (*Daphnia*) and 2 outgroup (*Moina* and *Cyzicus*) taxa. GenBank Accession Nos. U13902–U13911 have been assigned to these (and other, see Fig. 6) sequences in the following order: *D. pulex*-Arctic (melanic), Desert-DWA, Dunes-K8, *D. pulicaria*-Mendota, *D. curvirostris, D. galeata, D. magna, D. middendorffiana, D. obtusa, D. similis.* Position 1 in these sequences lies at position 110 from the 3′ end of the 12S gene (total length in *Daphnia pulex* is 753 nt; Van Raay and Crease, 1994). The tree is rooted using *Cyzicus* (clam shrimp) as a designated outgroup. Bootstrap values greater than 50% (1000 replicates) are indicated along branches. Total tree length is 302 steps; consistency index is 0.66. Branch lengths are proportional to the number of unambiguous reconstructed changes along the branch. For scale, the *D. pulex–D. pulicaria* split represents one change. In this tree, the *D. pulex* taxon is the Amana, Iowa reference sequence (Van Raay and Crease, 1994) and the *D. pulciaria* taxon is the Lake Mendota, Wisconsin sequence. Numbers in circles are diploid chromosome numbers (Trentini, 1980; Beaton and Hebert, 1994).

pulex-like samples. This suggests that the deletion is an autapomorphic character unique to most of the melanic dune clones. Additionally an insertion of 9–11 nucleotides was present in all three Oregon "Valley" clones included in this study and a similar 12-nucleotide insertion was present in the Arctic unpigmented pulex sample. Although these insertions were not 100% identical, they were coded as such in the data matrix because all nucleotide substitutions in this region of sequence were autapomorphic.

With these insertions/deletions coded as additional presence/absence characters having a weight equal to substitutions, maximum-parsimony analyses could be performed. Exhaustive searches were made with various 11-taxa choices and heuristic searches were performed with the entire set of taxa. One cladogram from an 11-taxa exhaustive search is shown in Fig. 5, which is but 1 of 12 equiparsimonious trees with length 102 steps. However, some branch nodes are found in all trees, including those supporting the clade grouping the Amana and the Arctic unpigmented pulex, the clade containing the Valley clones, and the clade grouping the Arctic pigmented pulex with the Dunes clone K8 (Fig. 5). It should be noted that despite the consistent grouping of the Arctic pigmented pulex and Dunes clone K8, these two types are very distinct from each other, differing by 35 character-state changes. The g_1 statistic for these trees is -0.56, indicating significant phylogenetic signal ($P < 0.01$). Although the reconstructed unambiguous transitions outnumber transversions by 1.7 : 1, increasing the character-state change weight *a posteriori* to reflect a 2 : 1 bias did not affect the tree topologies, only the branch lengths.

Because the number of taxa was too large and there were too few obvious clusters to justify excluding enough taxa, a maximum-likelihood analysis would not be feasible with the control-region data. However, the distance-matrix-based methods did suggest that certain groupings may be valid. Clades supported in both UPGMA and neighbor-joining reconstructions were the groupings of the Amana and the Arctic unpigmented pulex, the three Valley clones, and the Arctic pigmented pulex with the Dunes clones K8 and X20. All of these groups were also supported by the parsimony analyses. An additional group supported by the distance methods, but not parsimony, was a {(Dunes-S89, Lava), ((Dorena, Desert-RCK), elbow)} clade. Regardless of reconstruction method, paraphyly is evident in both the clones described as *D. pulex* (Amana, Portland, Arch, and Arctic) and those described as *D. pulicaria* (Mendota, Lava, Dorena, Elbow, and Desert-RCK).

The sum total of the control-region analyses can be combined to create a cross-method consensus cladogram for the PPSM samples. This cladogram in turn can be combined with the 12S rRNA consensus cladogram to produce a hierarchically derived phylogenetic tree for all of the samples in this study (Fig. 6). Despite the *D. pulicaria* paraphyly, the Oregon samples characterized as *D. pulicaria* based on allozyme genotype could be monophyletic.

DISCUSSION

To date, few investigations of *Daphnia* systematics have not been based on comparative morphology. Korpelainen (1986) used genetic distances derived from allozyme allele frequencies to determine the amount of genetic differentiation within and among four European species, *D. magna, D. cucullata, D. longispina,* and *D. pulex.* She discovered a high degree of genetic differentiation within these species but a relatively low level of divergence between *D. pulex* and *D. longispina.* She also noted that although *D. magna,* the sole *Ctenodaphnia* of the group, was an outgroup to the other

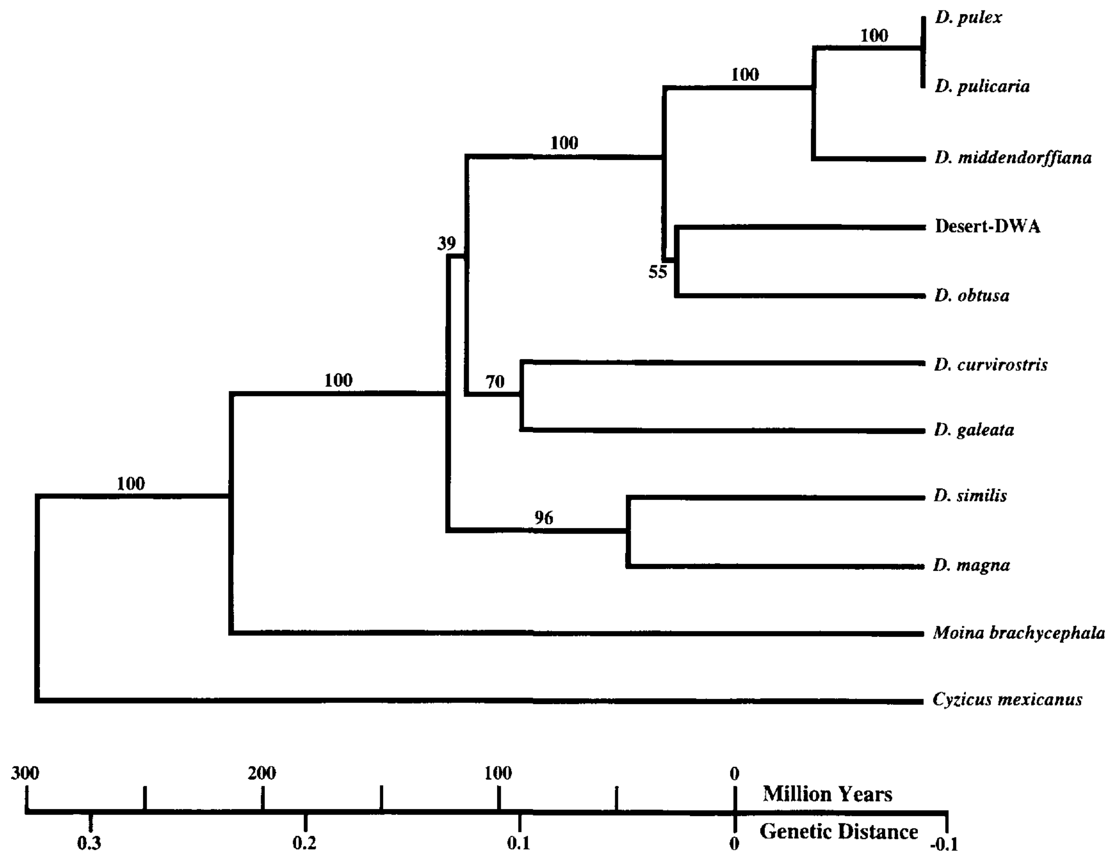

FIG. 4. UPGMA tree reconstructed from the 12S rRNA distance matrix. Neighbor-joining tree topology is the same. Bootstrap values (100 replicates) are indicated along branches. Scale of divergence times is based on the molecular-clock calibration technique of Lynch and Jarrell (1993), using the 288-nucleotide region sequenced for these taxa. Divergence times between pairs of species (upper scale) are estimated from the formula $\hat{T}_{ij} = \hat{D}_{ij}/2\delta$, where δ is the estimated substitution rate in this region, 0.55 substitutions per site per billion years. D_{ij} (lower scale) are genetic distances corrected for multiple hits, unequal nucleotide frequencies, and baseline heterozygosity (see Materials and Methods).

three, its genetic distances to the *Daphnia s. str.* species were only slightly higher than the other between-species values (except pulex to longispina). Schwenk (1993) used cytochrome *b* gene sequences from the mtDNA to determine branching orders within the longispina group and estimated that these species radiated from a common ancestor approximately 6 Mya. Hebert (1987) presented an allozyme-based phylogenetic reconstruction of North American members of the genus in which the branching order was given as {magna (similis (curvirostris (obtusa ((galeata, PPSM)))))}. This topology maintains the *Ctenodaphnia* as an outgroup but undermines the cohesiveness of the pulex and longispina groups (Table 1). Hebert and Wilson (1994) recently investigated the relationships among several *Ctenodaphnia* species but did not address the placement of the subgenera.

The results of this study confirm the general trends of earlier work on higher-level groupings. The *Ctenodaphnia* species are unequivocally monophyletic, but their outgrouip status is ambiguous. In an 11-taxa parsimony analysis, 1000 bootstrap replicates fail to support either a significant (>95%) ingroup or outgroup status of the *Ctenodaphnia* (Fig. 3). Although the most parsimonious arrangement places the *Ctenodaphnia* as an ingroup with respect to the curvirostris/galeata clade, only six additional steps are required to force them to the outgroup position. Both distance-based methods of reconstruction suggest that the *Ctenodaphnia* may, despite lack of statistical significance, deserve outgroup status. In any event, resolution of this issue must await additional phylogenetically informative characters and a broader taxonomic scope.

The estimated *Cyzicus–Moina* divergence time of 292 million years ago is in agreement with the branchiopod fossil record, which places the cladocera–conchostraca interorder split during the Permian, 225–280 Mybp (reviewed in Kerfoot and Lynch, 1987). Benzie (1987) argued that biogeographical evidence indicates that the genus *Daphnia* is old, with divergences among species within subgenera dating back at least 70 My. The estimated divergence time found here of 120 Mya for the genus as a whole agrees qualitatively (Fig. 4).

The 12S rRNA data also indicate unequivocal phylo-

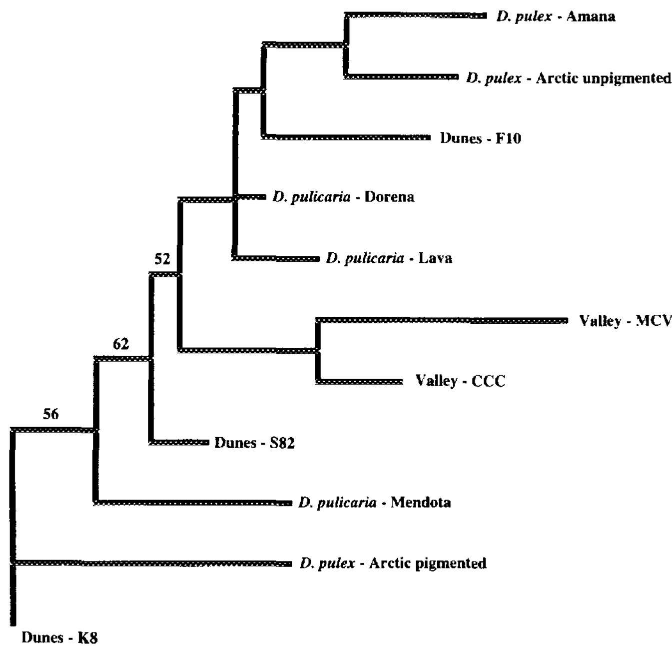

FIG. 5. Maximum-parsimony tree for PPSM samples, using data from approximately 330 nucleotides of the control region. The tree is rooted using the Dunes-K8 clone as a designated outgroup. Bootstrap values greater than 50% (1000 replicates) are indicated along branches. This is 1 of 12 equiparsimonious trees with length 102 steps and consistency index 0.66. Branch lengths are proportional to the number of unambiguous reconstructed changes along the branch.

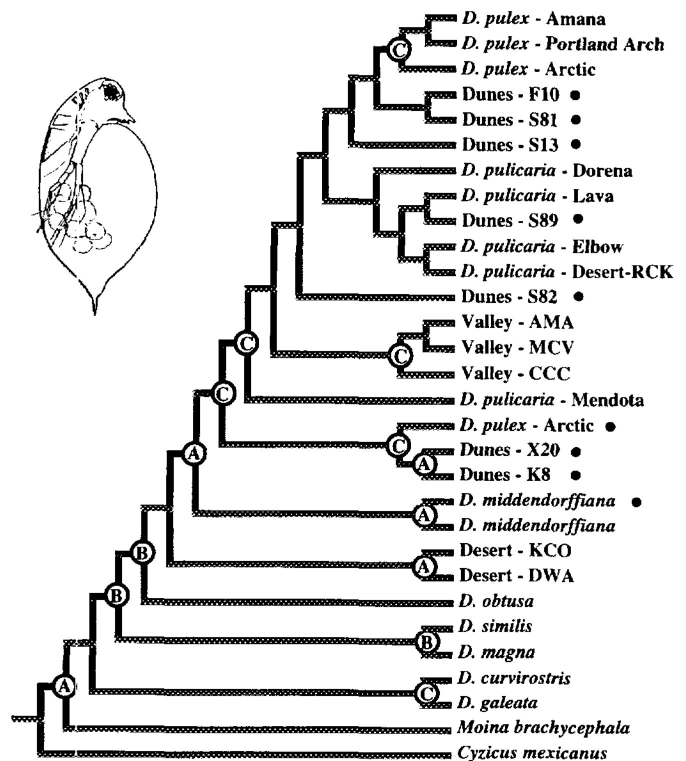

FIG. 6. Hierarchical composite cladogram for all samples in this study. PPSM members based on control-region data; higher-order relationships based on 12S rRNA data. Branching orders depicted are those most consistent with all analyses (see Figs. 4 and 5). Four broad categories of confidence of clade unity are; (A) clade found with all methods of reconstruction *and* bootstrap values >95%, (B) clade found with all methods of construction *and* bootstrap values 60–95%, (C) clade found in all methods of reconstruction, and (no letter) clade found in the majority of reconstruction techniques. Branch lengths do not reflect actual distances. Samples indicated by dots are melanic forms.

genetic placements for the *D. obtusa* and *D. middendorffiana* species, as well as for the Desert populations DWA and KCO. The pigmented and unpigmented samples of *D. middendorffiana* cluster together, a result that is corroborated by a recent allozyme study of the PPSM complex (Van Raay and Crease, 1995). The distinct Desert samples represent either a range expansion of a described North American species not included in this survey (e.g., *D. catawba* or *D. thorata*) or an undescribed species. Also of interest is the clustering of *D. curvirostris* with *D. galeata,* at odds with the traditional placing of *D. curvirostris* in the pulex group and *D. galeata* in the longispina group. The conventional arrangement has been questioned on both morphological (Hrbacek, 1959) and karyotypic (Trentini, 1980; Beaton and Hebert, 1994) grounds; the diploid chromosome number of *D. curvirostris* is 20, while other pulex group members all possess 24 (Fig. 3).

However, the 12S rRNA data provide no support for a pulex/pulicaria distinction. At this lower level, numerous recent genetic studies have generated evidence of poor resolution within the PPSM complex. Hebert and Loaring (1986) concluded that *D. pulex* and *D. schoedleri* are members of a single apomictic complex. Conversely, Hebert *et al.* (1989b), in a study of allozyme allele frequencies of *Daphnia* populations in the former Czechoslovakia concluded that diagnostic allele substitutions at the *Pgi* and *Amy* loci supported the distinctiveness of *D. pulex* from *D. pulicaria* in Europe. In Canada, the *Ldh* and *Pep* loci have been used in the same way (c.f. Hebert *et al.,* 1993). This distinction was also made in a survey of U.S. populations by Lynch and Spitze (1994). Yet *D. pulicaria* is now essentially defined by allozyme differentiation and will necessarily display genetic separation from *D. pulex* when measured allozymically.

Restriction-site surveys of the entire mtDNA genome depict a scenario of a highly diverse, reticulated complex of pulex- and pulicaria-like clones throughout the Holarctic (Crease *et al.,* 1989, 1990). Clonal diversity in this group is maintained in large part by a particular duality of mode of reproduction that is manifested at two evolutionary scales. On the scale of the individual, the presumed ancestral mode of reproduction is cyclical parthenogenesis. Individuals will produce several parthenogenetic clutches of female clones until an environmental cue triggers male production that usually leads

to a bout of sex and diploid, diapausing eggs. On the scale of the population (or deme), cyclically parthenogenetic populations can suddenly generate obligately asexual clones by the introduction of a sex-limited meiotic suppressor gene into the population (Hebert, 1981). Obligately parthenogenic clones may still produce males, however, and these males can infect other sexual populations, resulting in a recruitment of genetic diversity. Accordingly, mtDNA data demonstrate the polyphyletic origin of obligate asexuality (Crease *et al.*, 1989). Countering this process is the decreased long-term probability of survival of asexual lineages, which must ultimately succumb to the accumulation of deleterious mutant alleles (Lynch *et al.*, 1993). In small, isolated populations the extinction of asexual lineages can be exacerbated by a synergism between population size and deleterious mutations, a mutational "meltdown" (Lynch and Gabriel, 1990). The multitude of temporary-pond populations of *Daphnia,* such as those created by the receding Pleistocene glaciation, provide an excellent milieu for reiterated clonal diversification and concomitant extinction.

This fluctuation in reproductive strategies may have combined with other ecological factors to affect the appearance of phylogenetic divergence in *Daphnia.* In North America, reproduction by obligate parthenogenesis is observed only in members of the PPSM complex. Similarly, marked melanism is displayed predominantly in this group. The pigmentation has been demonstrated to be an adaptation against ultraviolet insolation (Hebert and Emery, 1990; Cerny and Hebert, 1993). As with obligate parthenogenesis, pigmentation has arisen multiple times in recent *Daphnia* radiations, as shown by mtDNA RFLP and control-region sequencing examinations of Canadian populations (Van Raay and Crease, 1995). If local populations have experienced isolation for a relatively extended period of time, they may adopt a few diagnostic genetic characteristics (Lynch and Spitze, 1994), as is the case for some Valley, Desert, Dunes, and Arctic localities. But even in these cases, the genetic lability of pulex-like *Daphnia* could result in a rapid turnover in the future.

The only discrete characteristic supporting a distinction between *D. pulex* and *D. pulicaria* is the existence of obligately asexual clones that are *Ldh* heterozygotes. These clones have been described as pulex/pulicaria hybrids and their asexuality has been cited as evidence for interspecific reproductive isolation (Hebert *et al.*, 1993). This position is further supported by the fact that the F/S heterozygotes possess allele frequencies across six other loci that are intermediate between those of their putative parental genotypes. However, strict reproductive isolation is belied by evidence of allozyme gene flow between the pulex and the pulicaria forms (Hebert *et al.*, 1993). Moreover, such hybrids are found in very high frequencies in Canadian populations, often exceeding 50% and persisting in the absence of the pulicaria parental type. An unexplored alternative is that *many* genotype crosses in the PPSM complex lead to obligate asexuality, as substantiated by a ubiquity of both asexual pulicaria (*Ldh* F/F) and asexual pulex (*Ldh* S/S) (Cerney and Hebert, 1993; Hebert *et al.*, 1993). In this scenario, the conventional pulex and pulicaria forms may represent extremes of a multidimensional allozymic array.

Taken together, these data suggest that clones in the PPSM complex are cohabitating, interfertile, and engaged in a genetic struggle between the advantages of sexual and asexual reproduction. Furthermore, there are few consistent patterns of genetic differentiation, as exemplified by the population-level phylogenetic uncertainty in the current study. Consequently, the division between *D. pulex* and *D. pulicaria* (and perhaps *D. schoedleri*) is probably artificial. None of the criteria that have traditionally been employed to distinguish species applies convincingly in this case, including reproductive isolation, morphological distinctiveness, geographic discontinuity, microhabitat partitioning, and genetic distance. Instead, a complex web of ecological factors has fostered an abundance of genetic diversity that has obscured phylogenetic unity. Analogous processes occur in the *D. longispina* group and in the subgenus *Ctenodaphnia,* leading to the conclusion that the genus *Daphnia* may be composed of a limited number of fluid genetic complexes, rather than many discrete species.

ACKNOWLEDGMENTS

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