Biological Journal of the Linnean Society, 2003, **79**, 629–643. With 3 figures

The evolutionary history of the Chydoridae (Crustacea: Cladocera)

VERONIKA SACHEROVÁ[1]* and PAUL D. N. HEBERT[2]

[1]*Department of Hydrobiology, Charles University Prague, Viničná 7, Prague 2, CZ-128 44, Czech Republic*
[2]*Department of Zoology, University of Guelph, Ontario, N1G 2W1, Canada*

Received 24 May 2002; accepted for publication 29 January 2003

Although much is known about the evolutionary history of the pelagic 'cladocerans', there is little information on benthic families such as the Chydoridae. In this study, we examine the phylogenetic history of 37 chydorid species using sequence variation in two mitochondrial genes, COI and 16S rDNA, and one nuclear gene, 18S rDNA. The four recognized subfamilies of chydorids (Eurycercinae, Saycinae, Aloninae and Chydorinae) were well supported, being separated by large sequence divergences of 14.3–16.4%. By contrast, the existing taxonomic system appears to be less clear at a generic level, since many genera (e.g. *Alona*, *Chydorus*, *Pleuroxus*) consist of an amalgam of distantly related species. However, among those genera which are monophyletic, levels of divergence are very high, suggesting that they originated somewhere in the mid-Palaeozoic. The factors involved in promoting diversification in this group are discussed.

ADDITIONAL KEYWORDS: 16S rDNA – 18S rDNA – benthic – COI – diversification – littoral – phylogeny – taxonomy.

INTRODUCTION

The cladoceran crustaceans are becoming an increasingly important model system for studies that aim to elucidate processes underpinning ecological and morphological diversification. Pelagic lineages have been the focus of past studies, but it has become apparent that the evolutionary processes important in diversification of 'Cladocera' (the validity of using the term Cladocera to indicate a monophyly is not yet established) can only be clarified by extending analysis to benthic lineages (Colbourne & Hebert, 1996; Taylor, Hebert & Colbourne, 1996). The motivation for this work lies in the striking difference in taxonomic diversity between benthic and pelagic clades. For example, the two cladoceran families (Chydoridae and Macrothricidae) dominated by benthic forms include 40 and 16 genera, respectively, while the nine pelagic families of 'Cladocera' include only one to five genera (Smirnov, 1971, 1996; Dodson & Frey, 1991). This study examines the patterns of genetic diversity among chydorids to gain a better sense of their past history of morphological diversification.

Due to their great morphological diversity, chydorids have attracted attention since the 18th century (reviewed by Frey, 1995). Morphological study has indicated that chydorids can be partitioned into four subfamilies (Frey, 1967). Differences in the head pores, mandibular articulation, trunk limb setation and postabdominal morphology serve to delineate the Eurycercinae, Saycinae, Aloninae and Chydorinae (Fig. 1). The Eurycercinae are regarded as the most primitive and the Chydorinae as the most advanced, reflecting the view that the number of components in homologous structures decreases and becomes fixed in advanced lineages (Geodakyan & Smirnov, 1968; Smirnov, 1969). For example, there is a shift from large, variable clutches in the Eurycercinae and the Saycinae to a fixed clutch size of two in the Aloninae and the Chydorinae. These and other similar shifts in morphology are linked, as well, to a reduction in size

*Corresponding author. E-mail: vsach@natur.cuni.cz

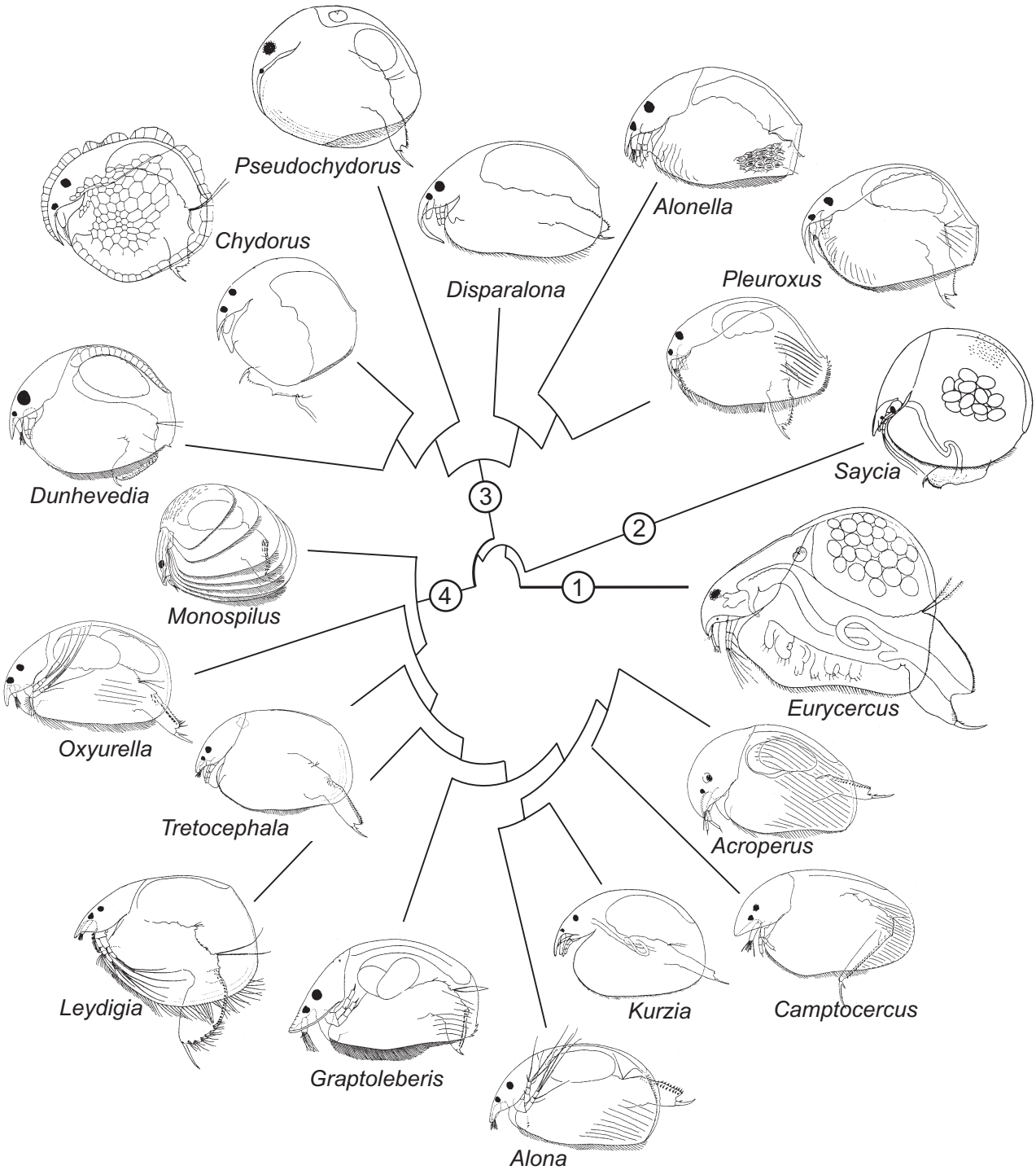

Figure 1. Morphological phylogeny of the chydorid genera (Smirnov, 1971). Drawings modified from Lilljeborg (1900), Smirnov (1971, 1996), Frey (1987) and Alonso (1996). The numbers indicate members of the four subfamilies: 1, Eurycercinae; 2, Saycinae; 3, Chydorinae; 4, Aloninae.

from a peak body length of 6 mm in *Eurycercus glacialis* (Eurycercinae) to about 0.2 mm in *Alonella nana* (Chydorinae). This study examines the phylogenetic relationships of 37 chydorid species to ascertain both the validity of the four subfamilies recognized on morphological grounds and their affinities.

Although chydorid species have been assigned to genera based on the form of their carapace, postabdomen, first and second antenna, these features do not provide any clear insight concerning generic affinities in each subfamily (Smirnov, 1966). Moreover, many of these morphological features are size-, age- or sex-dependent (Frey, 1982a). As a result, the phylogenetic relationships of genera within subfamilies have been based on general rules such as the assumption that the most advanced taxa are specialized forms (Fryer, 1968) or taxa that exploit rare or extreme niches (Frey, 1980). The study examines the validity of existing generic boundaries and the age of species lineages which compose them.

It is believed that the ancestors of modern 'Cladocera' were benthic or littoral animals which probably originated in the mid-Palaeozoic (Fryer, 1995). The Chydoridae share a large number of morphological and ecological features with the proposed ancestor of anomopods, a group of five 'cladoceran' families (Fryer, 1995). Although the first anomopod fossils date from the Mesozoic, these indicate that chydorids were well diversified by this time (Smirnov, 1992). Moreover, fossilized resting stages (ephippia), which resemble those of the more 'derived' anomopod genera (*Daphnia*, *Ceriodaphnia*, *Simocephalus* and *Moina*), date from the same age (Goulden, 1968; Fryer, 1991), suggesting an explosive diversification of the group. Molecular studies based on mitochondrial DNA have indicated very deep divergences within the genus *Daphnia* contrasting with many morphological similarities of its component species (Taylor *et al*., 1996; Colbourne, Hebert & Taylor, 1997). Both the molecular data and the fossil record suggest that this genus is as old as 200 Myr, supporting the evidence of a rapid origin of lineages. The Chydoridae, which were diversified at the time when the first daphniid ephippia appear in the fossil record, may well be even older. However, there has been no critical assessment of the age of chydorid lineages.

All members of the four chydorid subfamilies are benthic or littoral, living predominantly on and in macrophytes, plant detritus and organic sediments. They occur in a broad range of aquatic habitats and even in moist sphagnum moss (Frey, 1980). Their range in size and diversity of feeding behaviours probably played important roles in niche separation and possibly speciation (Fryer, 1995). The distribution and ecology of most species is tightly connected with the occurrence of aquatic plants, with species-specific plant assemblage associations (Hann, 1981; Chengalath, 1987; Tremel *et al*., 2000). Given this strong association, it remains unclear if diversification processes in aquatic plants and chydorids occurred simultaneously or if the family Chydoridae was well-diversified before the radiation of aquatic angiosperms. Both benthic origin and diversification patterns can be traced either by fossil record or phylogenetic relationships (Rigby & Milsom, 1996), and a molecular study of extant chydorids may shed light on the origin of contemporary taxa.

As a first step towards understanding the patterns of past evolutionary change in the Chydoridae, and the role of ecological associations in their diversity, we examined the extent and patterning of sequence divergences within three gene regions. The protein-coding mitochondrial cytochrome *c* oxidase subunit I (COI) gene and two ribosomal genes, the mitochondrial large subunit 16S rRNA (16S) gene and the nuclear small subunit 18S rRNA (18S) gene, were used to deduce phylogenetic relationships within chydorids. These three gene regions were chosen to enable resolution at the specific (COI), generic (16S) and subfamilial (18S) levels. COI is the slowest-evolving protein-coding gene within the mitochondrial genome (Lynch & Jarrell, 1993) and is suitable for dating late Mesozoic (Kumazawa & Nishida, 1993) or much more recent diversifications by examining amino acid or nucleotide substitutions, respectively. Sequence divergence in 16S rDNA can be used to examine diversification that occurred in the middle Mesozoic, while the conservative regions of 18S rDNA often provide useful information for Palaeozoic diversification (Hillis & Dixon, 1991). Combining information from these three gene regions with differing rates of evolution should provide insights into both the origins of the Chydoridae and the patterns of their radiation.

MATERIAL AND METHODS

Taxa studied

This study examines 37 chydorid species including representatives of 17 genera which were collected from Argentina, Canada, the Czech Republic and New Zealand during 1999 and 2000 (Appendix). Full details on localities for all taxa are available from the corresponding author. Samples were obtained using a 40 μm mesh net and preserved in 90% ethanol. To assure correct identification, several keys were used (Brooks, 1959; Smirnov, 1971, 1996; Pennak, 1989; Dodson & Frey, 1991). Representatives from all four recognized subfamilies within the family Chydoridae were included: Chydorinae (18 species, six genera), Aloninae (15 species, nine genera), Sayciinae (one species, one genus) and Eurycercinae (three species, one genus). The low diversity in the latter two subfamilies reflects the fact that the Saycinae contains only a single species, while the Eurycercinae contains just a single genus.

Six representatives from the family Daphniidae were chosen for use as outgroups. These included two

species from each of the three recognized subgenera: *Daphnia* (*D. pulex*, *D. parvula*), *Hyalodaphnia* (*D. laevis*, *D. dubia*) and *Ctenodaphnia* (*D. exilis*, *D. magna*) (Colbourne & Hebert, 1996). The Daphniidae is a well-established sister-group to the family Chydoridae (Taylor, Crease & Brown, 1999). These six daphniids were employed since the use of several outgroup taxa decreases long-branch attraction when using parsimony methods (Hillis, Moritz & Mabel, 1996).

Molecular protocols

Total DNA was isolated from up to ten individuals of each species using proteinase K methods (Schwenk, 1996). Two mitochondrial genes, COI and 16S, and one nuclear gene, 18S, were amplified via the polymerase chain reaction (PCR). The primers LCO1490 and HCO2198 (Folmer *et al*., 1994) were used to amplify a 658 base pair (bp) fragment of the COI gene. The primers 16Sch-a (5′-GAC TGT GCA AAG GTA GCA TAA TC-3′), and 16Sbr (Palumbi *et al*., 1991) were used to amplify a 412 bp fragment of 16S rDNA gene. The 16Sch-a primer was designed for the family Chydoridae based upon a comparison of 16S sequences from *Acroperus harpae*, *Alona affinis*, *Alonella exigua* and *Pleuroxus denticulatus* that were amplified using a standard primer pair 16Sar and 16Sbr (Palumbi *et al*., 1991). The primers 9F (5′-TGG GGA TCA TTG CAG TCC CCA ATC-3′), designed by T. Crease (pers. comm.), and 2004R (Crease & Colbourne, 1998) were used to amplify a 1995 bp fragment of the nuclear 18S. Each 50 μL PCR contained 10× PCR buffer, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 1 unit of Taq polymerase, 0.5 μM of each primer, and 4–10 μL of DNA template. The PCR protocols for COI and 16S consisted of 1 cycle of 1.5 min at 94°C; 35 cycles of 45 s at 93°C, 1 min at 50°C, and 1 min at 72°C, followed by one cycle of 5 min at 72°C. The PCR protocol for 18S consisted of 1 cycle of 1.5 min at 94°C; 35 cycles of 30 s at 93°C, 30 s at 50°C, and 3 min at 72°C, followed by one cycle of 5 min at 72°C. PCR products were purified using Qiaex II (Qiagen) and sequenced on an ABI 377 automated sequencer (Applied Biosystems) using the Taq FS dye rhodamine sequencing kit. For sequencing, we used amplification primers LCO1490, 16Sch-a and 9F, obtaining the whole sequence for COI and 16S products, and about 750 bp of the 18S product.

Sequence and phylogenetic analyses

The alignment of the COI sequences was carried out in Sequence Navigator (ABI). The alignment was unambiguous as no insertions or deletions were detected. The alignments for the 16S and 18S sequences were adjusted by eye using the proposed secondary sequence structure models of small and large subunit rRNA (De Rijk *et al*., 1997; Van de Peer *et al*., 1997; Crease & Colbourne, 1998; Taylor, Finston & Hebert, 1998). Sections of both ribosomal genes that could not be aligned unambiguously were excluded from subsequent analyses. The alignment is available from the corresponding author.

Preliminary phenetic analyses were performed using MEGA version 2.0 (Kumar *et al*., 2001). Phylogenies were reconstructed in PAUP* 4.0b8 (Swofford, 1998) using parsimony and likelihood criteria. To ascertain if nucleotide composition bias occurred among taxa, χ^2 goodness-of-fit tests were conducted on each gene region. The validity of combining characters from different gene regions was tested by the incongruence length difference (ILD) test (Farris *et al*., 1994; Huelsenbeck, Bull & Cunningham, 1996) in PAUP* using the partition homogeneity method with 100 replicates.

Phylogenies using maximum parsimony (MP) and maximum likelihood (ML) methods were determined for both combined data and for the single gene data. In MP, all characters were unordered and equally weighted. Character states were optimized using accelerated transformation (ACCTRAN). The MP trees were built using heuristic searches on parsimony-informative characters (500 replicates, ten trees held per replicate, sequences added at random, tree bisection-reconnection branch swapping (TBR). The ML trees were reconstructed using the HKY85 + G algorithm which assumes unequal base frequencies and two substitution types (Hasegawa, Kishino & Yano, 1985), and a gamma distribution allowing unequal substitution rates across sequences (Hillis *et al*., 1996). The ML trees were built using heuristic searches on informative characters (one replicate, one tree held per replicate, branch swapping by nearest neighbour interchanges (NNI), starting tree obtained by neighbour joining method). Confidence in recovered trees was assessed in PAUP* by bootstrap analysis with 1000 replicates for MP and 100 replicates for ML (Felsenstein, 1985). The combined data set (18S +16S + COI) was analysed using MP for both nucleotide and amino acid sequence of COI. ML analysis was performed for the combined data set using only the nucleotide sequences for COI. Since several taxa included in this study could not be amplified for 18S or COI, a separate data set of 16S gene sequences was analysed using both MP and ML criteria to reconstruct a more comprehensive phylogeny.

RESULTS

Sequence diversity

The 624 bp sequences of COI were aligned unambiguously as no insertions or deletions (indels) were

detected. The length of the 16S sequences varied from 406 bp in *Graptoleberis testudinaria* to 420 bp in *Eurycercus longirostris* reflecting frequent indels. Examination of the secondary sequence structure models (De Rijk *et al*., 1997; Taylor *et al*., 1998) indicated that regions E18′, G3 and G7 could not be aligned unambiguously and they were excluded from subsequent analyses. This resulted in a 340 bp alignment with 4–7 gaps. The sequences for the 18S fragment also showed length variation (from 720 bp in *Alona setulosa* to 757 bp in *Saycia cooki*) reflecting indels. Examination of the secondary sequence structure model for this gene (Van de Peer *et al*., 1997; Crease & Colbourne, 1998) indicated that most of these events occurred in the V2 and V4 regions. Helices V2-10, V2-E10-1 as well as four short regions corresponding to nucleotides 705–710, 717–718, 737–740 and 755–757 in V4-E23 helix of the *Daphnia pulex* 18S sequence (for codes see Crease & Colbourne, 1998) were excluded from subsequent analyses. This resulted in a 625 bp alignment with 4–10 gaps.

Table 1 shows that the number of parsimony-informative sites varied among genes. Of the 518 informative sites in the combined data set, there were 85 in 18S, 155 in 16S and 278 in COI. If translated to amino acids, the number of informative sites at COI collapsed to just 31. Base frequencies in Table 1 represent those at the informative sites that were used for analyses. The values were similar for the two mitochondrial genes, with an AT content of 70.8% in 16S and 70.9% in COI. The AT-bias was higher for informative sites than for the whole aligned fragment where AT content was 63.7% and 61.6% for 16S and COI, respectively. For 18S, nucleotide frequencies for the 85 informative sites were strongly C-biased, with an AT content of just 36%, vs. an AT content of 47% in the complete aligned fragment.

Table 2 shows pairwise mean uncorrected distances

Table 1. Summary statistics for the sequence data obtained for 28 chydorid species at 16S, 18S and COI, and 37 chydorid species for 16S

	Number of taxa		Length of alignment			Base composition (%) of P.I.S.					
Dataset	Ingroup	Outgroup	Total	Used	Number of P.I.S.	A	C	G	T	χ^2	*P*-value
All nt[a]	28	4	–	1602	518	28.6	18.2	16.2	36.9	239.4	0.000
nt + aa[b]	28	4	–	1176	270	–	–	–	–	–	–
16S[c]	28	4	426	338	155	35.6	14.2	15.0	35.2	130.4	0.006
18S[d]	28	4	750	625	85	13.6	36.4	27.5	22.4	38.8	0.999
COI(nt)[e]	28	4	639	639	278	28.8	15.7	13.4	42.1	321.1	0.000
COI(aa)[f]	28	4	213	213	31	–	–	–	–	–	–
Total 16S[g]	37	6	428	340	155	33.8	14.4	15.1	36.8	143.8	0.133

[a]data set combining all nucleotide sequences for 16S rDNA, 18S rDNA and COI mtDNA.
[b]data set combining nucleotide sequences for 16S rDNA and 18S rDNA and amino acid sequence for COI mtDNA.
[c]16S partition of combined data set.
[d]18S partition of combined data set.
[e]COI partition of combined data set.
[f]COI partition of combined data set.
[g]separate data set for 16S rDNA.
P.I.S. = parsimony-informative sites.

Table 2. Pairwise mean p-distances (per cent) based on nucleotide sequences for three gene regions (16S, 18S, COI) among four chydorid subfamilies and outgroup family Daphniidae

	Pairwise mean between group p-distances (%)			
	Daphniidae	Aloninae	Chydorinae	Saycinae
Aloninae	15.6			
Chydorinae	16.1	15.0		
Saycinae	16.0	15.4	16.4	
Eurycercinae	14.3	15.3	15.6	16.0

among the four chydorid subfamilies and the family Daphniidae calculated from the combined data set for all three gene regions. The distances range from a low of 14.3% between Daphniidae and Eurycercinae to a high of 16.4% between Saycinae and Chydorinae. Table 3 shows both pairwise distances within seven chydorid genera, as well as the greatest divergence within each genus. Distances calculated from partitions demonstrate differences in phylogenetic signal across genes used in this study. The highest divergence values were found for COI nucleotide sequences, which was about 1.5 times greater than those for 16S sequences and aproximately 15 times greater than those for 18S sequences. However, there was great variation among these divergence values among different taxa, suggesting that constraints varied among lineages. For example, although the aligned 18S gene-regions for *Acroperus harpae* and *Camptocercus* were identical, there was a 9-nucleotide insertion in the V2-10 helix (excluded from analysis) of the latter taxon. In the species pair *Chydorus sphaericus* and *Chydorus brevilabris*, there was no difference in COI amino acid sequence, but there was 16.3% divergence at the nucleotide level within the same gene. Similarly, only one amino acid substitution was found between *Chydorus faviformis* and *C. sphaericus*/*C. brevilabris* species pair at the same gene, but they showed 9.5% sequence divergence.

PHYLOGENETIC RECONSTRUCTIONS

There was incongruence for all data set partitions (results of ILD test shown in Table 4), indicating differences in phylogenetic signal in each partition. However, a total-evidence approach was applied as it has been shown that the best phylogenetic hypothesis results from combined partitions analysed simultaneously (Remsen & DeSalle, 1998). MP and ML anal-

Table 3. Mean pairwise percentage sequence distances among species belonging to each of chydorid genera and to the genus *Daphnia*. Figures in parentheses show the maximum divergence value among congeneric taxa. For a description of data sets see footnote of Table 1

	Pairwise mean intrageneric distances and the greatest value (in %)				
Genus	All nt[a] p-distance	16S[c,g] p-distance	18S[d] p-distance	COI(nt)[e] p-distance	COI(aa)[f] p-distance
Daphnia	10.7 (11.8)	9.4 (12.2)[c]	0.6 (1.0)	21.1 (22.6)	1.7 (1.9)
Alona	11.3 (13.1)	13.8 (17.9)[c]	2.3 (3.4)	18.8 (21.8)	2.6 (4.3)
Alonella	12.7	13.7[c]	0.2	22.5	3.8
Eurycercus	9.2 (9.9)	8.7 (10.7)[c]	0.4 (0.6)	17.7 (20.1)	3.3 (4.4)
Pleuroxus	9.5 (12.6)	9.6 (16.1)[c]	3.0 (4.5)	15.7 (19.7)	2.3 (3.4)
Chydorus	9.5 (13.5)	12.6 (18.8)[c]	2.2 (3.7)	15.2 (20.7)	1.4 (2.9)
Acroperus	–	14.4 [g]	–	–	–
Disparalona	–	12.3 [g]	–	–	–

Table 4. Tree statistics. For a description of data sets see footnote of Table 1

	ILD test	MP					ML			
Dataset	*P*-value	#MPT	Length	CI	HI	RI	#MLT	–ln L	Ti/Tv	α
All nt[a]	0.01	3	3509	0.27	0.73	0.39	1	13081.4	2.2	1.02
nt + aa[b]	0.01	1	1193	0.38	0.62	0.59	–	–	–	–
16S[c]	–	13	834	0.35	0.65	0.55	1	3377.8	1.5	1.35
18S[d]	–	28	200	0.60	0.40	0.82	1	1004.0	1.2	13.19
COI(nt) [e]	–	2	2260	0.24	0.76	0.31	1	7691.8	6.1	0.82
COI(aa) [f]	–	148	89	0.48	0.52	0.66	–	–	–	–
Total 16S[g]	–	9	1048	0.28	0.72	0.54	1	4284.5	1.8	1.19

ILD: incongruence length difference test, *P*-values lower than 0.05 indicate data incongruence.
MP: maximum parsimony reconstruction. #MPT: number of most-parsimonious trees. CI: consistency index. HI: homoplasy index. RI: retention index. ML: maximum likelihood reconstruction. #MLT: number of ML trees retained. –ln L: log-likelihood score of a tree. Ti/Tv: transition/transversion ratio. α: the shape parameter of gamma distribution.

yses were, however, also applied to each partition separately to ascertain how each data set contributed to the overall phylogeny. The tree statistics for all partition analyses are summarized in Table 4.

Figure 2 shows the trees which resulted from both MP and ML analyses of both total nucleotides (Fig. 2A,C) as well as MP analysis for the nucleotides for the ribosomal genes coupled with amino acids for COI (Fig. 2B). These trees provided strong support for the generic composition of each subfamily (Eurycercinae, Saycinae, Chydorinae, Aloninae), ranging from 66 to 100% (Fig. 2). The topology was best resolved and consistent within the subfamily Chydorinae.

The results of the MP analysis for the total 16S data set resulted in a number of unresolved nodes (Fig. 3A). The subfamilies Eurycercinae and Chydorinae formed a monophyletic clade with the exception of *Saycia cooki*, the sister-taxon to the subfamily Chydorinae. ML analysis of the same data set provided better resolution (Fig. 3B), with all subfamilies being monophyletic.

Several species were analysed for seven different genera, enabling a test of the monophyly of these groups. Only three of these genera (*Eurycercus*, *Acroperus* and *Alonella*) formed monophyletic clades in all combinations of data and analyses. The genus *Chydorus* formed a monophyletic clade with high bootstrap support in the combined data set and the MP tree of the total 16S data set, but formed two distinct clades with very low support in the ML tree for the total 16S data set. The genus *Pleuroxus* was paraphyletic with the genus *Chydorus* in MP analyses of the combined data (Fig. 2A,B). In ML analysis of the total 16S data, the same genus formed three distinctive clades (Fig. 3B), and was poorly resolved in MP analysis of the same data set (Fig. 3A). There was very deep divergence in 16S between the two *Disparalona* species (14.4%), and the genus was paraphyletic with the genus *Alonella* (Fig. 3). The genus *Alona* never formed a monophyletic clade in any of the analyses.

DISCUSSION

Phylogenetic hypothesis

This study provides the first molecular phylogeny for the cladoceran family Chydoridae. The results from the combined data set support both the recognition of four subfamilies and the genera assigned to them as a result of earlier morphological studies. All three reconstructions place the four subfamilies with very high bootstrap support in the same order: Eurycercinae, Saycinae, Chydorinae and Aloninae. Overall, maximum likelihood analysis provided the most resolved trees with the highest bootstrap support for both total-evidence analysis (Fig. 2) and separate 16S analysis (Fig. 3). The genetic distance values (Table 2) among the four chydorid subfamilies and the family Daphniidae ranged from 14.3% to 16.4%, showing that members of each subfamily are clearly separated from one another.

Despite the past uncertain taxonomic position of some chydorid genera (Smirnov, 1971, 1996), it is now apparent that sequencing studies can provide a clear answer regarding subfamilial assignments. For example, the taxonomic position of the genus *Monospilus*, based on morphology of head pores and trunk limbs, has been uncertain (Frey, 1959; Smirnov, 1966), but the 16S data place it firmly in the Aloninae. The same uncertainty extends to the generic level since many species have been allied on the basis of just a few morphological traits. In this study, we tested seven genera for monophyly. Only three of these genera (*Acroperus*, *Alonella*, *Eurycercus*) consisently formed monophyletic clades; the remainder showed signs of polyphyly. In fact three of the other genera (*Alona*, *Disparalona*, *Pleuroxus*) never formed a monophyletic assemblage in any of our analyses. The case of *Pleuroxus* was particularly interesting because prior morphological work has suggested that its members are partitioned into four subgenera (Frey, 1993). The present study included representatives of three of these groups: the subgenus *Picripleuroxus* (*P. denticulatus*, *P. procurvus*, *P. straminius*, *P. striatus*), the subgenus *Peracantha* (*P. truncatus*), and the subgenus *Tylopleuroxus* (*P. aduncus*, *P. scopuliferus*). Each appeared to be monophyletic and divergent from the others based on morphology of rostrum, antennule, postabdomen and ventral setae on the carapace (Frey, 1993). Our genetic data confirm their divergence and, based on this evidence, it appears that *Pleuroxus* should be partitioned into three or perhaps four genera.

Cases of character convergence have clearly also clouded the definition of generic boundaries in the chydorids (Fryer, 1968; Frey, 1982b). A host of characters have evolved on several occasions, including traits such as body form, carapace sculpturing, carapace melanization and the fate of exuviae (shed or retained). For example, there are several species within the genus *Chydorus* that possess a honeycombed carapace (Frey, 1987). However, they may not be closely related to each other as their males show significant differences in the traits that delineate species boundaries (Frey, 1982b, 1987). In *Chydorus faviformis*, the male loses its shell structure completely and closely resembles the male of *C. sphaericus* (Frey, 1987). Our results (Fig. 3) indeed show a closer genetic affinity of species with (*C. faviformis*) and without (*C. sphaericus*) this trait, than between species which share it (*C. faviformis*, *C. bicornutus*). In other cases, instances of supposed convergence may actually

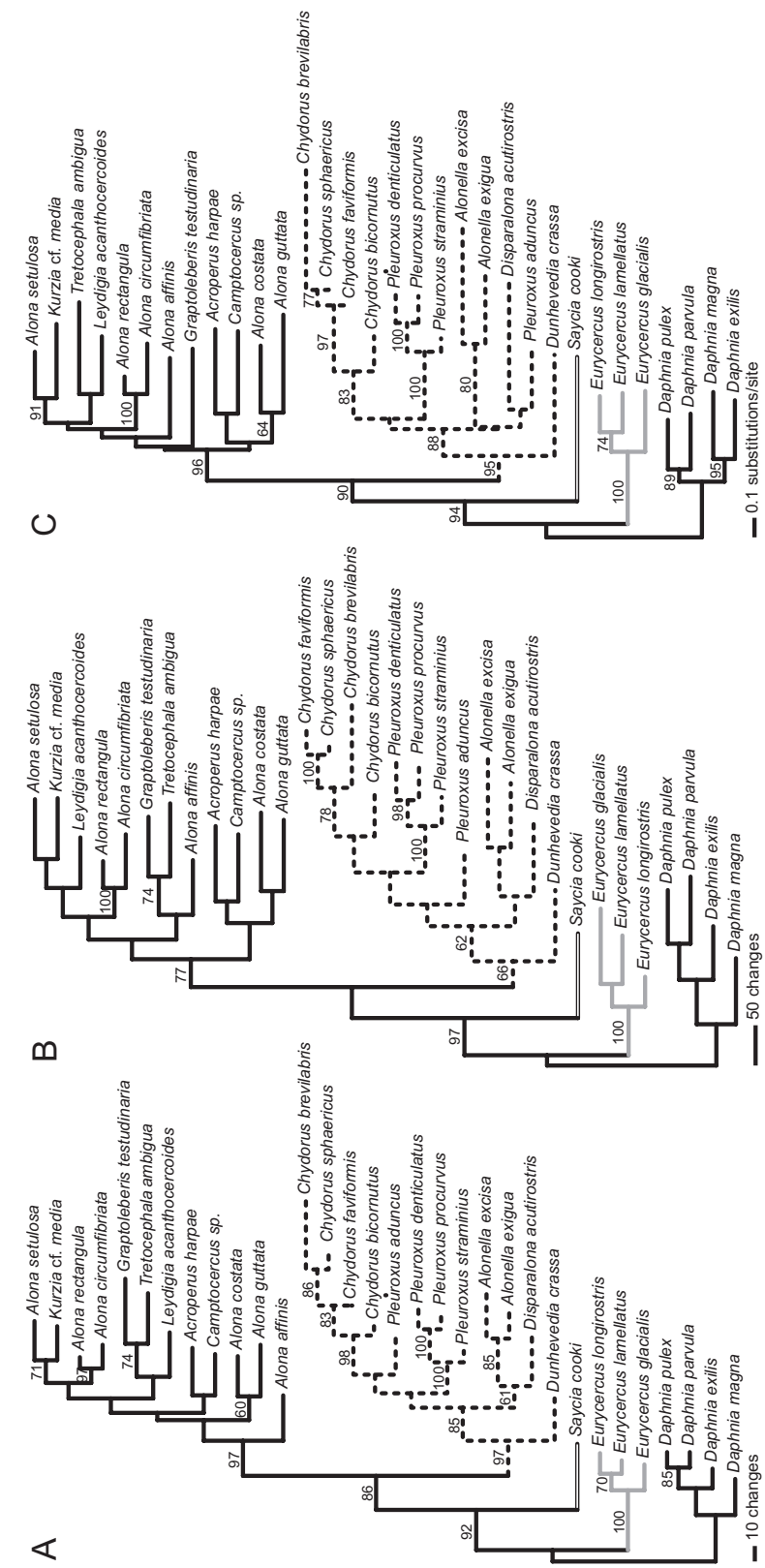

Figure 2. Phylogenetic reconstructions for combined data set of COI, 16S and 18S. (A) Maximum parsimony analysis of COI amino acid sequence and nucleotide sequences for 16S and 18S. (B) Maximum parsimony analysis of nucleotide sequences for all three gene regions. (C) Maximum likelihood analysis of nucleotide sequences of all three gene regions. Bootstrap support (500 replicates) higher than 60% shown above the branches. Grey line = subfamily Eurycercinae; dashed line = subfamily Chydorinae; double line = subfamily Saycinae (*Saycia cooki*), solid lines = subfamily Aloninae and outgroup (genus *Daphnia*).

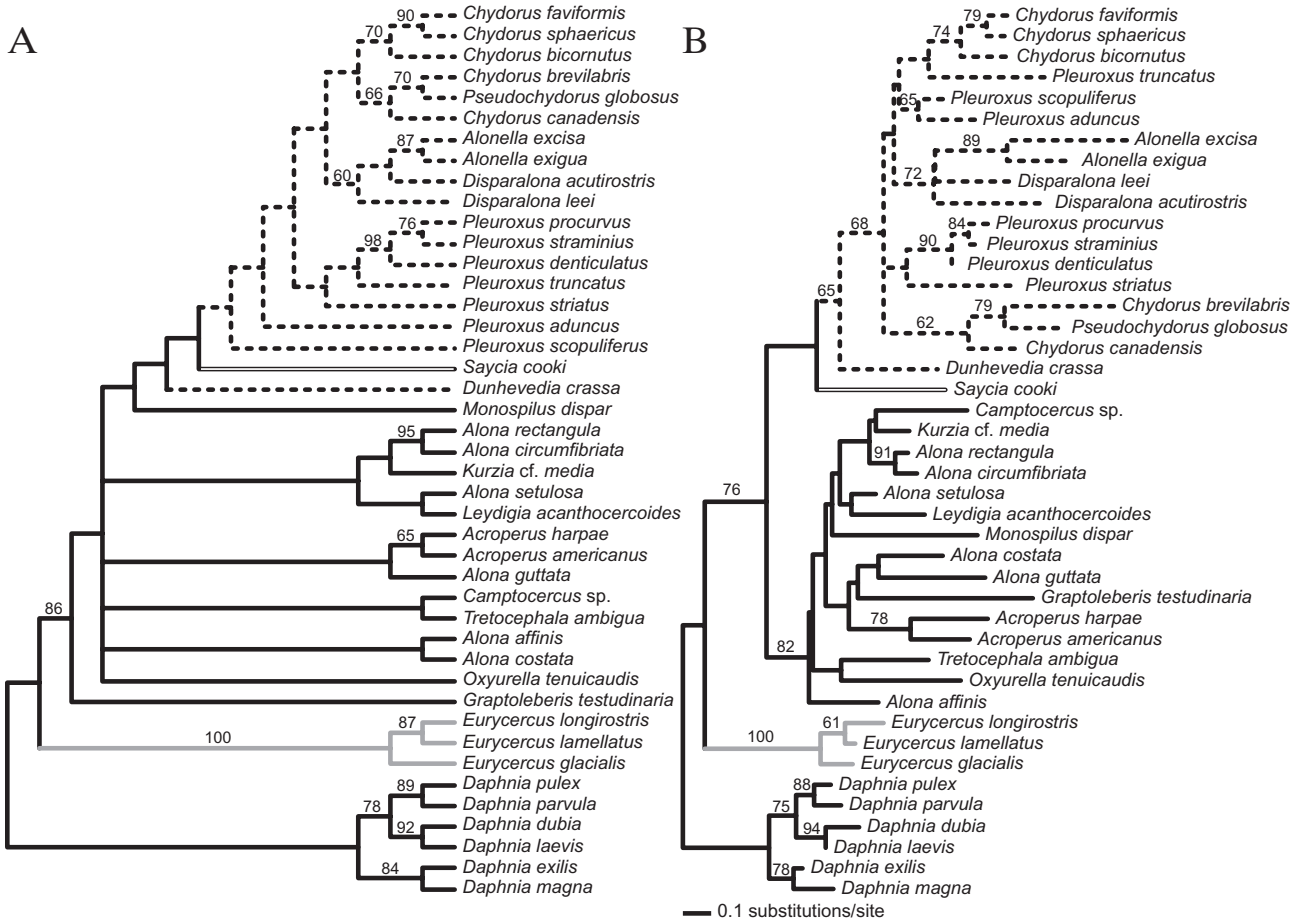

Figure 3. Phylogenetic reconstruction for total 16S data set. (A) 50% majority rule consensus tree of all nine equally parsimonious trees. (B) Maximum likelihood analysis. Grey line = subfamily Eurycercinae; dashed line = subfamily Chydorinae; double line = subfamily Saycinae, solid line = subfamily Aloninae and outgroup (genus *Daphnia*).

reflect cases of shared ancestry. For example, it has been argued that the resemblance of *Pseudochydorus globosus* to certain members of the genus *Chydorus* owes its origin to convergence (Fryer, 1968). Although morphology reveals a clear difference between these two genera, our data indicate that *P. globosus* is closely allied to two species of *Chydorus* (*C. brevilabris*, *C. canadensis*). However, *P. globosus* was included in the analysis of 16S rDNA only since we were not able to obtain sequence data for 18S rRNA and COI genes. The molecular evidence of affinity between *Chydorus* and *Pseudochydorus* is thus questionable, although it certainly cannot be ignored. Admittedly, the genus *Chydorus* itself shows evidence of fragmentation into two clades as three other members of the genus are quite divergent (*C. sphaericus*, *C. faviformis*, *C. bicornutus*), showing that the genus *Chydorus* is paraphyletic.

The situation in *Alona* was most complex. Although this genus was clearly polyphyletic, some species pairs were identified in most phylogenetic analyses (e.g. *A. rectangula* and *A. circumfimbriata*, *A. costata* and *A. guttata*). The latter two species often coexist, but the other pair has allopatric distributions with *A. rectangula* restricted to Europe and *A. circumfimbriata* to North America. Another species, *A. affinis*, was always genetically divergent from other *Alona*. Based on our preliminary genetic analysis there are probably two or more genera within the genus *Alona*. Collectively, these results raise interesting problems and make it clear that the existing generic classification system for chydorids based on morphology is not fully confirmed by genetic analysis. Moreover, the results suggest that generic diversity is substantially higher than that recognized by prior morphological study.

The origins of the Chydoridae

The thick chitinous carapaces and head shields of chydorids are often fossilized, facilitating study of the

group's history. Based on the fossil record, chydorids were well-diversified in the Mesozoic (Smirnov, 1992), although they probably evolved as early as the Palaeozoic, 250 Myr ago (Fryer, 1995; Frey, 1998). Thus, when Pangea started to break up 180 Myr ago, a similar set of 'protospecies' was probably left on each newly established continent (Frey, 1962, 1987, 1998). The modern chydorid subfamilies Eurycercinae, Aloninae and Chydorinae are present on all continents, excepting the Australian region where the Eurycercinae is replaced by the Saycinae. More than 50% of Australian chydorid genera and species are endemic (Frey, 1998), which is higher than on any other continent. Frey (1971), however, noted that *Saycia* inhabits temporary water bodies, while *Eurycercus* occupies permanent ones, so a more likely explanation of its absence is that it did not find favourable conditions in the Australian region. Based on 18S evidence, in which approximately 30% of informative nucleotide sites were indicative of subfamily status, the chydorid subfamilies were defined in the early Palaeozoic (Hillis & Dixon, 1991). This suggests that protospecies of all subfamilies were present on all continents and that their further radiation was constrained by availability of habitats. Thus, *Saycia* might be a relict of an ancient lineage that can only be found today in temporary ponds of eastern Australia and New Zealand, areas which share a similar geological history (Cattermole, 2000). As shown previously, the set of precise habitat parameters for each species is crucial for their occurrence (Frey, 1980), and thus *Eurycercus*, contrary to *Saycia*, might not have found habitats fulfilling its ecological requirements in Australia.

The relatively shallow branch lengths separating genera and the deep divergences within genera (up to 13% in the combined data set) typically reflect explosive radiations (Fu, 2000). In the case of the Chydoridae, speciation has been shown to be interconnected with shifts between substrates (see Fryer, 1968). Thus, the radiation of the major lineages might have coincided with the radiation of aquatic plants or the development of freshwater bodies, as documented in the case of Ostracoda, whose ecological history was connected with development of Permian lakes (Molostovskaya, 2000). Angiosperm diversification began in the middle-to-late Cretaceous (Futuyma, 1986; Cattermole, 2000). Angiosperm lineages invaded aquatic environments independently at least 50 times (Cook, 1996) and recent molecular work has shown that some of these are ancient lineages (Soltis *et al*., 2000). By the Cretaceous, the major generic lineages of Chydoridae were already established and so there was enough variation at a local scale to allow habitat shifts to newly developing niches. Interestingly, aquatic plants and small branchiopods show a number of similar adaptations to aquatic environments, such as the prevalence of clonal reproduction (Grace, 1993) and high levels of phenotypic plasticity (Les & Philbrick, 1993), although it is not clear if these features enabled organisms to invade water environments or if they were developed after invasion as they became advantageous (Grace, 1993). Thus, the radiation of aquatic plants clearly provided an opportunity for diversification of chydorid species.

Planktonic forms of many groups originated in the benthos of ancient oceans (Rigby & Milsom, 1996, 2000; Darling *et al*., 1997). Although crustacean clades have primarily marine origins (Vermeij & Dudley, 2000), 'Cladocera' were probably not derived directly from marine ancestors (Pennak, 1985). The transition to freshwater most likely occurred in groups of organisms that lived near the transition zones (Vermeij & Dudley, 2000), and thus the first freshwater 'Cladocera' very likely inhabited littoral zones and became associated with substrates and vegetation. Also, the pelagic environment, where the environmental–biotic interactions are more important than biotic–biotic (Rigby & Milsom, 1996), probably did not provide sufficient nutrients at the beginning and was much more variable and unpredictable than littoral/benthic environments (Pennak, 1985). Invasion of the pelagic zone then became more advantageous when competition and predation pressure in the benthos increased, and most likely happened many times (Rigby & Milsom, 1996). The radiation of forms in the benthic vs. planktic environments was then probably shaped by different mechanisms. Chydorids show a lot of morphological novelties associated with habitat requirements (e.g. *Graptoleberis*, *Leydigia*, *Camptocercus*). On the other hand, in the frequently open-water sister-group Daphniidae, there is long-term morphological stasis with a high level of phenotypic plasticity (Colbourne, 1999). Pond-dwelling lineages of *Daphnia* are more speciose than lake-dwelling ones due to the higher variety of habitats in ponds (Colbourne, 1999). This variety of habitats seems to be even more pronounced in littoral/benthic environments and chydorids. The magnitude of such habitat-dependent radiations is reflected by the fact that planktonic daphniids diversified mostly within the genus *Daphnia* at the water-body level (Taylor *et al*., 1996), while chydorids diversified into many different genera within a single water body.

Based on evidence of 12S rDNA, *Daphnia* is an ancient genus and its subgeneric differentiation occurred about 180 Myr ago (Colbourne & Hebert, 1996). Evolutionary divergence is even higher within the chydorid genera (Figs 2,3). Our molecular data, coupled with fossil and morphological evidence, reinforce the hypothesis that the major chydorid lineages originated as far back as the mid-Palaeozoic, approximately 400 Myr ago.

In summary, this study provides the first molecular-based phylogenetic hypothesis for the Chydoridae. Given the patterns of radiation discussed above, it is doubtful that collecting more sequence data (both sequence length and additional genes) would improve the resolution of the phylogeny. Instead, future efforts should be directed towards examining additional taxa and towards resolving relationships within genera, as shown in a recent study on lizards (Fu, 2000). However, our study gives strong support for the ancient origin of major chydorid lineages, both subfamilies and genera, and provides a solid framework for further studies of littoral microcrustaceans.

ACKNOWLEDGEMENTS

We are especially thankful to Sarah Adamowicz for stimulating comments on earlier drafts of the manuscript. We would like to thank John Green for specimens of *Saycia* from New Zealand, and Sarah Adamowicz for chydorid specimens from Argentina. Melania Cristescu, Jeremy DeWaard, Rob Dooh and Michelle Stuckey assisted with North American collections, while Martin Cerny, Petra Psenakova and Ota Strunecky assisted with Czech Republic collections. We thank A. Holliss for assistance with DNA sequencing. Two anonymous referees provided valuable comments on an early version of this manuscript. Funding for this research was provided by a Natural Sciences and Engineering Research Council grant to P.D.N.H and a NATO Science Fellowship to V.S.

REFERENCES

Alonso M. 1996. Crustacea, branchiopoda. In: Ramos MA *et al*., eds. *Fauna Iberica, Volume 7*. Museo Nacisnal de Ciencias Naturales, CSIC, Madrid.

Brooks JL. 1959. Cladocera. In: Edmondson WT, ed. *Freshwater biology*. New York: Wiley, 587–656.

Cattermole P. 2000. *Building Planet Earth, five billion years of Earth history.* Cambridge: Cambridge University Press.

Chengalath R. 1987. The distribution of chydorid Cladocera in Canada. *Hydrobiologia* **145:** 151–157.

Colbourne JK. 1999. A documentary on the evolutionary history of *Daphnia*. Unpublished D. Phil Thesis, Ontario: University of Guelph.

Colbourne JK, Hebert PDN. 1996. The systematics of North American *Daphnia* (Crustacea: Anomopoda): a molecular phylogenetic approach. *Philosophical Transactions of the Royal Society of London, Series B* **351:** 349–360.

Colbourne JK, Hebert PDN, Taylor DJ. 1997. Evolutionary origins of phenotypic diversity in *Daphnia*. In: Givnish TJ, Sytsma KJ, eds. *Molecular evolution and adaptive radiation.* Cambridge: Cambridge University Press, 163–188.

Cook CDK. 1996. *Aquatic plant book.* Location: SPB Academic Publishing, The Hague: The Netherlands.

Crease TJ. 1999. The complete sequence of the mitochondrial genome of *Daphnia pulex* (Cladocera: Crustacea). *Gene* **233:** 89–99.

Crease TJ, Colbourne JK. 1998. The unusually long small-subunit ribosomal RNA of the crustacean, *Daphnia pulex*: Sequence and predicted secondary structure. *Journal of Molecular Evolution* **46:** 307–313.

Darling KF, Wade CM, Kroon D, Brown AJL. 1997. Planktic foraminiferal molecular evolution and their polyphyletic origins from benthic taxa. *Marine Micropaleontology* **30:** 251–266.

De Rijk P, Caers A, Van de Peer Y, De Wachter R. 1997. Database on the structure of large ribosomal subunit RNA. *Nucleic Acids Research* **27:** 174–178.

Dodson SI, Frey DG. 1991. Cladocera and other Branchiopoda. In: Thorpe JH, Covich AP, eds. *Ecology and classification of North American freshwater invertebrates.* San Diego, San Francisco, New York, Boston, London, Sydney, Tokyo: Academic Press, Inc, 723–786.

Farris JS, Kallersjo M, Kluge AG, Bult C. 1994. Testing significance of incongruence. *Cladistics* **10:** 315–319.

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39:** 783–791.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. 1994. DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* **3:** 294–299.

Frey DG. 1959. The taxonomic and phylogenetic significance of the head pores of the Chydoridae (Cladocera). *Internationale Revue der Gesamten Hydrobiologie* **44:** 27–50.

Frey DG. 1962. Cladocera from the Eemian Interglacial of Denmark. *Journal of Paleontology* **36:** 1133–1154.

Frey DG. 1967. Phylogenetic relationships in the family Chydoridae (Cladocera). *Marine Biology Association of India, Symposium Part* **1:** 29–37.

Frey DG. 1971. Worldwide distribution and ecology of *Eurycercus* and *Saycia* (Cladocera). *Limnology and Oceanography* **16:** 254–308.

Frey DG. 1980. The non-swimming chydorid Cladocera of wet forests, with descriptions of a new genus and two new species. *Internationale Revue der Gesamten Hydrobiologie* **65:** 613–641.

Frey DG. 1982a. Questions concerning cosmopolitanism in Cladocera. *Archiv für Hydrobiologie* **93:** 484–502.

Frey DG. 1982b. The honeycombed species of *Chydorus* (Cladocera, Chydoridae): comparison of *C. bicornutus* and *C. bicollaris* n. sp. with some preliminary comments on *faviformis*. *Canadian Journal of Zoology* **60:** 1892–1916.

Frey DG. 1987. The North American *Chydorus faviformis* (Cladocera, Chydoridae) and the honeycombed taxa of other continents. *Philosophical Transactions of the Royal Society of London, Series B* **315:** 353–402.

Frey DG. 1993. Subdivision of the genus *Pleuroxus* (Anomopoda, Chydoridae) into subgenera worldwide. *Hydrobiologia* **262:** 133–144.

Frey DG. 1995. Changing attitudes toward chydorid anomopods since 1769. *Hydrobiologia* **307:** 43–55.

Frey DG. 1998. Expanded description of *Leberis aenigmatosa* Smirnov (Anomopoda: Chydoridae): a further indication of the biological isolation between western and eastern Australia. *Hydrobiologia* **367:** 31–42.

Fryer G. 1968. Evolution and adaptive radiation in the Chydoridae (Crustacea: Cladocera): a study in comparative functional morphology and ecology. *Philosophical Transactions of the Royal Society of London, Series B* **254 (795):** 221–385.

Fryer G. 1991. A daphniid ephippium (Branchiopoda: Anomopoda) of Cretaceous age. *Zoological Journal of the Linnean Society* **102:** 163–167.

Fryer G. 1995. Phylogeny and adaptive radiation within the Anomopoda: a preliminary exploration. *Hydrobiologia* **307:** 57–68.

Fu J. 2000. Toward the phylogeny of the family Lacertidae. Why 4708 base pairs of mtDNA sequences cannot draw the picture. *Biological Journal of the Linnean Society* **71:** 203–217.

Futuyma DJ. 1986. *Evolutionary biology*, 2nd edn. Sunderland, MA: Sinauer Associates.

Geodakyan VA, Smirnov NN. 1968. Sexual dimorphism and evolution in lower Crustacea. *Problems of Evolution* **1:** 30–36. [In Russian].

Goulden CE. 1968. The systematics and evolution of the Moinidae. *Transactions of the American Philosophical Society* **58:** 1–101.

Grace JB. 1993. The adaptive significance of clonal reproduction in angiosperms: an aquatic perspective. *Aquatic Botany* **44:** 159–180.

Hann BJ. 1981. Occurrence and distribution of littoral Chydoridae (Crustacea, Cladocera) in Ontario, Canada, and taxonomic notes on some species. *Canadian Journal of Zoology* **59:** 1465–1474.

Hasegawa M, Kishino H, Yano T. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution* **22:** 160–174.

Hillis DM, Dixon MT. 1991. Ribosomal DNA molecular evolution and phylogenetic inference. *Quarterly Review of Biology* **66:** 411–454.

Hillis DM, Moritz C, Mabel BK, eds. 1996. *Molecular systematics*. Sunderland, MA: Sinauer.

Huelsenbeck JP, Bull JJ, Cunningham CW. 1996. Combining data in phylogenetic analysis. *Trends in Ecology and Evolution* **11:** 152–158.

Kumar S, Tamura K, Jakobsen IB, Nei M. 2001. MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics* **17:** 1244–1245.

Kumazawa Y, Nishida M. 1993. Sequence evolution of mitochondrial tRNA genes and deep-branch animal phylogenetics. *Journal of Molecular Evolution* **37:** 380–398.

Les DH, Philbrick CT. 1993. Studies of hybridization and chromosome number variation in aquatic angiosperms: evolutionary implications. *Aquatic Botany* **44:** 181–228.

Lilljeborg W. 1900. *Cladocera Sueciae*. Nova acta regiae societas scientiarum upsaleinsis seriei 3, Upsala.

Lynch M, Jarrell PE. 1993. A method for calibrating molecular clocks and its implication to animal mitochondrial DNA. *Genetics* **135:** 1197–1208.

Molostovskaya II. 2000. The evolutionary history of Late Permian Darwinulocopina Sohn, 1988 (Ostracoda) from the Russian Plate. *Hydrobiologia* **419:** 125–130.

Palumbi SR, Martin AP, Romano S, McMillan WO, Stice L, Grabowski G. 1991. *The simple fool's guide to PCR*. Honolulu: Special Publication of the Department of Zoology, University of Hawaii.

Pennak RW. 1985. The fresh-water invertebrate fauna: problems and solutions for evolutionary success. *American Zoologist* **25:** 671–687.

Pennak RW. 1989. *Fresh-water invertebrates of the United States. Protozoa to Mollusca.* Location: John Wiley & Sons, Inc, New York.

Remsen J, DeSalle R. 1998. Character congruence of multiple data partitions and the origin of the Hawaiian Drosophilidae. *Molecular Phylogenetics and Evolution* **9:** 225–235.

Rigby S, Milsom CV. 1996. Benthic origins of zooplankton: An environmentally determined macroevolutionary effect. *Geology* **24:** 52–54.

Rigby S, Milsom CV. 2000. Origins, evolution, and diversification of zooplankton. *Annual Review of Ecology and Systematics* **31:** 293–313.

Schwenk K. 1996. *Evolutionary genetics of Daphnia species complex: hybridism in syntopy.* Nieuwersluis, The Netherlands: Netherlands Institute of Ecology, Centre for Limnology. Publication 2231.

Smirnov NN. 1966. The taxonomic significance of the trunk limbs of the Chydoridae (Cladocera). *Hydrobiologia* **27:** 337–347.

Smirnov NN. 1969. Morpho-functional grounds of mode of life of Cladocera. III. Oligomerization in Cladocera. *Hydrobiologia* **34:** 235–242.

Smirnov NN. 1971. *Chydoridae Fauny Mira. Fauna SSSR.* Nov Ser, no. 101. *Rakoobraznyye*, 1. vyp. 2. [In Russian], Nauta, Leningrad.

Smirnov NN. 1992. Mesozoic Anomopoda (Crustacea) from Mongolia. *Zoological Journal of the Linnean Society* **104:** 97–116.

Smirnov NN. 1996. *Cladocera: the Chydorinae and Sayciniae (Chydoridae) of the world.* Belgium: SPB Academic Publishing.

Soltis DE, Soltis PS, Chase MW, Mort ME, Albach DC, Zanis M, Savolainen V, Hahn WH, Hoot SB, Fay MF, Axtell M, Swensen SM, Prince LM, Kress WJ, Nixon KC, Farris JS. 2000. Angiosperm phylogeny inferred from 18S rDNA, rbcL, and atpB sequences. *Botanical Journal of the Linnean Society* **133:** 381–461.

Swofford DL. 1998. *PAUP*: phylogenetic analysis using parsimony (*and other methods), Version 4.0b8.* Sunderland, MA: Sinauer Associates.

Taylor DJ, Crease TJ, Brown WM. 1999. Phylogenetic evidence for a single long-lived clade of crustacean cyclic parthenogens and its implications for the evolution of sex.

Proceedings of the Royal Society of London Series B, Biological Sciences **266:** 791–797.

Taylor DJ, Finston TL, Hebert PDN. 1998. Biogeography of a widespread freshwater crustacean: pseudocongruence and cryptic endemism in the North American *Daphnia laevis* complex. *Evolution* **52:** 1648–1670.

Taylor DJ, Hebert PDN, Colbourne JK. 1996. Phylogenetics and evolution of the *Daphnia longispina* group (Crustacea) based on 12S rDNA sequence and allozyme variation. *Molecular Phylogenetics and Evolution* **5:** 495–510.

Tremel B, Frey SE, Yan ND, Somers KM. 2000. Habitat specificity of littoral Chydoridae (Crustacea, Branchiopoda, Anomopoda) in Plastic Lake, Ontario, Canada. *Hydrobiologia* **432:** 195–205.

Van de Peer Y, Jansen J, DeRijk P, De Wachter R. 1997. Database on the structure of small ribosomal subunit RNA. *Nucleic Acids Research* **25:** 111–116.

Vermeij GJ, Dudley R. 2000. Why are there so few evolutionary transitions between aquatic and terrestrial ecosystems? *Biological Journal of the Linnean Society* **70:** 541–554.

APPENDIX

List of the six daphniid species and 37 chydorid species examined in this study, together with localities and/or references for the sequence data. CZ = Czech Republic. CA = Canada

	Locality/reference		
Species	16S	18S	COI
ORDER: CLADOCERA			
SUBORDER: ANOMOPODA			
Family: Daphniidae			
Daphnia pulex Leydig, 1860	Crease (1999)	Crease & Colbourne (1998)	Crease (1999)
Daphnia parvula Fordyce, 1901	Adamowicz, pers. comm.	Adamowicz, pers. comm.	Adamowicz, pers. comm.
Daphnia dubia Herrick, 1883	Taylor *et al*. (1998)	–	–
Daphnia laevis Birge, 1878	Taylor *et al*. (1998)	–	–
Daphnia magna Straus, 1820	Colbourne, pers. comm.	Adamowicz, pers. comm.	Colbourne, pers. comm.
Daphnia exilis Herrick, 1895	Colbourne, pers. comm.	Adamowicz, pers. comm.	Colbourne, pers. comm.
Family: Chydoridae			
Acroperus americanus Kubersky, 1977	Ditch, Bonavista Peninsula, Newfoundland, CA	–	–
Acroperus harpae (Baird, 1834)	Sasajewun Lake, Ontario, CA 45°35′N 78°31′W	Burntwood River, Manitoba, CA 55°49′N 97°55′W	Sasajewun Lake, Ontario, CA 45°35′N 78°31′W
Alona affinis Leydig, 1860	Sasajewun Lake, Ontario, CA 45°35′N 78°31′W	Sasajewun Lake, Ontario, CA 45°35′N 78°31′W	Sasajewun Lake, Ontario, CA 45°35′N 78°31′W
Alona circumfi—briata Megard, 1967	Ditch, Manitoba, CA 54°21′N 100°16′W	Ditch, Manitoba, CA 54°21′N 100°16′W	Ditch, Manitoba, CA 54°21′N 100°16′W
Alona costata Sars,1862	Little Lake, Ontario, CA 43°25′N 80°16′W	Doe Lake, Ontario, CA 44°56′N 79°17′W	Little Lake, Ontario, CA 43°25′N 80°16′W
Alona guttata Sars, 1862	Sasajewun Lake, Ontario, CA 45°35′N 78°31′W	Sasajewun Lake, Ontario, CA 45°35′N 78°31′W	Sasajewun Lake, Ontario, CA 45°35′N 78°31′W
Alona rectangula Sars, 1862	Pond near Golcuv Jenikov, CZ 49°49′N 15°29′E	Pond near Golcuv Jenikov, CZ 49°49′N 15°29′E	Pond near Golcuv Jenikov, CZ 49°49′N 15°29′E

APPENDIX *Continued*

Species	Locality/reference		
	16S	18S	COI
Alona setulosa Megard, 1967	Ditch, Manitoba, CA 54°21′N 100°16′W	Ditch, Manitoba, CA 54°21′N 100°16′W	Ditch, Manitoba, CA 54°21′N 100°16′W
Alonella excisa (Fischer, 1854)	Starling Lake, Ontario, CA 45°34′N 78°30′W	Starling Lake, Ontario, CA 45°34′N 78°30′W	Starling Lake, Ontario, CA 45°34′N 78°30′W
Alonella exigua (Lilleborg, 1853)	Starling Lake, Ontario, CA 45°34′N 78°30′W	Starling Lake, Ontario, CA 45°34′N 78°30′W	Starling Lake, Ontario, CA 45°34′N 78°30′W
Camptocercus rectirostris complex Schoedler, 1862	Pond near Ottawa, Ontario, CA 45°22′N 75°52′W	Burntwood River, Manitoba, CA 55°49′N 97°55′W	Pond near Ottawa, Ontario, CA 45°22′N 75°52′W
Chydorus bicornutus Doolittle, 1909	Starling Lake, Ontario, CA 45°34′N 78°30′W	Starling Lake, Ontario, CA 45°34′N 78°30′W	Starling Lake, Ontario, CA 45°34′N 78°30′W
Chydorus brevilabris Frey, 1980	Pinehurst Lake, Ontario, CA 43°16′N 80°23′W	Pinehurst Lake, Ontario, CA 43°16′N 80°23′W	Sasajewun Lake, Ontario, CA 45°35′N 78°31′W
Chydorus canadensis Chengalath and Hann, 1981	Pond near Ottawa, Ontario, CA 45°22′N 75°52′W	–	–
Chydorus faviformis Birge, 1893	Sasajewun Lake, Ontario, CA 45°35′N 78°31′W	Sasajewun Lake, Ontario, CA 45°35′N 78°31′W	Sasajewun Lake, Ontario, CA 45°35′N 78°31′W
Chydorus sphaericus (Mueller, 1785)	Burntwood River, Manitoba, CA 55°49′N 97°55′W	Burntwood River, Manitoba, CA 55°49′N 97°55′W	Burntwood River, Manitoba, CA 55°49′N 97°55′W
Disparalona acutirostris (Birge, 1879)	Sasajewun Lake, Ontario, CA 45°35′N 78°31′W	Sasajewun Lake, Ontario, CA 45°35′N 78°31′W	Sasajewun Lake, Ontario, CA 45°35′N 78°31′W
Disparalona leei (Chien, 1970)	Weismuller lake, Ontario, CA 44°56′N 79°13′W	–	–
Dunhevedia crassa King, 1853	Pond near Ottawa, Ontario, CA 45°22′N 75°52′W	Pond near Ottawa, Ontario, CA 45°22′N 75°52′W	Pond near Ottawa, Ontario, CA 45°22′N 75°52′W
Eurycercus glacialis Lilljeborg, 1887	Tundra pond, Manitoba, CA 58°46′N 93°52′W	Tundra pond, Manitoba, CA 58°46′N 93°52′W	Tundra pond, Manitoba, CA 58°46′N 93°52′W
Eurycercus lamellatus (Mueller, 1776)	Smyslov pond, CZ 49°25′N 13°48′E	Smyslov pond, CZ 49°25′N 13°48′E	Smyslov pond, CZ 49°25′N 13°48′E
Eurycercus longirostris Hann, 1982	Burntwood River, Manitoba, CA 55°49′N 97°55′W	Burntwood River, Manitoba, CA 55°49′N 97°55′W	Burntwood River, Manitoba, CA 55°49′N 97°55′W
Graptoleberis testudinaria (Fischer, 1851)	Little Lake, Ontario, CA 43°25′N 80°16′W	Little Lake, Ontario, CA 43°25′N 80°16′W	Little Lake, Ontario, CA 43°25′N 80°16′W

APPENDIX *Continued*

Species	Locality/reference		
	16S	18S	COI
Kurzia cf. *media* (Hudec, 2000)	Pond near Gravenhurst, Ontario, CA 44°56′N 79°19′W	Pond near Gravenhurst, Ontario, CA 44°56′N 79°19′W	Pond near Gravenhurst, Ontario, CA 44°56′N 79°19′W
Leydigia acanthocercoides (Fischer, 1854)	Pond near Las Varillas, Cordoba, Argentina 32°25′S 62°41′W	Pond near Las Varillas, Cordoba, Argentina 32°25′S 62°41′W	Pond near Las Varillas, Cordoba, Argentina 32°25′S 62°41′W
Monospilus dispar Sars, 1862	Weismuller lake, Ontario, CA 44°56′N 79°13′W	–	–
Oxyurella tenuicaudis (Sars, 1862)	Smyslov pond, CZ 49°25′N 13°48′E	–	–
Pleuroxus aduncus (Jurine, 1820)	Pond near Golcuv Jenikov, CZ 49°49′N 15°29′E	Pond near Golcuv Jenikov, CZ 49°49′N 15°29′E	Pond near Golcuv Jenikov, CZ 49°49′N 15°29′E
Pleuroxus denticulatus (Birge, 1879)	Pinehurst Lake, Ontario, CA 43°16′N 80°23′W	Pinehurst Lake, Ontario, CA 43°16′N 80°23′W	Pinehurst Lake, Ontario, CA 43°16′N 80°23′W
Pleuroxus procurvus Birge (1879)	Sasajewun Lake, Ontario, CA 45°35′N 78°31′W	Sasajewun Lake, Ontario, CA 45°35′N 78°31′W	Sasajewun Lake, Ontario, CA 45°35′N 78°31′W
Pleuroxus scopuliferus Ekman, 1900	Farm dam, Chubut, Argentina 43°44′S 70°2′W	–	–
Pleuroxus straminius (Birge, 1879)	Starling Lake, Ontario, CA 45°34′N 78°30′W	Starling Lake, Ontario, CA 45°34′N 78°30′W	Starling Lake, Ontario, CA 45°34′N 78°30′W
Pleuroxus striatus (Schoedler, 1863)	Sasajewun Lake, Ontario, CA 45°35′N 78°31′W	–	–
Pleuroxus truncatus (Mueller, 1785)	Sandpit near Masovice, CZ 48°51′N 15°58′E	–	–
Pseudochydorus globosus (Baird, 1843)	Pond near Ottawa, Ontario, CA 45°22′N 75°52′W	–	–
Saycia cooki (King, 1866)	New Zealand, not specified	New Zealand, not specified	New Zealand, not specified
Tretocephala ambigua (Lilljeborg, 1900)	Jevisovka River, CZ 48°51′N 16°16′E	Jevisovka River, CZ 48°51′N 16°16′E	Jevisovka River, CZ 48°51′N 16°16′E