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Affinities among Anostracan (Crustacea: Branchiopoda) Families Inferred from Phylogenetic Analyses of Multiple Gene Sequences

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To gain insights into the relationships among anostracan families, molecular phylogenetic analyses were performed on nuclear (28S D1–D3 ribosomal DNA) and mitochondrial (16S rDNA, COI) gene regions for representatives of seven families and an outgroup. Data matrices used in the analyses included 951 base pairs (bp) of aligned sequences for 28S, 465 bp for 16S, and 658 bp (219 amino acids) for COI. Maximum-parsimony and maximum-likelihood methods were used to construct phylogenetic trees, enabling the evaluation of both previous hypotheses of taxonomic relationships among families based on morphology, and of the relative merits of independent versus simultaneous analyses of multiple data sets for phylogeny construction. Data from various combinations of the gene regions produced relatively congruent patterns of phylogenetic affinity. In most analyses, two monophyletic groups were resolved: one cluster included the families Polyartemiidae, Chirocephalidae, Branchinectidae, Streptocephalidae, and Thamnocephalidae, while the other contained the Artemiidae and Branchipodidae. Comparative analyses showed that combining gene regions in a single matrix generally resulted in increased resolution and support for each cluster relative to those obtained from single-gene analyses. Statistical tests demonstrated that morphology-based hypotheses of relationships among families had poorer support than those determined from molecular data, reflecting the homoplasy in characters used to differentiate families. © 2000 Academic Press

Key Words: **Anostraca; phylogeny; nuclear DNA; mitochondrial DNA; rDNA; COI.**

INTRODUCTION

Anostracan crustaceans are a common faunal element of ephemeral freshwater and saline ponds. Also known as fairly or brine shrimps, the order includes at least 258 species that are currently assigned to 21 genera and eight families (Belk, in Dodson and Frey, 1991; Belk and Brtek, 1995). Although anostracans are well-defined from other crustacean orders by a suite of morphological characters, the delineation of familial and generic boundaries has proven difficult (Belk and Brtek, 1995), and relationships among anostracan families are very poorly understood. Establishing a robust hypothesis of relationships for anostracans is important because the group is believed to comprise the most basal lineage of branchiopod crustaceans (Schram and Hof, 1998). Furthermore, certain anostracans, especially the brine shrimp *Artemia*, have gained importance as model systems in genetic, physiological, ecological, and aquaculture studies.

Morphological studies on anostracans date back more than a century. By reexamining morphological diversity in the group, Linder (1941) revised their taxonomy, relying heavily on male anatomical features (e.g., penial morphology and number of thoracic legs) and proposed a tentative scheme of relationships among the families (Fig. 1a). Dodson and Frey (1991) augmented these characters with details of the second (II) antennae to produce a different taxonomic scheme (Fig. 1b). Their conflicting hypotheses of taxonomic relationships agree only in depicting the Polyartemiidae as the least related to the other anostracan families because of its unique number of leg-bearing thoracic segments. Owing to the lack of phylogenetic context on which these taxonomies were derived, there is no understanding (and therefore no assumptions are made) as to which families are basal, intermediate, or derived.

A critical reassessment of anostracan relationships is overdue and it is clear from work on other crustacean groups (e.g., Spears *et al.*, 1992; Colbourne and Hebert, 1996; Crandall and Fitzpatrick, 1996; Taylor *et al.*, 1999) that molecular phylogenetic studies are the best approach. While the use of sequence data for phylogeny construction is now routine, concerns have been raised that conclusions derived from analysis of a single segment of the genome (either nuclear or organellar) may not be reliable (Avise, 1994; Page and Holmes, 1998). This awareness has stimulated efforts to base phylogenies on a broader survey of genetic diversity. The

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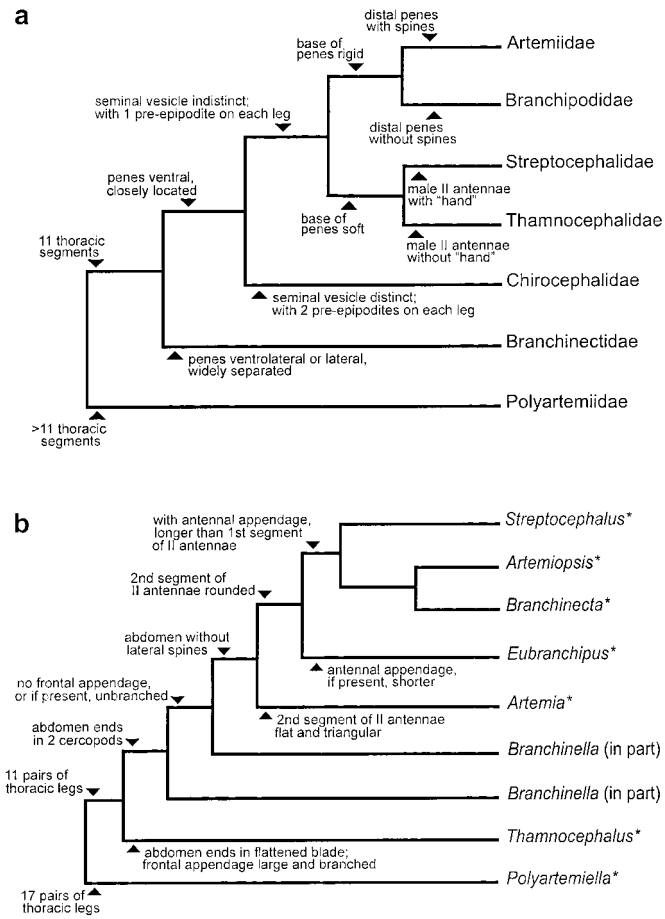

FIG. 1. Hypothesis of taxonomic relationships based on morphology among (a) anostracan families (Linder, 1941) or (b) representative anostracan genera (Dodson and Frey, 1991). The characters shown were extracted from each of the author's diagnostic keys, which we graphically reproduced for convenience and clarity. "Hand" refers to the "complex branched medial process attached at the inner corner of the apex of the 1st segment of the II antennae" of males. Asterisks (*) indicate taxa sequenced in this study.

merits of independent versus simultaneous analyses of data sets for phylogenetic inference and the conditions under which each type of analysis is appropriate are contentious (Swofford, 1991; De Queiroz *et al.*, 1995; Huelsenbeck *et al.*, 1996). However, it is generally accepted that phylogenetic hypotheses are most convincing when supported by data from a variety of sources.

We sought to provide an estimate of familial relationships in anostracans by analyzing sequence variation from the nuclear 28S rRNA gene, and the mitochondrial 16S rRNA and cytochrome *c* oxidase I (COI) genes. Analysis of the 28S rRNA gene targeted the 5′ region which includes variable domains (also termed expansion segments) 1 to 3 (D1–D3) and their conserved flanking regions, while a portion of the 3′ end of the 16S gene was examined. Because both gene segments contain variable regions set in a highly conserved core, they are informative at various levels of taxonomic divergence. The 28S rDNA molecule ordinarily evolves at rates appropriate for dating divergences as far back as 250 mya, whereas the 16S rRNA gene, which evolves more rapidly, is best suited for taxa that diverged within the last 150 million years (Mindel and Honeycutt, 1990; Hillis and Dixon, 1991). Because COI exhibits a slower evolutionary rate than other protein-coding genes in the mitochondrial genome (Jacobs *et al.*, 1988), it is also useful for examining relatively deep evolutionary divergences (~100 mya) (Kumazawa and Nishida, 1993). As anostracans are thought to have originated in the Lower Cretaceous (~145 mya) (Fryer, 1987; but see Walossek and Müller, 1998), the limits of resolution for these three target molecules are appropriate for phylogenetic studies on these organisms.

Here we report the results of phylogenetic analyses performed on species representing seven of the eight known anostracan families. Phylogenetic trees inferred from independent and combined analyses of molecular data sets were used to test previous hypotheses of relationships among families based on morphology, and to examine the relative virtues of separate versus simultaneous analyses of multiple data sets for phylogeny construction.

MATERIALS AND METHODS

Taxa Studied

A single species was examined for five of the eight known anostracan families, while two species of Chirocephalidae from different genera and two congeneric species of Branchipodidae were sequenced (Table 1); no representative of the monotypic Linderiellidae was available. As there is no existing phylogenetic hypothesis for the Anostraca, our primary focus was to determine (1) which genes provide useful information at various levels of taxonomic divergence, (2) which families form stable monophyletic groups, and (3) which lineage is basal or derived. The present study aimed to answer these questions using a limited number of genera and species from the families examined. This approach has provided sufficient baseline information on the main relations within the Anostraca (see Results), without entailing the immediate survey of large numbers of taxa. Our analyses of several genes helped to reduce any impact of limited taxonomic sampling on phylogenetic analyses. The notostracan *Lepidurus* sp., a putative sister taxon of anostracans (Walossek and Müller, 1998), was used as an outgroup.

Molecular Protocols

Genomic DNA was prepared using standard or CTAB-based phenol-chloroform extraction methods (Sambrook *et al.*, 1989; Doyle, 1991). Primer sequences and procedures for PCR amplification of gene fragments from both nuclear (28S D1–D3 rDNA) and mt (16S rDNA and COI) genomes are available at http://

TABLE 1

Information for Specimens Examined

Taxonomy[a]	Locality/source	GenBank-EMBL accession numbers[b]/Reference
Family Artemiidae		
Artemia franciscana	U.S.A.[c]	Taylor *et al.* (1999), AF209057, Valverde *et al.* (1994)
Family Branchipodidae		
Parartemia contracta	Western Australia	AF209042, AF209048, AF209059
Parartemia longicaudata	Western Australia	AF209043, AF209049, AF209060
Family Chirocephalidae		
Eubranchipus sp.	Ontario, Canada	AF209044, AF209052, AF209061
Artemiopsis stefanssoni	Northwest Territories, Canada	AF209045, AF209053, AF209062
Family Polyartemiidae		
Polyartemiella hazeni	Alaska, U.S.A.	Taylor *et al.* (1999), AF209054, AF209063
Family Branchinectidae		
Branchinecta paludosa	Northwest Territories, Canada	Taylor *et al.* (1999), AF209055, AF209064
Family Streptocephalidae		
Streptocephalus dorothae	Triops Educational Science, Florida	Taylor *et al.* (1999), AF209056, AF209065
Family Thamnocephalidae		
Thamnocephalus platyurus	Triops Educational Science, Florida	AF209046, AF209057, AF209066
Outgroup		
Lepidurus sp.	Northwest Territories, Canada	AF209047, AF209058, AF209067

[a] Family level classification follows Belk (in Dodson and Frey, 1991).
[b] 28S, 16S, COI.
[c] The sample used to obtain the 16S sequence was from Great Salt Lake, Utah.

www.cladocera.uoguelph.ca/tools/. The primers amplified segments corresponding to nucleotides (nt) 3336–4402 of nuclear 28S rDNA of the fruitfly *Drosophila melanogaster* (GenBank Accession Nos. M21017 and M29800; Tautz *et al.*, 1988), and nt 12169–12662 of mt 16S rDNA and nt 1385–2042 of mt COI of the brine shrimp *Artemia franciscana* (X69067, Valverde *et al.*, 1994). Purified PCR products were subjected to dye terminator cycle-sequencing reactions and sequenced on an ABI 377 (Applied Biosystems). Each of the three gene regions was sequenced in both directions.

Sequence and Phylogenetic Analyses

CLUSTAL W (Thompson *et al.*, 1994) was used to initially align the rDNA sequences using default parameters (pairwise alignment set to slow mode, with a gap opening penalty of 10 for both pairwise and multiple alignment procedures, and gap extension penalties of 0.1 and 0.05 for pairwise and multiple sequence alignments). A secondary sequence structure model of the large subunit rRNA (De Rijk *et al.*, 1999) was used as a guide to align hypervariable sites in both 28S and 16S. Prior to phylogenetic analyses, segments where alignments were ambiguous and sites which contained gaps were omitted from the data matrix. For COI, nt sequences were aligned in XESEE (Cabot and Beckenbach, 1989); no gaps were required to produce an unambiguous alignment. After determining their reading frame and using the invertebrate mtDNA code (available from GenBank), they translated to 219 amino acids. All sequences obtained in this work have been submitted to GenBank, with their accession numbers given in Table 1. The final alignment lengths for each gene region, as well as those used in combined analyses, are presented in Table 3. The aligned data matrices used in the analyses are available at www.cladocera.uoguelph.ca/tools/.

All analyses were conducted using PAUP 4.0b2 (Swofford, 1998). A χ^2 goodness-of-fit test was performed on the sequence data for each gene region to determine if nucleotide composition bias occurred among taxa. The suitability of pooling sequence data from two or all three genes for phylogenetic reconstruction was assessed by the incongruence length difference (ILD) test (Farris *et al.*, 1995), an efficient predictor of phylogenetic accuracy for analysis of combined data sets (Cunningham, 1997). This test was done in PAUP using the partition homogeneity method. The ILD is the difference in length between the shortest tree derived from two or more combined data sets (e.g., 28S + 16S) and the most-parsimonious tree inferred separately from each data set. The ILD for the original data sets is compared with those from replicated versions, with all 11 possible combinations of the data sets (see Table 2) analyzed using 1000 replicates. The number of times the ILD from replicated data sets was greater than that from the original analysis is given a *P* value, with those lower than 0.05 indicating data incongruence.

Maximum-parsimony (MP) and maximum-likelihood (ML) methods were utilized to search for optimal trees in both separate and combined data sets. For MP, exhaustive tree searches were done to ensure that only the shortest tree was found. All characters were treated as unordered and equally weighted (except in

one of the COI analyses), and character states were optimized using accelerated transformation (ACCTRAN) so that changes are placed as close to the origin as possible. For COI data, three independent analyses were performed to evaluate the effect of character weighting on tree topology: analysis 1 was based on all codon positions equally weighted, analysis 2 on transversions given twice as much weight as transitions, and analysis 3 on inferred amino acids. Skewness or g_1 statistics resulting from exhaustive tree searches were obtained to determine if there was significant phylogenetic information in each data set (Hillis and Huelsenbeck, 1992). ML procedures were carried out using the HKY85 algorithm which allows for unequal base frequencies and two substitution types (Hasegawa *et al.*, 1985), assumptions which are compatible with the mechanics of evolution for many genes (Goldman, 1993). Another rationale for using ML is that it allows the incorporation of estimates of among-site rate variation in the analysis, and is therefore a useful tool to counter the confounding effects of long-branch attraction which may arise in studies which survey a small number of taxa (Cunningham *et al.*, 1998). Aside from among-site heterogeneity, additional parameters (base frequencies, transition/transversion ratio, proportion of invariable sites) were estimated from actual data since they also have the potential to affect the outcome of phylogenetic analysis (Swofford *et al.*, 1996). Heuristic searches were employed to find the optimal tree. Each data set was analyzed to completion, ensuring discovery of the single tree with the best score. Only the COI nt sequences were used in ML analyses of separate and combined data, as amino acid sequences are not amenable to this type of examination. Two methods were used to assess the stability of groups generated by the tree-building algorithms: (1) bootstrap analyses (Felsenstein, 1985) of 1000 or 100 replicates for MP or ML methods using the full heuristic algorithm (starting tree obtained by stepwise addition, random addition sequence, TBR branch-swapping algorithm), with groups appearing in ≥70% of the replicates considered as well-supported (Hillis and Bull, 1993), and (2) Bremer support analysis which calculates the number of extra steps at which clades fail to be resolved by comparing tree lengths between the shortest tree and successively longer trees (Bremer, 1994), with high numerical scores indicating good support. The latter procedure was performed in association with Autodecay 4.0 (Eriksson, 1996).

Constraint analyses, which involved comparisons of trees inferred from single-gene analyses, were carried out using the Wilcoxon signed-ranks test (Templeton, 1983) to determine which tree(s) (if any) differed significantly from the best estimates of phylogeny. MACCLADE 3.04 (Maddison and Maddison, 1992) was used in conjunction with this analysis to alter the branching order of taxa in trees.

TABLE 2

Results of Partition-Homogeneity Tests between or among Gene Regions[a]

Data set partitions	*P* values[b]
28S rDNA vs 16S rDNA	1.00
28S rDNA vs COI	0.30 (0.99)
28S rDNA vs 16S rDNA + COI	0.84 (1.00)
28S rDNA + 16S rDNA vs COI	0.17 (1.00)
16S rDNA vs COI	0.54 (0.99)
28S rDNA vs 16S rDNA vs COI	0.50 (0.99)

[a] *P* values in parentheses are for tests which used COI amino acid sequences.

[b] Not significant for all data set partitions.

RESULTS

Sequence Diversity

Base content varied among the gene regions; both mitochondrial genes (16S and COI) had a substantially higher A-T content (mean = 59.9 and 58.1%) than the nuclear 28S rRNA gene (42.3%). Among the ingroup taxa, there were no significant differences in nt composition for the rDNA sequences (28S: $\chi^2 = 12.2$, $df = 24$, $P = 0.98$; 16S: $\chi^2 = 35.5$, $df = 24$, $P = 0.06$), but significant differences existed for COI ($\chi^2 = 57.0$, $df = 24$, $P < 0.001$). In particular, codon position had a significant effect on nt content ($\chi^2 = 504.3$, $df = 6$, $P < 0.001$), with third codon sites exhibiting extreme compositional bias across taxa relative to the other sites. Levels of sequence divergence (uncorrected *P* distances in %) among families ranged from 2.7 to 18.9% for 28S, 14.8 to 31.8% for 16S, and 16.4 to 27.5% for COI.

Partition-homogeneity tests (Table 2) showed that sequences from the two rRNA genes showed strong congruence ($P = 1.00$), while the lowest congruence involved the combined 28S + 16S sequences with COI ($P = 0.17$). However, there was no evidence of significant incongruence for any of the data set partitions, justifying subsequent simultaneous analyses of multiple data matrices for phylogenetic inference. Table 3 summarizes the results of the MP analyses on all data sets. The frequency distributions of lengths of all possible trees generated by exhaustive searches revealed significant cladistic structure in each data set ($g_1 = -0.54$ to -1.22, $P < 0.01$; critical values range from -0.27 to -0.39 (from Table 2 in Hillis and Huelsenbeck, 1992)). g_1 values were lowest for COI nt and amino acid sequences, whereas the highest value was from the 28S data alone and the combined 28S + 16S data. Among the three gene regions, COI nt sequences had the most parsimony-informative sites (244). Each data set yielded a single most-parsimonious tree, except the COI amino acid sequences which produced two trees. Comparisons of tree indices derived from MP analyses of all data matrices are also given in Table 3.

TABLE 3

Sequence and Tree Properties Resulting from MP Analyses[a]

Data sets	Length[b]	#Var	#Pinf	g_1	#Mpt (Length)	CI[c]	HI[c]	RI
Single-gene								
28S rDNA	951	357	199	−1.22	1 (580)	0.75	0.25	0.77
16S rDNA	465	235	174	−1.01	1 (576)	0.61	0.39	0.49
COI (nt)	658	288	244	−0.63	1 (882)	0.53	0.47	0.34
COI (aa)	219	38	21	−0.54	2 (60)	0.64	0.36	0.72
Two-gene								
28S + 16S	1416	592	373	−1.22	1 (1156)	0.67	0.33	0.64
28S + COI (nt)	1609	645	443	−1.16	1 (1465)	0.60	0.40	0.52
28S + COI (aa)	1170	390	219	−1.18	1 (631)	0.74	0.26	0.76
16S + COI (nt)	1123	523	418	−0.98	1 (1462)	0.56	0.44	0.40
16S + COI (aa)	684	268	194	−0.96	1 (627)	0.60	0.40	0.51
Three-gene								
28S + 16S + COI (nt)	2074	880	617	−1.18	1 (2043)	0.60	0.40	0.51
28S + 16S + COI (aa)	1635	625	393	−1.19	1 (1207)	0.67	0.33	0.64

[a] #Var, number of variable sites; #Pinf, number of parsimony-informative sites; g_1, skewness statistic; #Mpt, number of most-parsimonious trees; CI, consistency index; HI, homoplasy index; RI, retention index; nt and aa refer to COI nucleotide and amino acid sequences, respectively.

[b] Refers to the final alignment length in base pairs (bp), amino acids, or combination of bp and amino acids, and upon which all sequence and phylogenetic analyses were based.

[c] Excluding uninformative sites.

Affinities among Anostracan Families

Three-Gene Analyses

The single most-parsimonious tree derived from the combined analysis of the three-gene data divided anostracans into two distinct monophyletic groups (Fig. 2a). This total molecular evidence tree (TMT) consisted of one clade (A) which included the Artemiidae and Branchipodidae, while the other five families composed the second clade (B). Among the families comprising the latter, the Polyartemiidae and Chirocephalidae formed a monophyletic clade (D) that was sister to the Branchinectidae, while the Streptocephalidae and Thamnocephalidae (E) were grouped together. Relationships at all levels of taxonomic divergence were well supported by both bootstrap and decay analyses (Table 4), including those between the two genera of Chirocephalidae (*Artemiopsis* and *Eubranchipus*) and the two species of *Parartemia*. Bootstrap and Bremer support values for each clade derived from the three-gene analyses (and relative to the single-gene and two-gene studies) are given in Table 4, while the nature and extent of support for the various clades in both separate and combined analyses of data sets are illustrated in Fig. 3. The three-gene analysis based on COI nt sequences yielded a tree identical to the TMT, but support for clade C was weaker. ML analysis of the three-gene data set also produced a tree (Fig. 2b) which was congruent with the TMT. The log-likelihood score for each of the best ML tree and bootstrap support for various clusters based on separate and combined analyses of data sets are given in Table 5.

Two-Gene Analyses

(a) 28S + 16S. The single shortest tree obtained from this data set was identical to the TMT (see Fig. 2a). This phylogeny had one of the highest levels of support for all clusters of families, comparable to those obtained using pooled data from all three genes (Table 4). The ML tree based on this data set also generated a single best tree similar to the TMT.

(b) 28S + COI, 16S + COI. Each of the single shortest trees (not shown) derived from both data sets using COI nt sequences resembled the tree (see Fig. 2f) found by weighted transversion parsimony analysis [i.e., clade C not resolved due to switched positions of the (Streptocephalidae + Thamnocephalidae) clade relative to the Branchinectidae]. Use of amino acids, however, resulted in the resolution of clade C, but with varying levels of support (Table 4). The ML tree (not shown) derived from analysis of 28S + COI had the same topology as the TMT, with all clusters of families receiving high support, except Streptocephalidae + Thamnocephalidae (Table 5). In contrast, the 16S + COI ML tree did not resolve cluster B, as the group containing Streptocephalidae and Thamnocephalidae was united with the cluster composed of Artemiidae and Branchipodidae. As a group, these four families were poorly supported (Table 5).

Single-Gene Analyses

(a) 28S, 16S. Because MP trees constructed from separate analyses of the 28S and 16S data were identical to the TMT, they are not illustrated. For 28S, all

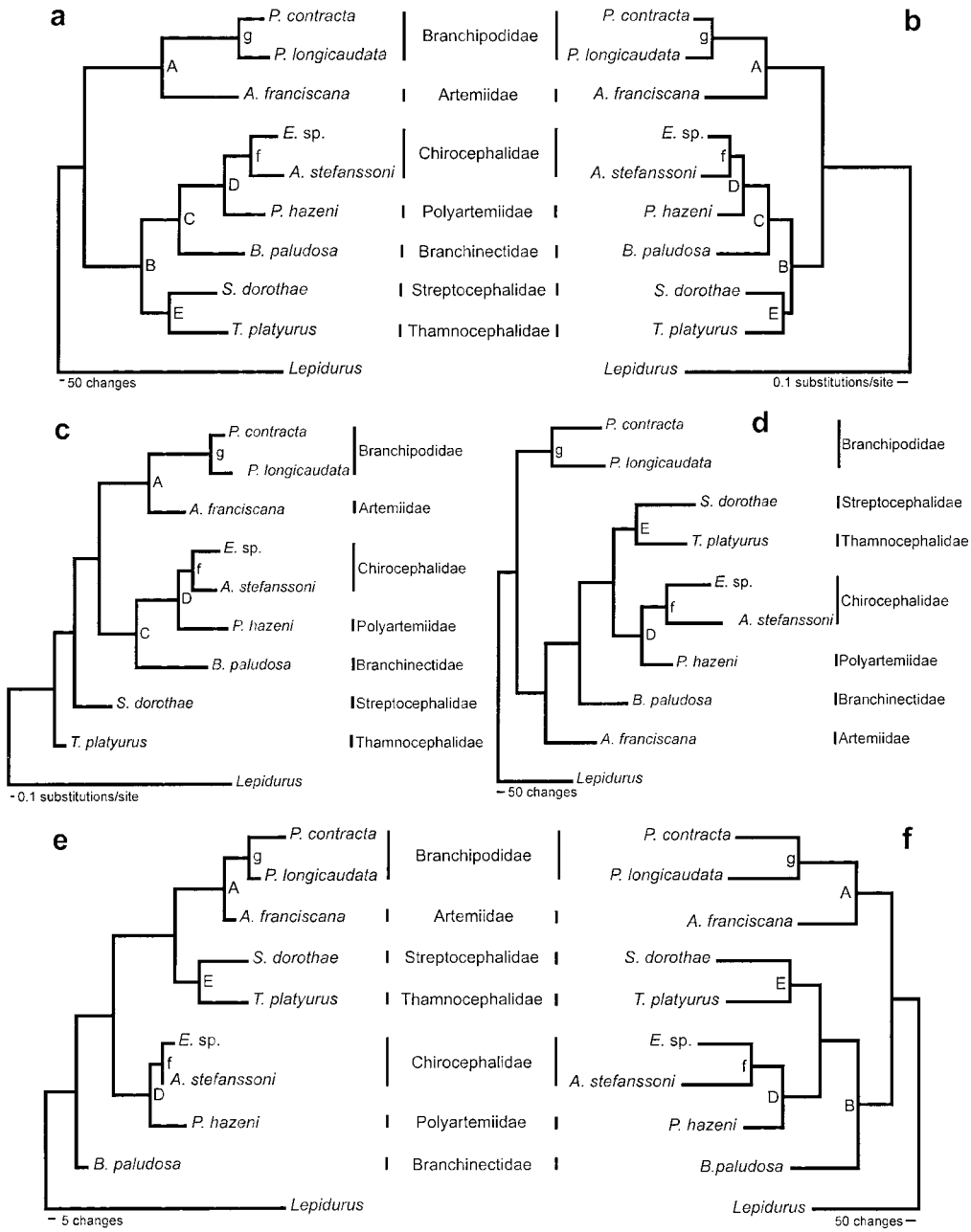

FIG. 2. Phylogenetic relationships among anostracan lineages based on various gene combinations and analytical approaches. Letters at internodes refer to monophyletic groups of families (A–E), confamilial genera (f), and congeneric species (g) that were resolved. For Figs. a–e, tree topologies are identical to those obtained from bootstrap 50% majority-rule consensus trees; details of tree properties and levels of support are shown in Tables 3–5. (a) Relationships inferred from an exhaustive MP analysis of the combined 28S + 16S + COI amino acid data. (b) Relationships inferred from ML analysis of the combined 28S + 16S + COI nucleotide sequence data (−ln likelihood = 11251.5). The parameters used for tree construction were derived from ML estimates of actual data (base frequencies: A = 0.26, C = 0.21, G = 0.21, T = 0.32; t_s/t_v ratio = 1.8; gamma shape parameter = 0.5; proportion of invariable sites = 0.3). (c) Relationships based on an independent ML analysis of the 16S rDNA data (−ln likelihood = 2886.3). The parameters used for tree construction were derived from ML estimates of actual data (base frequencies: A = 0.32, C = 0.14, G = 0.16, T = 0.38; t_s/t_v ratio = 2.0; gamma shape parameter = 0.4; proportion of invariable sites = 0.2). (d) Relationships based on an exhaustive MP analysis of the COI nucleotide sequence data. (e) One of two shortest trees inferred from an exhaustive MP analysis of COI amino acid data. (f) One of two shortest trees inferred from weighted transversion parsimony analysis of COI nucleotide sequences using an exhaustive MP search (tree length = 928; CI excluding uninformative characters = 0.53; RI = 0.35).

groups had high support values (Table 4) except the branch leading to clade C. The 16S data generated substantially lower support for various clades in the MP analysis (Table 4). ML analysis of the 28S data produced a tree congruent with the TMT. However, the 16S tree found by ML (Fig. 2c) differed from the TMT

TABLE 4

Bootstrap Scores (in %) and Bremer Support Values (d) for each Clade Resolved[a]

Data sets	Clades[b] A	B	C	D	E	f	g
Single-gene							
28S D1–D3 rDNA	100, d = 24	100, d = 15	69, d = 0	100, d = 8	89, d = 4	99, d = 5	100, d = 30
16S rDNA	99, d = 11	58, d = 3	65, d = 2	87, d = 6	59, d = 0	93, d = 5	100, d = 19
COI (nt)	—	—	—	93, d = 9	34, d = 1	72, d = 3	99, d = 15
COI (aa)	76, d = 1	—	45, d = 0	84, d = 2	64, d = 1	60, d = 1	63, d = 1
Two-gene							
28S + 16S	100, d = 33	100, d = 16	82, d = 0	100, d = 17	92, d = 2	100, d = 8	100, d = 48
28S + COI (nt)	100, d = 21	100, d = 21	—	100, d = 19	90, d = 7	99, d = 11	100, d = 50
28S + COI (aa)	100, d = 24	100, d = 14	75, d = 0	100, d = 11	92, d = 4	99, d = 5	100, d = 29
16S + COI (nt)	95, d = 11	77, d = 6	—	100, d = 17	63, d = 2	97, d = 10	100, d = 39
16S + COI (aa)	99, d = 11	66, d = 2	73, d = 2	94, d = 9	66, d = 0	94, d = 5	100, d = 19
Three-gene							
28S + 16S + COI (nt)	100, d = 36	100, d = 24	57, d = 0	100, d = 28	90, d = 7	100, d = 17	100, d = 71
28S + 16S + COI (aa)	100, d = 33	100, d = 15	87, d = 0	100, d = 19	94, d = 2	100, d = 8	100, d = 47

[a] Dashes (—) indicate that clade was not resolved in the most-parsimonious tree. nt and aa refer to COI nucleotide and amino acid data, respectively.

[b] Composition of clades (A–E are familial, f are confamilial generic, and g are congeneric species groupings): A, (Artemiidae + Branchipodidae); B, (((Chirocephalidae + Polyartemiidae) + Branchinectidae) + (Streptocephalidae + Thamnocephalidae)); C, ((Chirocephalidae + Polyartemiidae) + Branchinectidae); D, (Chirocephalidae + Polyartemiidae); E, (Streptocephalidae + Thamnocephalidae); f, (*Eubranchipus* + *Artemiopsis*); g, (*P. contracta* + *P. longicaudata*).

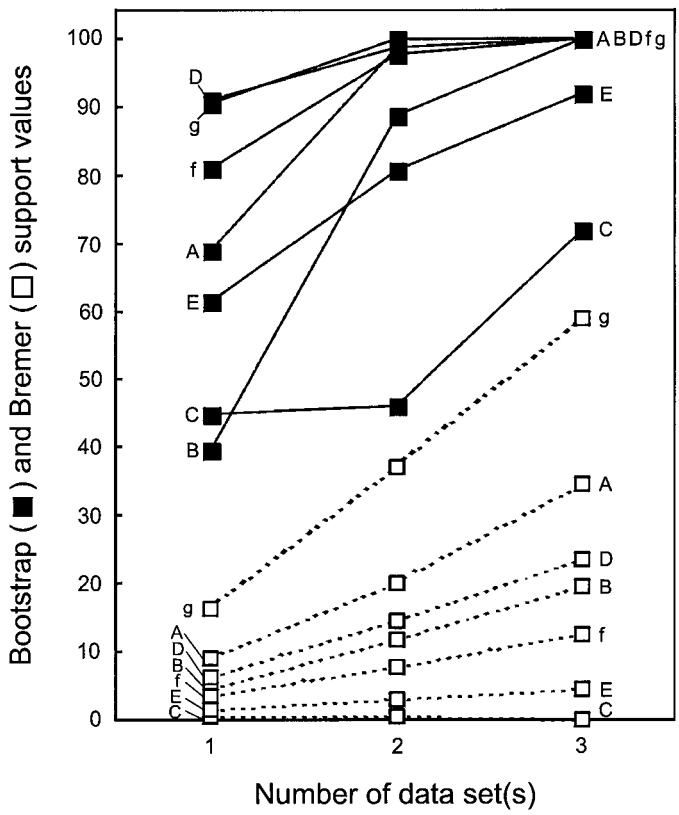

FIG. 3. Patterns of average bootstrap and Bremer support (based on data in Table 4) for monophyletic groups of families (A–E), confamilial genera (f), and congeneric species (g) in separate and combined analyses of data sets.

in one aspect: it did not recognize Thamnocephalidae and Streptocephalidae as monophyletic but instead positioned each of them as successive sister families at the base of the tree.

(b) COI. Phylogenetic trees based on this gene differed markedly from the TMT in terms of topology, tree indices, and levels of support, depending on the type of data set and phylogenetic method used (Figs. 2d–2f, Tables 3–5). Bootstrap analyses using all nt sites provided significant support for only one monophyletic group of families: the Polyartemiidae and Chirocephalidae (Tables 4 and 5). There was, however, substantial support for the monophyly of the two chirocephalid genera as well as the two species of *Pararartemia.* In contrast, one of two shortest MP trees (Fig. 2e) based on amino acid sequences only differed from the TMT by failing to resolve clade B; instead, it depicted the (Streptocephalidae + Thamnocephalidae) clade as sister to (Artemiidae + Branchipodidae), as well as positioning Branchinectidae as the most-basal family. The weighted transversion parsimony analysis yielded two minimum-length trees, one of which (Fig. 2f) differed from the TMT only in that the placement of Branchinectidae relative to the (Streptocephalidae + Thamnocephalidae) clade was interchanged. The COI ML tree based on nt sequences was identical to Fig. 2e.

Tests of Alternative Molecular Phylogenies

Table 6 presents the results of constraint analyses which enforced various familial groupings resolved from independent sequence analysis of each gene region. The most-parsimonious 28S and 16S trees (tree I)

TABLE 5

Log-Likelilhoods (−ln L) of Best Trees and Bootstrap Support (in %) Using ML Analysis[a]

Data sets	−ln L	Clusters						
		A	B	C	D	E	f	g
Single-gene								
28S rDNA	3855.1	100	52	52	60	44	64	100
16S rDNA	2886.3	94	—	89	95	—	63	100
COI	4077.0	35	—	—	93	34	94	82
Two-gene								
28S + 16S	6972.8	100	49	89	90	39	89	100
28S + COI	8288.4	100	77	70	97	60	96	100
16S + COI	7022.9	84	—	82	99	62	94	99
Three-gene								
28S + 16S + COI	11251.5	100	81	96	99	72	99	100

[a] The composition of clusters and symbol (—) definition are as in Table 4.

(and by inference the TMT) emerged as the best estimate of anostracan phylogeny. Against the best 28S tree, all COI trees (along with the 16S ML phylogeny (tree VII, Fig. 2c)) were significantly worse, except one of two shortest trees derived from the weighted transversion parsimony analysis (tree V, Fig. 2f). In contrast, several of the longer COI trees could not be rejected as poorer hypotheses of anostracan relationships when matched against the best 16S phylogeny. These included the tree found by ML based on the entire nt sequence (tree IV, Fig. 2e), the two trees obtained by MP analyses of amino acids (trees III and IV), and one of two shortest trees derived from weighted transversion parsimony analysis (tree V, Fig. 2f). The same result held for the 16S ML tree (tree VII, Fig. 2c).

DISCUSSION

Although past morphological studies have not resulted in an explicit phylogenetic hypothesis for anostracans, cases of divergence and affinity among families have been noted. For example, because of their unique number of thoracic appendages, the Polyartemiidae have been recognized as a divergent lineage (Linder, 1941; Dodson and Frey, 1991). Conversely, the Artemiidae and Branchipodidae have been thought to be closely allied because of their common possession of penes with rigid proximal parts (Linder, 1941). The present study has employed sequence data on two mitochondrial and one nuclear gene to clarify the phylogenetic affinities among anostracan families in general. The single-gene analyses indicated significant phylogenetic signal for all three gene regions. The 28S sequences were best in recovering deep nodes whereas the 16S was best for the shallow nodes, while the COI sequences were least informative. These findings were consistent with estimates of sequence divergence for each gene, being lowest in the 28S and higher in 16S and COI. Interestingly, the latter gene had the most parsimony-informative sites, but there was a high level of homoplasy, especially at the third codon positions

TABLE 6

Comparisons between the Best Estimate and Alternative Hypotheses of Anostracan Relationships[a]

Tree topology[b]	28S			16S		
	TL	*z* value	*P* value	TL	*z* value	*P* value
Tree I ((((1,2),3),((((4,5),6),7),(8,9))),10); (Fig. 2a)	best: 580			best: 576		
Tree II (((1,2),((((8,9),((4,5),6)),7),3)),10); (Fig. 2d)	606	3.41	0.0010*	594	2.85	0.007*
Tree III (((((1,2),3),(8,9)),(((4,5),6),7)),10)	597	3.16	0.0016*	580	0.94	0.346
Tree IV ((((((1,2),3),(8,9)),((4,5),6)),7),10); (Fig. 2e)	604	3.62	0.0003*	582	1.00	0.317
Tree V ((((1,2),3),(((8,9),((4,5),6)),7)),10); (Fig. 2f)	582	0.50	0.6171	579	0.77	0.439
Tree VI (((((1,2),(9,(8,((4,5),6)))),3),7),10)	657	7.23	<0.0001*	604	3.24	0.001*
Tree VII ((((((1,2),3),(((4,5),6),7)),8),9),10); (Fig. 2c)	612	5.06	<0.0001*	586	1.72	0.086

[a] Results were based on Wilcoxon signed-ranks test (Templeton, 1983). Tree I is the best estimate while trees II–VII are alternative hypotheses of anostracan relationships. Tree IV is also the ML tree based on COI nucleotide sequences alone. Trees III and VI are the other shortest trees based on amino acid sequences only and weighted transversion parsimony analysis. *P* values with asterisk indicate significant difference relative to the best tree at $P < 0.05$. TL, tree length.

[b] Definition and familial designation of taxon numbers in trees: 1, *P. contracta* 2, *P. longicaudata:* Branchipodidae; 3, *A. franciscana:* Artemiidae; 4, *Eubranchipus* sp., 5, *A. stefanssoni:* Chirocephalidae; 6, *P. hazeni:* Polyartemiidae; 7, *B. paludosa:* Branchinectidae; 8, *S. dorothae:* Streptocephalidae; 9, *T. platyurus:* Thamnocephalidae; 10, *Lepidurus:* outgroup.

where most of these sites resided. As a result, nt sequences for this gene were of little value in clarifying relationships among distantly related families, although they were useful in revealing associations among more closely allied lineages. The ability of 28S to resolve deeper nodes relative to the other genes appears to support the hypothesized origin of anostracans in the early Cretaceous (Fryer, 1987), as the resolution limits of this gene encompass their estimated age. An alternative hypothesis, that anostracans evolved much earlier, based on the recent discovery of the Upper Cambrian genus *Rehbachiella* (Walossek and Müller, 1998), remains possible and is testable using 18S sequences (but see Schram and Hof (1998) for arguments against the purported affinity of this fossil to anostracans, derived from parsimony analyses of morphological characters).

Assessing levels of saturation and compositional bias are important as their presence can obscure phylogenetic relationships. The COI nt sequences (and to a lesser extent amino acids) provided the poorest resolution of clusters of families that otherwise formed distinct groupings from separate analyses of 28S or 16S. This can be explained in part by the poorer quality of phylogenetic signal in COI (both nt and amino acid sequences), as indicated by lower g_1 values relative to 28S and 16S. To recover a tree (see Fig. 2f) which closely resembled the best estimate of phylogeny, it was necessary to perform character weighting, i.e., assigning greater weight to transversions relative to transitions, as the former accumulated in a linear fashion with sequence divergence in the data set (data not shown). Substantial noise in COI nt data also explains why its inclusion in the combined gene analyses resulted in some loss of congruence, as evidenced by the partition homogeneity test. However, this conflict of data did not have a negative effect on levels of resolution and support as combined analyses generally resulted in increased values for each cluster. Use of amino acids instead of nt sequences produced strongly congruent data sets. An attempt to find a better estimate of relationships based on the nt sequences, using the Log determinant method of Lockhart *et al.* (1994) which corrects for deviation from stationarity in base composition among taxa, was unsuccessful, as it yielded the same tree shown in Fig. 2d.

Despite their differing resolutions, there was no significant discordance in the phylogenies obtained from the separate single-gene analyses. In fact, tree topologies generally remained consistent despite variation in the methods (MP, ML) used in their construction or the genes analyzed. It is worth emphasizing that these concordant phylogenies were derived from genetic markers with different evolutionary histories (nuclear vs mitochondrial), functions (rRNA vs protein-coding), and evolutionary rates. Furthermore, despite the non-independent evolution of some sites (stems vs loops) in rDNA sequences (Wheeler and Honeycutt, 1988; Dixon and Hillis, 1993), trees produced using these data, alone or in various combinations with other genes, resolved similar patterns of familial affinities, indicating that estimates of relationships are robust.

Multiple gene studies are ordinarily viewed as a safeguard against stochastic errors which may confound the analysis of data from a single source (Li, 1997), or as a way of checking for the inadvertent analysis of pseudogenes (Zhang and Hewitt, 1996). Likewise, the analysis of multiple gene data can strengthen confidence in phylogenetic hypotheses. The present analysis confirmed this fact as trees constructed from the analysis of multiple genes showed more support for clades than those based on single genes. However, the nature of this increased support varied between the tests (see Table 4 and Fig. 3). Bootstrap scores indicated that poorly supported groups benefited most from the addition of data, whereas those with high support in the single-gene analyses gained much less. A different picture emerged from Bremer support analyses: clades with high support from single-gene analysis continued to accumulate increased support with more data, whereas weakly supported groups showed little or no increment. An extreme case of the latter pattern was exemplified by clade C, whose support did not improve with the addition of data.

Aside from improved resolution, multi-gene analyses provide protection against the effects of local rate heterogeneity on tree topology. The poor resolution for the clade including the Streptocephalidae and Thamnocephalidae in analyses of 16S rDNA derived from its tendency to group with the Artemiidae and Branchipodidae, apparently because of long-branch attraction. There is evidence for rate heterogeneity in these groups, with the latter two families showing accelerated rates of sequence divergence (unpublished data). However, this effect was reduced by the inclusion of data from the other two genes. This finding, along with those derived from ML to mitigate the confounding effects of long-branch attraction which may result from limited taxon sampling, places greater confidence on phylogenetic hypothesis in which rate heterogeneity had been considered.

The phylogenetic results indicate that the seven anostracan families examined in this study are divided into two major groups, clade A consisting of the two families (Artemiidae, Branchipodidae) characteristic of saline waters, and clade B which includes the five families (Branchinectidae, Chirocephalidae, Polyartemiidae, Streptocephalidae, Thamnocephalidae) from freshwaters. These results confirm the close affinity of the two halophilic families which had been suspected on morphological grounds (Linder, 1941). Although prior morphological studies have not led to any consensus concerning the affinities of the freshwater families, the present results suggest that they are partitioned into two major groups: clade C (Branchinectidae, Poly-

artemiidae, Chirocephalidae) and clade E (Streptocephalidae and Thamnocephalidae). The alliance of clade E is consistent with Linder's (1941) assessment based on penial morphology, but differs from Dodson and Frey's (1991) view that the streptocephalids are closely allied to the chirocephalids (but to *Artemiopsis* only) and branchinectids because of their similar antennal appendages. The latter hypothesis is in conflict with the molecular data, requiring 54 more steps for 28S and 50 more steps for 16S. The pairing of Streptocephalidae with Thamnocephalidae in the molecular trees also does not support the inclusion of the thamnocephalids in the Chirocephalidae as suspected on morphological grounds (Daday, 1910), since rearranging these taxa to match this hypothesis needed 24 and 28 additional changes for the 28S and 16S, respectively. The remaining freshwater clade C includes an internal branch leading to the Polyartemiidae and Chirocephalidae, the most strongly supported clade in all analyses. The close association of these two families conflicts with morphological assessments which have separated the Polyartemiidae from the other families because of their divergent number of thoracic segments. However, an effort to enforce the monophyly of the other freshwater families relative to the Polyartemiidae required 50 extra steps at 28S and 20 steps at 16S. The monophyly of clade C was itself supported by high bootstrap values (73–87%) but Bremer decay indices provided little support, reflecting the propensity of the Branchinectidae to resolve basally (see Figs. 2e and 2f). A clearer picture of their relationships will require the acquisition of sequence data for other members of these families. The relationships of families comprising clade C are in conflict with the morphology-based taxonomy of Linder (1941), but support Dodson and Frey's (1991) view that *Branchinecta* and *Artemiopsis* are closely related (both possess an unbranched antennal appendage attached near the proximal end of the basal (1st) segment of the II antennae). Based on details of their antennal appendages, the latter authors also suggested that *Artemiopsis* and *Eubranchipus* were not closely allied, but this proposal conflicts with the molecular data. Apart from the clarification of familial relations, the present study confirmed the confamilial status of two genera, *Artemiopsis* and *Eubranchipus*.

Among the alternative hypotheses of relationships generated from molecular data which did not differ significantly from the best estimates of phylogeny, only that linking Artemiidae and Branchipodidae with Streptocephalidae and Thamnocephalidae (tree IV in Table 6; Fig. 2e) supports the relationship proposed by Linder (1941). He united all four families because of their common possession of an indistinct seminal vesicle and a single preepipodite on each leg. Further work is needed to clarify their relationships, as Templeton's test gave inconclusive results on whether the monophyly of these families represents a good fit of the data. Imposing the morphology-based taxonomic groupings of Linder (1941) on the 28S and 16S analyses required 47 and 19 additional changes, respectively. These tree length differences were statistically significant for both genes according to Wilcoxon signed-ranks tests ($N = 61$, $z = 6.02$, $P < 0.001$ for 28S; $N = 49$, $z = 2.71$, $P = 0.007$ for 16S). The groupings proposed by Dodson and Frey (1991) also turned out as poor hypotheses of familial relationships, requiring 101 and 60 statistically significant extra steps ($N = 80$, $z = 7.85$, $P < 0.001$ for 28S; $N = 81$, $z = 4.93$, $P < 0.001$ for 16S) when matched against the best estimates of phylogenies.

Support for the monophyly of all clades was greatest in two of the combined data matrices: (1) 28S + 16S and (2) 28S + 16S + COI. Each of these data gave similar degrees of bootstrap support for clade C. For the three-gene analyses, however, greater support for this clade was obtained when COI amino acids were used instead of nt sequences. Simultaneous analysis of the two rDNA sequences thus appears to be adequate for obtaining high resolution and support for familial relationships. Although data from other gene regions will not likely improve levels of support, broader sampling of taxa is imperative to obtain a comprehensive view of anostracan relationships. The inferred familial groupings may slightly change as more samples are examined, and it will be interesting to ascertain the affinities of the Linderiellidae. Nonetheless, the present phylogeny of anostracans has provided the framework for future, more detailed studies crucial to the establishment of a stable taxonomy. For example, the branching order of taxa serves as a valuable guide on which outgroup is appropriate for studies aimed to discriminate among genera and species within families.

We made no attempt to carry out phylogenetic analyses based on either morphology alone, or upon combined molecular and morphological data. We adopted this position because there is no well-supported morphological phylogeny and our molecular analyses suggest that many cases of trait congruence reflect convergence rather than shared ancestry. The poor performance of morphological traits in identifying monophyletic taxa indicates the need for renewed efforts aimed to discover morphological characters with high phylogenetic utility. Such characters, in conjunction with molecular data, will increase our ability to resolve phylogenetic affinities in the Anostraca. However, the present analysis has provided new insights on character state evolution in the Anostraca. For instance, the scattered occurrence of frontal and antennal appendages and abdominal outgrowths in distantly related families implies the recurrent loss or acquisition of these traits. As such, these traits are of little value in assessing familial affinities. The phylogeny does establish that one unique morphological innovation, the gain of six thoracic segments, along with the

six pairs of legs associated with these segments, by the Polyartemiidae occurred late in anostracan evolution. Most classes of arthropods, such as insects or arachnids, have shown stasis in limb count since their origin, despite enormous taxonomic diversification. In contrast, variation in thoracic limb count is common at an ordinal level in crustaceans, occurring not only in anostracans but also in such groups as the conchostracans and cladocerans (Olesen, 1998). Although there has been no mechanistic explanation of this remarkable morphological flexibility, studies on a number of arthropod homeotic (Hox) genes provide growing evidence for the important role of these genes in modulating the repression or development of body segments and their associated appendages. Specifically, recent work implicating the repressive effects of certain Hox genes on "leg-making" genes in insects (Lewis *et al.*, 2000, and references therein) suggests possible genetic mechanisms underpinning the limb diversity apparent in crustaceans. The possibility that shifts in Hox gene expression are responsible for the change in limb number in polyartemiids merits further investigation.

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Note added in proof. Since the acceptance of the manuscript for publication, we have obtained 16S and COI sequences among members of the genus *Branchinella* from Australia. On morphological grounds, there is a great deal of controversy about the familial placement of this genus. Combined analyses of 16S and COI amino acid sequences depicted *Branchinella* and *Streptocephalus* as sister taxa, with the two forming a monophyletic group with *Thamnocephalus*. The same type of relationship was recovered from independent analysis of the 16S data; however, that based on COI amino acid sequences only indicated that *Branchinella* is more closely related to *Thamnocephalus* than it is to *Streptocephalus*.

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