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The systematics of North American *Daphnia* (Crustacea: Anomopoda): a molecular phylogenetic approach.

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SUMMARY

Despite extensive studies on the ecology and evolution of the freshwater microcrustacean *Daphnia*, there is little understanding of the evolutionary history of the genus. Past attempts at reconstructing phylogenetic relationships among *Daphnia* species have been highly controversial, mainly because of the poor taxonomy of the genus. However, following a revised taxonomy of the daphniid fauna of North America, we conducted a comprehensive appraisal of systematic relationships within the genus through the analysis of sequence diversity in 503 b.p. of the 12S rRNA gene of the mtDNA. The large sequence divergence among its 34 North American members indicates that the genus *Daphnia* originated during the Mesozoic, even though many lineages exhibit extreme morphological stasis. Results from both cladistic and phenetic analyses indicate the presence of three subgenera comprised of 15 species complexes. Only four of these lineages have shown active speciation over the past 3 Ma, suggesting that cladogenesis in the genus has been constrained. Our study also reveals that interspecific hybridization occurs between taxa which show very large sequence divergence (up to 14%), suggesting that reproductive isolation within the genus evolves slowly.

1. INTRODUCTION

Although daphniids are an increasingly important target for ecological and evolutionary studies, knowledge of systematic relationships within the genus *Daphnia* remains primitive. Systematic studies have been seriously constrained by the volatile taxonomy of the group, which has resulted from both the occurrence of dramatic phenotypic plasticity (Hebert 1978; Havel 1987; Dodson 1989; Lampert 1994) and the prevalence of interspecific hybrids (Taylor & Hebert 1992; Schwenk 1993). Efforts to explicate species boundaries using a conventional morphological approach have had limited success and the application of multivariate analysis produced few new insights (Edwards 1980; Dodson 1981). However, over the last decade, studies on closely allied species of *Daphnia* have indicated that genetic analyses might resolve taxonomic problems (Hebert & Finston 1993, 1995; Taylor & Hebert 1994). Recently, this approach has been employed to revise the taxonomy of the entire North American fauna (Hebert 1995), setting the stage for a thorough evaluation of systematic relationships within the genus.

Although morphological studies have been of limited value in defining species boundaries, they have provided some insight into the affinities of species within the genus, but not without controversy. Species of *Daphnia* are ordinarily partitioned into two subgenera which show clear morphological divergence (Brooks 1957*a*; Schwartz & Hebert 1984). Members of the subgenus *Ctenodaphnia* dominate the fauna of the southern continents, whereas the nominate subgenus is dominant in North America and Eurasia. This biogeographical pattern suggests an ancient origin (Hebert 1978; Benzie 1987) and fossil records confirm that the genus has been in existence for at least 60 Ma, and that closely related genera have existed for at least 100 Ma (Fryer 1991*a*).

The relationships of species belonging to each subgenus are less well defined. The subgenus *Ctenodaphnia* is normally recognized to include two species groups (*atkinsoni*, *similis*) that show a number of morphological differences, but some species are not easily assigned to either group. Also, researchers have argued that many species now assigned to the genus *Daphniopsis* should be assigned to this subgenus (Hrbácek 1987; Fryer 1991*b*). Traditionally, members of the subgenus *Daphnia* have been assigned to either the *pulex* or *longispina* groups, primarily based on the size of the medial pecten on the post-abdominal claw. A few 'orphan' taxa have unclear affinities. For example, *D. curvirostris* has the prominent pecten typical of the *pulex* group, but its chromosome number (Beaton & Hebert 1994) and certain morphological features suggest a closer relationship with the *longispina* group. Clearly, alternative characters are required to verify species affinities.

Allozyme analyses have been used to distinguish closely related species pairs from more distantly related taxa (Hebert 1987). Studies of allozyme divergence have also provided a first indication of the interval over which modern lineages have been extant. Available

data suggest that even the most closely related species pairs generally have separate evolutionary histories of a million or more years, whereas members of different species groups within a subgenus show genetic divergences that suggest their origin more than 20 Ma BP (Hebert & Finston 1993). However, as allozyme characters are of limited value in cladistic analyses (see Murphy 1993), these studies have provided only limited information on species relationships.

Two recent studies have employed sequence analysis of the 12S rRNA mtDNA gene to extend understanding of relationships within the genus. A comprehensive study of the Holarctic members of the *longispina* group provided evidence for both its monophyly, and for the ancient origin of its component lineages (Taylor *et al.* 1995). Focusing on a smaller number of North American species, Lehman *et al.* (1995) found evidence for the polyphyletic origin of the *pulex* group, noting the closer relationship of one species (*D. curvirostris*) to a member of the *longispina* group. Their results also challenged the validity of subgeneric boundaries by showing that members of the *longispina* group were more closely related to species of *Ctenodaphnia* than to other members of the subgenus *Daphnia*.

This study provides the first comprehensive appraisal of systematic relationships among the species of *Daphnia* found in North America. Conclusions are based upon the analysis of sequence diversity in the 12S rRNA gene of the mitochondrial genome. The extent of sequence divergence among taxa is also used to estimate both the age of individual taxa, and of distinct clades within the genus. Furthermore, a new systematic classification is proposed reflecting the evolutionary history of *Daphnia*.

2. MATERIALS AND METHODS

(*a*) *DNA amplification and sequencing*

Isolates were obtained for 32 of the 33 *Daphnia* species which inhabit North America. The sole species excluded from our study, *D. brooksi*, is known from only nine individuals and may represent an introduced species, as it closely resembles *D. barbata*, which is common in Africa (Dodson 1985). For the remaining species, DNA was isolated either from isoclonal laboratory cultures, or from cryopreserved individuals from field collections. Before DNA isolation, *Daphnia* species were identified morphologically and taxonomic assignments were confirmed by allozyme analyses. Collection sites for these species are provided in table 1. In most cases, only a single isolate of a species was analysed, because previous studies have shown that intraspecific variation in the 12S rRNA gene of *Daphnia* is very limited (Taylor *et al.* 1995). However, two clones of *D. tenebrosa* were included in the analysis, one a polyploid and the other a diploid. Two populations of *D. pulicaria* from different areas of North America were also analysed. *Daphniopsis ephemeralis*, the only known North American member of this genus (Schwartz & Hebert 1984), was included in our study because of its potential membership in the subgenus *Ctenodaphnia* (Fryer 1991*b*). A member of the genus *Simocephalus*, another daphniid, was used as an outgroup for parsimony analysis. The 12S rDNA sequence data for members of the conventional *longispina* group were obtained from Taylor *et al.* (1995).

Total DNA was extracted by placing single individuals in 30 μl of 6% Chelex 100 (Bio-Rad). Samples were then incubated at 60 °C for 3 h, vortexed, and boiled at 100 °C for 9 mins, cooled to room temperature, centrifuged at 14000 r.p.m. for 1 min, and left overnight at 4 °C. Polymerase chain reaction (PCR) amplifications were conducted in volumes of 50 μl containing 6 μl of template DNA within the Chelex supernatant, 0.4 μM of each primer, 200 μM each dATP, dCTP, dGTP and dTTP, buffer (10 mM Tris-HCl (pH 8.3); 1.5 mM $MgCl_2$; 50 mM KCl), 1 unit of Taq polymerase (Perkin Elmer), and 0.5 μl dimethyl sulfoxide. The primers (5′_ATGCACTTTCCAG-TACATCTAC_3′; 5′_AAATCGTGCCAGCCGTC-GC_3′) were designed from conserved regions within the aligned 12S rRNA genes of *Daphnia pulex* and *Drosophila yakuba* (Genbank accession nos. Z15015 and X03240). PCR amplification involved a 1 min denaturation at 94 °C, followed by 10 cycles of 1 min at 94 °C, 1.5 mins at 53 °C and 1 min at 72 °C, and subsequently 30 cycles of 45 s at 92 °C, 1 min at 53 °C and 1 min at 72 °C. The products of two PCR reactions were combined and purified using Geneclean II glassmilk (Bio-101), then sequenced directly by dideoxynucleotide chain termination (Sanger *et al.* 1977) using T7 DNA polymerase (Pharmacia). An average of 482 b.p. of the 12S rRNA gene (the gene is 753 b.p. long in *D. pulex*; Van Raay & Crease 1994) were analysed from each species by sequencing both DNA strands.

(*b*) *Sequence analysis*

The DNA segment approximately corresponding to nucleotides 221–711 of the *D. pulex* 12S rRNA gene (Van Raay & Crease 1994), was aligned for all species using ClustalW (Thompson *et al.* 1994) with a gap penalty of 10 and with transitions (TS) weighted over transversions (TV) by 2:1. The alignment was then adjusted by eye using the SeqApp 1.9a sequence editor (Gilbert 1992). Nucleotide composition, frequency of gaps and pairwise comparisons of the number of transitions and transversions were calculated by MEGA, version 1.02 (Kumar *et al.* 1993).

Phylogenetic analysis of the sequences was conducted using both distance-based (phenetic) and character-based (cladistic) methods which differ in their use of the data set. Briefly, the phenetic approach reduces variation to a single divergence value for all pairwise comparisons of the taxa. Although this transformation decreases the usable information, it does not ignore autapomorphic and invariant characters. In contrast, cladistic methods use traits that are shared by two or more taxa to infer patterns of ancestry. This sequence data contained 270 variable sites for phenetic analysis and 228 of these characters were informative for cladistic analysis.

Estimates of sequence divergence between all pairs of taxa were corrected using the Kimura two-

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Table 1. *List of* Daphnia *species included in the study, and their collection site*

taxon	waterbody	site
subgenus *Daphnia*		
pulex group		
arenata	pond near Florence	Oregon, U.S.A.
catawba	Wren Lake near Dorset	Ontario, Canada
cheraphila	pond near Buffalo	South Dakota, U.S.A.
latispina	pond near Lakeview	Oregon, U.S.A.
melanica	pond near Zoil	Oregon, U.S.A.
middendorffiana	pond on Longstaff Bluff	Baffin Isld, N.W.T. Canada
minnehaha	pond near Sault St. Marie	Ontario, Canada
neo-obtusa	pond near Bend	Oregon, U.S.A.
obtusa	pond near Chandler	Oklahoma, U.S.A.
oregonensis	pond near Bend	Oregon, U.S.A.
pileata	pond near Atwater	Texas, U.S.A.
prolata	pond near Amarillo	Texas, U.S.A.
pulex	pond near Windsor	Ontario, Canada
pulicaria	Guelph Lake	Ontario, Canada
pulicaria Arctic	pond near Flint Lake	Baffin Isld, N.W.T. Canada
tenebrosa BL[a]	tundra pond near Churchill	Manitoba, Canada
tenebrosa CL[b]	tundra pond near Churchill	Manitoba, Canada
villosa	pond near Soap Lake	Washington, U.S.A.
longispina group		
dubia	Wren Lake near Dorset	Ontario, Canada
mendotae	Center Lake	Indiana, U.S.A.
laevis	pond in Rondeau Park	Ontario, Canada
longiremis	lake on the Melville Peninsula	N.W.T. Canada
dentifera	Old Lake	Indiana, U.S.A.
umbra	pond near Richards Bay	N.W.T. Canada
thorata	Flathead Lake	Montana, U.S.A.
orphan taxa		
ambigua	pond	Florida, U.S.A.
curvirostris	pond near Tuktoyaktuk	N.W.T. Canada
parvula	Columbia Lake in Kitchener	Ontario, Canada
retrocurva	Crooked Lake	Indiana, U.S.A.
subgenus *Ctenodaphnia*		
ephemeralis	pond near Guelph	Ontario, Canada
exilis	pond near Amarillo	Texas, U.S.A.
lumholtzi	Pomme de Terre Lake	Missouri, U.S.A.
magna	pond near Crescent Lake	Nebraska, U.S.A.
salina	Shoe Lake	Saskatchewan, Canada
similis	pond near Soap Lake	Washington, U.S.A.

[a] BL designates a melanic *tenebrosa*.
[b] CL designates an unpigmented *tenebrosa*.

parameter model (Kimura 1980). Phenetic analysis of the resulting distance matrix was carried out by the unweighted pair-group method with arithmetic means (UPGMA; Sneath & Sokal 1973) and by neighbour-joining (N-J; Saitou & Nei 1987) using MEGA 1.02 (Kumar *et al.* 1993). The two methods make different assumptions about the molecular evolutionary process. UPGMA assumes that the data are ultrametric. Although this property is seldom met in sequence analyses of divergent taxa (or even among some closely related taxa), UPGMA reconstructions are reasonably accurate (Sourdis & Krimbas 1987). This method also facilitates investigations into the ages of species groups when coupled with information on rates of sequence divergence. In contrast, the N-J method does not rely on the clock-like behaviour of base substitutions. Confidence is increased when these two methods converge on a single topology of phylogenetic relationships (Kim 1993). All nucleotides were included in this analysis, but gap sites were deleted. Analyses were also done by using a Log determinant (LogDet) transformed distance matrix to assess the effects of shifts in nucleotide composition on tree structure (Lockhart *et al.* 1994). Other analyses eliminated stem sites based on the secondary structure model of the *D. pulex* 12S rRNA (Van Raay & Crease 1994). A molecular clock for the 12S rRNA gene was adopted from Taylor *et al.* (1995), calibrated according to methods described by Lynch & Jarrell (1993), or by Brower (1994) for closely related taxa.

Cladistic analysis was performed by maximum parsimony (MP) using PAUP, version 3.1.1 (Swofford 1993) and Hennig86 (Farris 1988). Parsimony procedures search for phylogenetic topologies that minimize the number of evolutionary events (or tree length) required to interpret the data set. This approach is valid when homoplasies within the data set do not outweigh the phylogenetic signal. To examine

the impact of homoplasy without eliminating too many characters, transversions were weighted over transitions by 1:1, 2:1, or 4:1, depending upon the divergence of the lineages analysed. Homoplasy was measured using the consistency index (CI; see Wiley *et al.* 1991), although it is sensitive to autapomorphies and symplesiomorphies, and is not consistent across data sets (Archie 1989). Gaps were coded as missing characters because of difficulties in modeling insertions and deletions (indels). After generating phylogenetic hypotheses which included all taxa, the nodes within monophyletic groupings were further analysed by choosing functional outgroups within sister clades. Choosing less distantly related species as an outgroup reduces the probability of multiple nucleotide substitutions per site (noise obscuring phylogeny). The confidence in each grouping was assessed by: (i) plotting 100000 trees drawn at random from all possible topologies as a function of tree length to obtain a g_1 kurtosis statistic (Hillis & Huelsenbeck 1992); (ii) the PTP (Permutation and Tail Probability) test using 1000 randomized datasets generated by character state permutation (Faith & Cranston 1991); and (iii) bootstrapping using 200 pseudo-replicates (Felsenstein 1985). Following the cladistic analyses, g_1 statistics were calculated using PAUP and PTP tests were performed using Random Cladistics, version 2.1.0 (Siddall 1994). Because identical taxa distend PTP values (Faith & Cranston 1991), only a single representative of species with less than 1% sequence divergence was included in the tests. The outgroup character states were not randomized. Bootstrapping was also done using Random Cladistics, which provided bootstrap proportions on the phylogenetic hypothesis, even though the validity of using bootstrap analysis to assess confidence has been questioned (see Hillis & Bull 1993). Character state changes were investigated using MacClade, version 3.04 (Maddison & Maddison 1992), so to infer patterns and rates of molecular evolution in *Daphnia* mtDNA.

3. RESULTS

(*a*) *12S rDNA sequence diversity*

Compositional variation was apparent within the 503 b.p. fragment of the 12S rRNA gene compared for the 32 species of *Daphnia*. The 12S rRNA gene was A-T rich in all species, with an average A-T content of 67.2%. However, significant mean deviations from stationarity were observed between species in the *pulex* and *longispina* groups, whereas small differences were detected even among closely allied taxa. For example, *D. latispina* possessed the lowest A-T value (61.5%) for members of the *pulex* group, whereas *D. retrocurva* had the highest value at 69.2%. Species of *Ctenodaphnia* showed variation ranging from 67.7% in *D. magna* to 70.4% in *D. lumholtzi*. Members of this subgenus also showed a greater substitution bias than members of the *pulex* and *longispina* groups with, for example, 62.9% of transversions within *Ctenodaphnia* sequences being A-T, versus 58.1% in the *pulex* group. The above observations may be important to phylogenetic analyses, for compositional bias within ancient clades, associated with long periods of anagenesis and a rapid rate of nucleotide substitution, can induce state-specific homoplasy using MP and confound phylogenetic inferences (Lockhart *et al.* 1992; Collins *et al.* 1994).

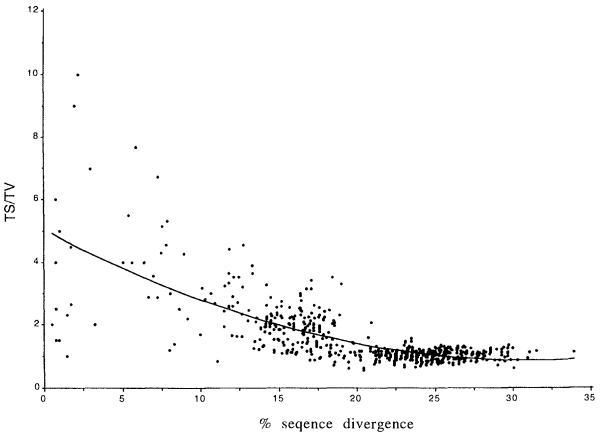

Figure 1. Plot of the transition/transversion (TS/TV) ratio and sequence divergence for all pairwise combinations of the 33 taxa of *Daphnia*. A second order regression curve is drawn.

Corrected pairwise sequence divergence estimates among the 32 species ranged from 0.2–33.0%. The largest distance, between members of the *longispina* group and *Ctenodaphnia*, was 30.8% whereas members of the *pulex* and *longispina* groups showed 29% divergence on average. Interestingly, the maximum sequence divergence between extant species within each of the two groups of *Daphnia* and within *Ctenodaphnia* were very similar, ranging from 18.8–22%. At this point (ca. 20%), pairwise divergences approximated transitional saturation (where TS/TV = 1; see figure 1). Within the *pulex* group, only sequence comparisons involving *D. ambigua* approached saturation. By contrast, comparisons involving *D. curvirostris*, *D. laevis* and *D. dubia* each approached saturation within the *longispina* group. Within the subgenus *Ctenodaphnia*, all sequence comparisons, except those with *D. exilis* were saturated with transitions.

(*b*) *Phenetic analysis of 12S rDNA sequence divergence*

The UPGMA dendrogram of Kimura distances showed that North American members of the genus *Daphnia* were divisible into three lineages displaying more than 20% sequence divergence (figure 2). One group included all the members of the subgenus *Ctenodaphnia*, as well as *Daphniopsis ephemeralis*. We therefore suggest that this species be reassigned to this subgenus. The other two groups were comprised of species that have in the past been assigned to the subgenus *Daphnia*. Because of their marked genetic divergence, we suggest that these species be partitioned into two subgenera (*Daphnia* and *Hyalodaphnia*) as first suggested by Schödler (1866). We also propose that taxa in each of these subgenera be assigned to species complexes that delineate species which are either known to hybridize, or are likely able to hybridize. The most genetically divergent species that regularly produce hybrids displayed less than 14% sequence

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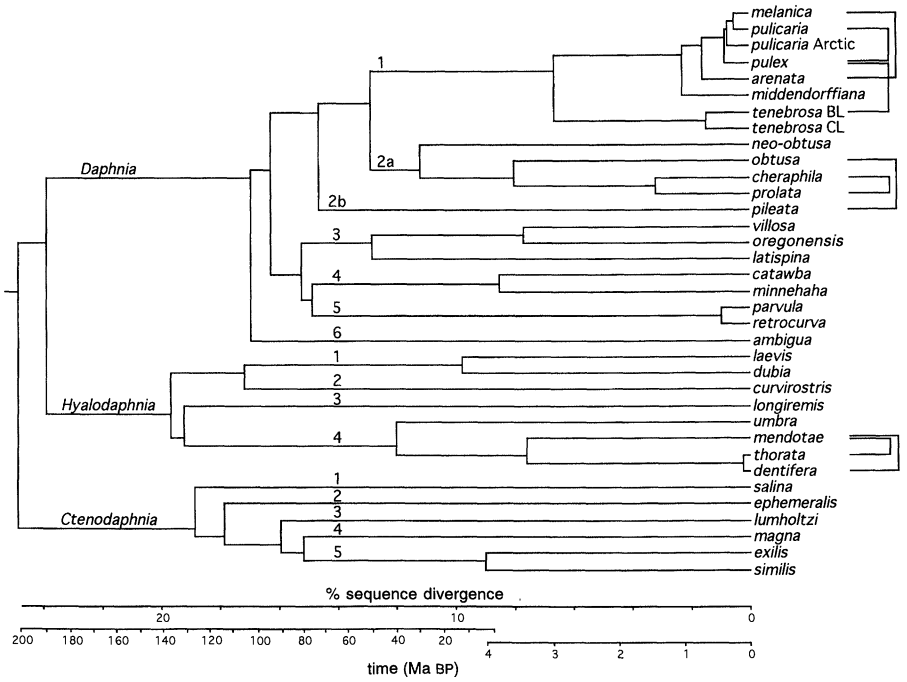

Figure 2. A UPGMA tree of 12S rRNA gene nucleotide variation in *Daphnia*. The arthropod mtDNA clock (Brower 1994) of 2.3% sequence divergence/Ma was used for closely related taxa (up to 9% divergence). For divergence values in excess of 10%, Lynch & Jarrell's (1993) estimated substitution rate of 0.489%/Ma was employed, where the asymptotic identity (I∞) for our data was 0.281. Lines join taxa known to hybridize. Numbers indicate species complexes within each of the three subgenera proposed in this new classification of the genus *Daphnia*. The six complexes within the subgenus *Daphnia* are: *pulex* (1), *obtusa* (2), *villosa* (3), *catawba* (4), *retrocurva* (5), *ambigua* (6). Complexes within the subgenus *Hyalodaphnia* are: *laevis* (1), *curvirostris* (2), *longiremis* (3), *longispina* (4). *Ctenodaphnia* complexes are: *atkinsoni* (1), *ephemeralis* (2), *lumholtzi* (3), *magna* (4), *similis* (5).

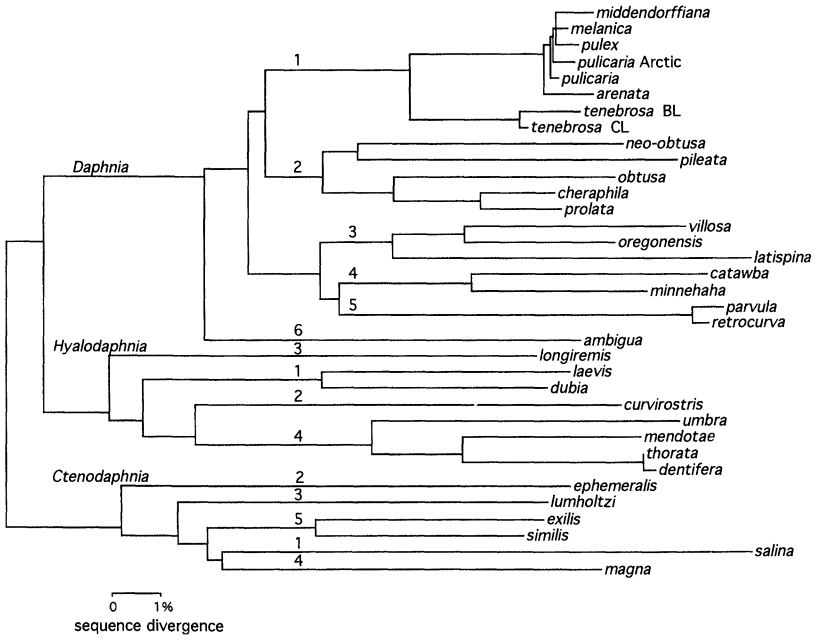

Figure 3. A neighbour-joining tree of 12S rRNA gene variation in the genus *Daphnia*. Numbers on branches denote species complexes within the three subgenera, with names corresponding to those of figure 2.

divergence within the 12S rRNA gene (figure 2). Based on this criterion, the North American fauna includes six species complexes within the subgenus *Daphnia*, five species complexes of *Ctenodaphnia* and four of *Hyalodaphnia*. Although *D. pileata* slightly exceeds this divergence criterion, we place it in the *obtusa* complex for reasons that are detailed later.

Both the UPGMA and N-J analyses, using the same unweighted data set (figure 2 and figure 3), showed that the six members of *Ctenodaphnia* formed a monophyletic group, ancestral to *Daphnia* and *Hyalodaphnia*. Furthermore, the N-J tree confirmed the existence of the same 15 species complexes within the genus and agreed with UPGMA estimates of the large sequence divergences between groupings. The two trees were largely congruent regarding relationships among these

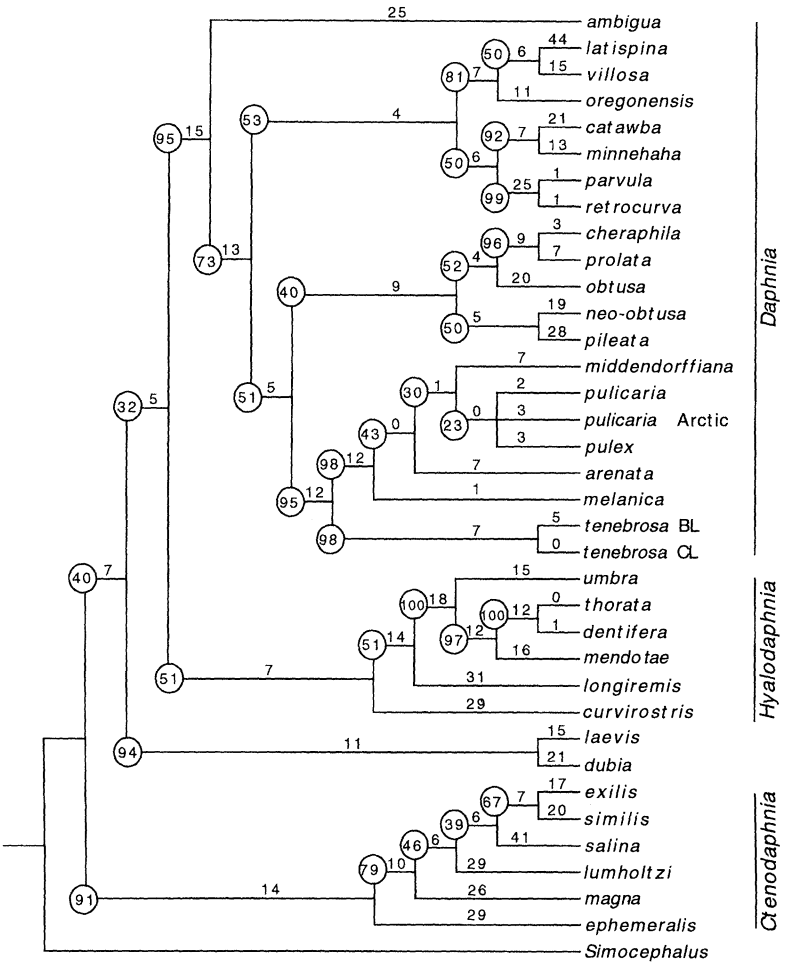

Figure 4. Fifty percent majority rule consensus of seven most parsimonious trees (length 1186, CI = 0.41, RI = 0.60 with 270 characters). Transversions and transitions were weighted equally. Trees were resolved from an heuristic search; taxa were added using the SIMPLE option with MULPARS and steepest descent options invoked and with branch swapping by the tree bisection-reconstruction algorithm. No other MP trees were found when taxa were added randomly with 25 replications and with 10 trees being held at each step. Within the *pulex* complex, four of the seven trees placed *D. melanica* at the basal node and placed *D. arenata* at the next higher branch. *D. middendorffiana* was the sister species to the unresolved (*pulex*, *pulicaria*) clade in 5 of the 7 trees. The number of characters changing unambiguously, including all variable characters, are shown on the branches. Bootstrap percentages from 200 pseudo-replicates are shown in circles. Tree length using informative characters only was 1135 steps. Aligned sequences using all nucleotides are available upon request.

species complexes but, there were some differences. In contrast to UPGMA, the N-J algorithm indicated that the *obtusa* complex included *D. pileata*. As well, it suggested that *D. curvirostris* was not a sister group to the *laevis* complex and that *longiremis* was the ancestral *Hyalodaphnia*. Also, between both analyses, there was no common assemblage of species complexes within *Ctenodaphnia*.

The effect of differing nucleotide frequencies on tree topologies was assessed by constructing a UPGMA tree using LogDet transformed distances (tree not shown). The tree was congruent with figure 2, except that *D. ambigua* was recognized as a more derived complex within the subgenus *Daphnia*, and *D. ephemeralis* was the least derived species of *Ctenodaphnia*. This analysis confirmed that shifts in nucleotide composition have little impact on phenetic tree structure.

To test the effects of character non-independence, stem sites were eliminated from the data set using the 12S rRNA secondary structure model proposed by Van Raay & Crease (1994). In *Daphnia*, 60% of the nucleotide characters from the 12S rRNA fragment are thought to be non-independent due to Watson-Crick base pairing (see Wheeler & Honeycutt 1988). Neighbour-joining only loop sites produced a tree largely congruent with figure 3, displaying only two minor topological differences among the species complexes (not shown). The *villosa* complex was indicated as a sister group to *retrocurva*, whereas the *similis* complex was the least derived member of *Ctenodaphnia*.

(c) *Cladistic analysis of 12S rDNA sequence divergence*

Maximum parsimony analysis of the 12S rDNA sequences using an heuristic search found seven equally parsimonious trees (figure 4). These trees were identical at all nodes, except for the positioning of branch points describing the relationships among five members of the closely related *pulex* complex. There were only 26 variable sites within the sequences from the latter group and only two were informative. Clearly, more

characters are needed to resolve relationships within this clade.

Using *Simocephalus* as an outgroup, four divisions within the genus *Daphnia* were suggested, with members of the subgenus *Ctenodaphnia* forming a monophyletic group that was ancestral to *Daphnia*, *Hyalodaphnia* and the divergent *laevis* complex. There was, however, little support for the node showing a polyphyletic origin of *Hyalodaphnia*. Only 32% of the bootstrap pseudo-replicates placed *laevis* and *dubia* outside of the clade, and more importantly, only one extra step in the tree forced the *laevis* complex back within the subgenus. *Daphniopsis* was confidently grouped with the *Ctenodaphnia* and orphan taxa were clearly assigned membership within the two other subgenera. Hence, *D. curvirostris* is a member of the subgenus *Hyalodaphnia*, whereas the other three species (*ambigua*, *parvula* and *retrocurva*) belong to the subgenus *Daphnia*. The g_1 statistic (-0.49) of 100000 random trees indicated strong phylogenetic signal from the total data ($P < 0.01$; Hillis & Huelsenbeck 1992).

(*d*) *Relationships within the subgenus* Daphnia

Cladistic analysis identified *D. ambigua* as the ancestral species of the subgenus *Daphnia*, and provided support for the group memberships of the other five species complexes (figure 4). The two clones of *D. tenebrosa* formed the basal group of the first clade, the *pulex* complex. Support for its monophyletic origin was very strong. PTP testing revealed non-significant patterning of the data (PTP < 0.01), and the bootstrap resampling value at the basal node of the clade was 95%. Difficulties in resolving relationships within the *pulex* complex were linked to the small number of characters distinguishing its members. Interestingly, the two clones of *D. tenebrosa* showed substantial divergence with five unambiguous character changes (including three transversions). Evidently, the melanic polyploid clone of the taxon is as divergent from its diploid unpigmented conspecific as other species of the *pulex* complex are from one another.

The *obtusa* complex, the sister clade to the *pulex* complex, was comprised of five species (*cheraphila*, *prolata*, *obtusa*, *neo-obtusa*, *pileata*). From the number of character state changes among these taxa, it is clear that members of the *obtusa* complex are far more divergent than the species comprising the *pulex* complex. Although PTP testing indicated non-random associations and added support for the monophyletic origin of the clade (PTP < 0.01), the result from bootstrap replicates did not confidently authenticate this lineage. The species topology was, however, consistent for all weightings of transversions and transitions.

All of the seven equally parsimonious trees supported the third, fourth and fifth sister complexes (*villosa*, *catawba*, *retrocurva*) within the subgenus *Daphnia* (PTP $= 0.01$). However, exhaustive searches using *D. ambigua* as the functional outgroup revealed three equally parsimonious trees when transversions were weighted 2:1 over transitions. One tree was identical to the topology of the clades in figure 4, with *D. oregonensis* as the ancestral species of the *villosa* complex. A second tree did not support the node grouping the *catawba* and *retrocurva* clades. The third revealed an association between *D. villosa* and *D. oregonensis* and shared the same topology as the single most parsimonious tree with transversions weighted 4:1 over transitions. Overall, the bootstrap resampling (95%) and the PTP test (PTP < 0.01) indicated that the subgenus *Daphnia* is monophyletic. The g_1 statistic for the group was -0.58 ($P < 0.01$).

(*e*) *Relationships within the subgenus* Hyalodaphnia

Although there was no structural noise in the data (PTP < 0.01), there was only weak support for the most basal nodes of the subgenus *Hyalodaphnia* (see bootstrap values in figure 4). The cladistic analysis suggested as well that *Hyalodaphnia* could be polyphyletic, even when transversions were weighted 2 times transitions using all species. However, as only one extra step to the tree length is needed to place the *laevis* complex within the subgenus, there is little confidence for its present topological position. Furthermore, contrary to the above findings, an exhaustive search using *magna* and *lumholtzi* as outgroups showed two equally parsimonious trees (length = 404; CI = 72; RI = 63). Both trees assigned *D. curvirostris* as the sister group to an (*umbra* (*mendotae* (*dentifera*, *thorata*))) clade but differed by placing the *laevis* or *longiremis* complex at the ingroup node. However, when these topologies were imposed onto the phylogenetic hypothesis of figure 4, the trees were four and five steps longer. Homoplasy may account for this instability, as the sequences for the *laevis* and *curvirostris* complexes, as well as those of the *Ctenodaphnia* have attained transitional saturation. Although trees with extra steps do not meet the criterion of maximum parsimony, they account for the low bootstraps at basal nodes and suggest possible alternative relationships among species. The g_1 statistic indicated a strong phylogenetic signal from the data set ($P < 0.01$).

(*f*) *Relationships within the subgenus* Ctenodaphnia

The seven most parsimonious trees revealed a single species topology describing relationships among the six members of *Ctenodaphnia* and placed *D. ephemeralis* as the ancestor of the subgenus (figure 4). As ten additional steps to the tree length were needed to branch *D. ephemeralis* outside of the genus, its inclusion is robust. The g_1 skewness statistic for the clade (-0.33) indicated that there was no significant correlation among characters beyond that expected at random ($P > 0.05$). PTP testing at the ingroup node also suggested that no significant cladistic covariation existed between characters (PTP > 0.78). However, when transversions were weighted 2, 4 and 6 times transitions, the same tree was found, with a consistency index of 0.78 and a retention index of 0.4. Weighting transversions was appropriate for members of *Ctenodaphnia*, because pairwise comparisons of nucleotide frequencies suggested that most sequences had attained transitional saturation. The g_1 statistic for the

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weighted data set was -0.37. This number was equal to the critical value ($P = 0.05$), and suggested that there was significantly more phylogenetic signal using weighted characters than within the unweighted data set. Weak confidence indices for the species relationships within the group no doubt stem from extensive homoplasy in the sequence data, compounded by the extremely long branches between taxa. A slower evolving gene is required to provide a defensible MP phylogeny of the group. Even though the topology was consistent for all two-parameter weightings, overall, there is little support for the affinities among species excepting the clear association of *D. exilis* and *D. similis*.

(*g*) *Age of* Daphnia

Sequence divergence among the North American lineages of *Daphnia* is depicted below the UPGMA dendrogram in figure 2, along with a 12S rRNA clock. For divergence values under 9%, the calibration is based on the arthropod mtDNA clock of Brower (1994) whereas Lynch & Jarrell's (1993) calibration is used for higher divergences. The latter clock suggests that the genus *Daphnia* is over 200 Ma old and that speciation in all three subgenera has been occurring for more than 100 Ma. More surprising are the ages of taxa within the subgenus *Daphnia*, especially between taxa which until very recently were treated as conspecific. For example, *D. villosa* and *D. latispina* are almost morphologically indistinguishable, yet they apparently last shared a common ancestor over 50 Ma BP! Within *Hyalodaphnia*, all complexes are ancient, apparently originating over 100 Ma BP. Within *Ctenodaphnia*, *D. salina* and *D. ephemeralis* also originated over 100 Ma BP.

(*h*) *Molecular evolution of the 12S rRNA gene*

The number of gaps introduced into the sequences during alignment varied from three to nine. Most (79%) of the indels were 1 b.p. long, and no gaps were greater than 3 b.p. (aligned sequences are available upon request). Interestingly, a number of indels were conserved and diagnostic for major species groups, and the majority supported species relationships identified through nucleotide comparisons. For instance, all species of *Ctenodaphnia* shared a plesiomorphic deletion of 3 b.p. with *Simocephalus* and, at a second site, they all possessed a gap of 1 b.p., excepting *D. similis*. These characters, which were shared with the outgroup, provided further evidence that the *Ctenodaphnia* are ancestral to the genus. All members of this subgenus also possessed a derived gap of 1 b.p., except for *D. ephemeralis*, supporting its position at the base of the subgenus. Similarly, all species of *Hyalodaphnia* shared a distinctive 2 b.p. deletion with *D. ambigua*, the least derived member of the subgenus *Daphnia*. And at another site, they also acquired a 1 b.p. gap, except the *laevis* complex which retained the ancestral state. Another single b.p. deletion grouped *laevis* with *dubia*. No gap sites were shared between all members of the *Daphnia* subgenus, but a 3 b.p. deletion occurred in the ancestor to the (*villosa*(*catawba*, *retrocurva*)) clade.

Table 2. *Percent nucleotide substitutions within the 12S rRNA gene derived from averages across the total tree shown in figure 2 and for species in each of the three subgenera as estimated using the state changes and stasis option of MacClade* 3.04

	to			
from	A	C	G	T
total tree				
A	–	6.0	22.2	14.4
C	2.2	—	1.0	9.8
G	5.3	0.4	—	0.8
T	12.6	21.7	3.6	—
Daphnia				
A	—	4.4	22.4	12.5
C	3.4	—	1.3	13.7
G	7.2	0.7	—	0.7
T	8.2	22.0	3.5	—
Hyalodaphnia				
A	—	6.3	20.8	12.8
C	3.5	—	1.5	13.3
G	8.2	0.5	—	1.1
T	13.4	16.1	2.5	—
Ctenodaphnia				
A	—	6.1	19.1	15.1
C	1.7	—	0.3	9.7
G	5.8	0.1	—	1.6
T	15.1	20.2	5.3	—

A detailed examination of sequence shifts across the phylogeny indicated important deviations from the Kimura 2-parameter model of molecular evolution (table 2). As expected, the most common base substitutions within the 12S rRNA gene were transitions (A → G, T → C), but surprisingly, A → T transversions were more common than either G → A or C → T transitions. Thus the probability of substitutions is strongly asymmetric. Furthermore, there were substantial differences in the relative frequency of T → A transversions among the subgenera (table 2). Because of the moderately uniform A-T bias among clades, we expected equivalence of the A → T and T → A substitution rate. Yet within the subgenus *Daphnia*, T → A transversions account for only 8.2% of all nucleotide substitutions (24.2% of all transversions), whereas within *Hyalodaphnia* and *Ctenodaphnia*, they represent 13.4 and 15.1% of the nucleotide changes respectively (32.3 and 33.4% of all transversions). By contrast, A → T transversions showed similarity in frequency among *Daphnia* and *Hyalodaphnia*.

Mutations from G → A and G → T were far less common than A → G, indicating that the number of G's within the evolving 12S rRNA gene should be increasing. This is an unexpected result because the sequences are A-T rich and display stationarity. On average, sequence divergence between taxa is smallest in the more speciose subgenus *Daphnia* and greatest in *Ctenodaphnia* suggesting that cladogenesis varies across the genus, increasing from *Ctenodaphnia* to *Daphnia*. The same pattern is present in the average TS/TV ratios for *Daphnia* (1.88), *Hyalodaphnia* (1.41) and *Ctenodaphnia* (1.27). These results imply an association of certain substitutions with branch lengths between species, and

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that mutation rates are not constant for all types of nucleotide changes, not even among transversions.

4. DISCUSSION

Attempts to understand phylogenetic relationships within the genus *Daphnia* have been limited by the constrained morphological diversity of its 100 or so species. Moreover, a high proportion of the few variable traits show exuberant levels of phenotypic plasticity which complicate character state assignments (Brooks 1957*b*; Dodson 1981; Benzie 1986). This study, although based upon the analysis of sequence diversity within a single gene, substantially extends the data available for phylogenetic inference. By examining 32 of the 33 species known from North America, it provides comprehensive information about relationships among taxa for the continent with the greatest species richness of daphniids, which also includes representatives of all major morphological groups within the genus.

Past morphological work has long suggested that species in the genus should be partitioned into two subgenera: *Ctenodaphnia* and *Daphnia* (see Hrbácek 1987). Members of the first subgenus differ considerably in morphology from other members of the genus (Brooks 1957*a*; Schwartz & Hebert 1984), but the nominate subgenus has long been recognized to include two lineages (*pulex*, *longispina*) whose taxonomic status has been unclear. Members of the two groups show consistent divergence in several morphological attributes (Hrbácek 1987). Furthermore, cytogenetic studies have shown that species within the *longispina* group have a diploid chromosome number of 20, whereas those of the *pulex* group have 24 (Trentini 1980; Beaton & Hebert 1994). This study provides additional evidence of their genetic divergence, and suggests that these two groups, together with *Ctenodaphnia*, should be assigned to different subgenera. Molecular support for a shift in the subgeneric classification derives, in large measure, from the similar level of divergence in the 12S rRNA gene for these three clades. However, distinctive patterns of nucleotide deletion in this gene also distinguish members of two of the three subgenera.

Previous morphological studies have supported the close affinity of *Daphniopsis* to *Ctenodaphnia*, and indeed Wagler (1936) transferred its type species to this subgenus. This study supports the reassignment of *Daphniopsis ephemeralis* to *Ctenodaphnia*, a conclusion supported by Fryer's (1991*b*) morphological analysis. There remains a need for a more comprehensive evaluation of the affinities of the other members of this genus, especially those from South America and Australia.

Our analyses indicated that *Ctenodaphnia* are ancestral to the other two subgenera of *Daphnia*. This conclusion disagrees with the results of another recent study of sequence variation in the 12S rRNA gene, which suggested that the *longispina* group (here *Hyalodaphnia*) may be the oldest clade (Lehman *et al.* 1995). Our results do indicate that the resolution of subgeneric relationships is complicated by the ancient age of all three lineages and by the close correspondence in the timing of their diversification. As their ancient origin results in transitional saturation for comparisons among subgenera, state-specific homoplasy from compositional bias constrains the success of maximum parsimony analysis (Collins *et al.* 1994). In such cases, the examination of longer segments of DNA ordinarily aids in the resolution of phylogenetic indeterminacy (Avise *et al.* 1994). The accuracy of maximum parsimony also increases with the number of taxa examined, so long as this enhances 'stemminess' of the tree (Rohlf *et al.* 1990). Our data set is better suited for resolving deep nodes within the genus, because it includes twice the number of characters and three times as many taxa as that of Lehman *et al.* (1995).

Aside from establishing the ancestry of its subgenera, this study permitted the assignment of all species of *Daphnia* in North America to a specific subgenus. This clarification of the phylogenetic positioning of 'orphan' taxa was of particular value. The assignment of *D. curvirostris* to *Hyalodaphnia* shattered evidence for the monophyletic origin of the prominent medial pecten on the post-abdominal claw in the genus, but this conclusion is not surprising given the frequent loss/gain of this trait in other anomopods. The placement of *D. parvula* and *D. retrocurva* within *Daphnia* suggests their origin through neoteny, as they couple a small adult size, with a medial pecten whose shape is similar to that of juveniles of other species in this genus (Hebert 1995). Finally, the placement of *D. ambigua*, which lacks a pecten, as the basal species of the same subgenus, suggests that the shift in prominence of the medial pecten followed the origin of this subgenus.

The extent of 12S rDNA sequence divergence among members of single subgenera showed much variation. Each species of *Ctenodaphnia*, the clade with the lowest taxonomic diversity, showed substantial divergence from its closest relative. The subgenus *Hyalodaphnia* also showed low species diversity, but consisted of one group of closely related species as well as a small number of divergent taxa. The most speciose subgenus, *Daphnia*, included two groups of closely related taxa as well as four other divergent lineages comprised of just one or two species. This variable pattern of divergence in the 12S rRNA gene reveals the value of recognizing species complexes. In establishing a meaningful boundary for membership in a complex, we have employed information on the occurrence of hybridization. Past studies have provided no evidence of hybridization between members of different subgenera or between distinct lineages within a subgenus. Instead, cases of interspecific hybridization occur between species showing less than 14% sequence divergence within the 12S rDNA (Taylor & Hebert 1992; Hebert *et al.* 1993; Hebert & Finston 1995). Although hybrids are not known between all species showing low levels of divergence, many of these exceptions involve taxa with allopatric distributions (see Hebert 1995). The application of this criterion led to the recognition of six species complexes in the subgenus *Daphnia*, four complexes in *Hyalodaphnia* and five in the subgenus *Ctenodaphnia*. As the North American fauna includes

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representatives of all the major morphological types of *Daphnia* and *Hyalodaphnia*, it seems likely that no further complexes await identification in these subgenera. However, because North America is less representative for *Ctenodaphnia*, the discovery of new complexes in this group seems possible.

Previous work on arthropods has shown that over intervals of a few million years or less, mtDNA shows sequence change at a rate of approximately 2.3% per Ma (Brower 1994). When considered over longer intervals (> 9 Ma), the apparent rate of sequence change within the 12S rDNA declines to approximately 0.5% per Ma (Lynch & Jarrell 1993), because of the invisibility of mutations at sites where multiple transitional events have occurred. Evidence of this effect was seen for the genus *Daphnia* with the transition/transversion ratio declining sixfold as sequence divergence rose to 20%. This ceiling for transitional saturation is similar to that of vertebrates, however, saturation of 12S rDNA is not apparent in single mammalian genera, but is instead evident in comparisons involving representatives from different orders (Mindell & Honeycutt 1990). This difference suggests that the three subgenera of *Daphnia* are ancient, and, based on a divergence rate of 0.5% per Ma, subgeneric differentiation occurred approximately 180 Ma BP. The North American fauna includes 15 species complexes which have persisted for at least 50 Ma. Most of these complexes include only one or two species in North America, and only four complexes (*longispina*, *obtusa*, *pulex*, *retrocurva*), show evidence of active speciation in the past 1–3 Ma. Although a global survey will undoubtedly enrich species diversity in several complexes, speciation in the genus has clearly been constrained (excluding possible extinctions).

Phylogenetic reconstructions utilizing information from mtDNA sequence divergence can be problematic when introgression is important. Although previous work has established that F_1 hybrids are common between some species of *Daphnia*, introgression appears rare, apparently as a result of the low fitness of F_2 and backcross individuals (Hebert 1985; Taylor & Hebert 1992). This conclusion has been reinforced by molecular studies on the *D. longispina* group (Taylor *et al.* 1995) which showed, with a single exception, close correspondence between phylogenies based on the analysis of mtDNA and nuclear genes. Moreover, the sole exception involved a case where hybridization led to nuclear rather than mitochondrial introgression. Although there is a need to verify these results with studies on other genes, there is no reason to suspect that conclusions from this study have been seriously influenced by the restriction of analysis to the mitochondrial genome.

Species phylogenies using mtDNA information from a single gene can be inaccurate because the data set may fail to recover the actual sequence of speciation events, providing instead, a legitimate gene pedigree (see Avise & Ball 1990). This problem arises when ancestral polymorphic mitochondrial haplotypes persist through a number of cladogenetic events. Some studies which have examined species phylogenies based on more than one mitochondrial gene yielded congruent trees (e.g. Funk *et al.* 1995), whereas others have not (e.g. Cameron *et al.* 1992). However, in all cases in which a number of daphniids from various North American conspecific populations were sequenced, they formed monophyletic groupings (Taylor *et al.* 1995; J. K. Colbourne, unpublished data). Therefore, this analysis provides a reasonable estimate of species relationships among *Daphnia*, not simply a gene genealogy.

This study has provided new insights into factors governing both the occurrence of hybridization and the evolution of reproductive isolation within the genus. Within *Hyalodaphnia*, hybrids are so far known only among species of the *longispina* complex (Taylor & Hebert 1992; Flössner 1993; Schwenk 1993). In this group, hybrids between *D. mendotae* and *D. dentifera*, species showing 7.6% sequence divergence at the 12S rRNA gene, are particularly common. Within the subgenus *Daphnia*, hybrids are known for only two of the six species complexes. Hybrids in the *pulex* complex are common between two species pairs (*pulex-pulicaria*, *arenata-melanica*; Hebert *et al.* 1989; Crease & Lynch 1991; P. D. N. Hebert, unpublished data) showing 1 and 2% sequence divergence respectively. Hybrids are also known between *D. pulex* and *D. tenebrosa* (Dufresne & Hebert 1994), but they are both rare and show unusual genetic attributes, which are perhaps associated with the more substantial divergence (5%) of these taxa. Within the *obtusa* complex, hybrids are most common between *D. prolata* and *D. cheraphila* (Hebert & Finston 1995), species showing 3% sequence divergence. *D. obtusa* and *D. pileata* are the most genetically divergent (14%) pair of species known to hybridize in North America, but their hybrids are both extremely rare and probably sterile (Hebert & Finston 1995). The restriction of hybridization to closely related taxa is reinforced by the absence of interspecific hybrids in the North American *Ctenodaphnia*. Interestingly, Australian members of the same subgenus show rampant hybridization (Hebert & Wilson 1994) coupled with limited (< 10%) sequence divergence of 12S rDNA (C. C. Wilson, J. K. Colbourne & P. D. N. Hebert, unpublished data). Collectively, these results suggest that prezygotic reproductive isolation is often absent between daphniid species whose level of divergence suggests their separate evolution for intervals of 4 Ma or less. However, in a few cases, species appear to lack prezygotic isolation, despite their apparent independent evolution for over 50 Ma! Daphniids are clearly slow to evolve reproductive isolation, but they are not unique in this regard. For example, Wilson *et al.* (1974) provided evidence, using an albumin clock, for hybridization between frog species which last shared a common ancestor some 21 Ma BP.

This study has provided an initial indication of the affinities among species groups which comprise each of the subgenera of *Daphnia*, but there is a need to better define these patterns of relationship. The close correspondence of results gained through phyletic and cladistic analysis suggests that the latter approach was not seriously affected by the violation of assumptions in relation to the probability of specific nucleotide

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changes. Moreover, the information obtained on the incidence of specific nucleotide substitution probabilities in 12S rDNA may aid in developing a more accurate description of taxonomic relationships, especially if other mitochondrial and nuclear genes show similar asymmetries in mutational events.

In summary, this study has provided the first comprehensive molecular information on systematic relationships within the genus *Daphnia*. The data suggest the need for a taxonomic revision that recognizes the existence of three subgenera and their component species complexes, which include taxa previously assigned to the genus *Daphniopsis*. The extent of 12S rDNA sequence divergence among members of the genus suggest its origin during the Mesozoic, a result congruent with the fossil record. The molecular data provide additional evidence for the antiquity of many lineages within the genus, an unexpected result given their limited divergence in gross morphology. The study also indicates that only four of the 15 North American clades have shown active speciation over the last 3 Ma. Three of these lineages show frequent interspecific hybridization, suggesting that reproductive isolation evolves both slowly, and as an incidental byproduct of genomic divergence.

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