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# Structural rRNA characters support monophyly of raptorial limbs and paraphyly of limb specialization in water fleas

**Timothy D. Swain† and Derek J. Taylor***

*Department of Biological Sciences, University at Buffalo, Buffalo, NY 14260, USA*

The evolutionary success of arthropods has been attributed partly to the diversity of their limb morphologies. Large morphological diversity and increased specialization are observed in water flea (Cladocera) limbs, but it is unclear whether the increased limb specialization in different cladoceran orders is the result of shared ancestry or parallel evolution. We inferred a robust among-order cladoceran phylogeny using small-subunit and large-subunit rRNA nuclear gene sequences, signature sequence regions, novel stem-loops and secondary structure morphometrics to assess the phylogenetic distribution of limb specialization. The sequence-based and structural rRNA morphometric phylogenies were congruent and suggested monophyly of orders with raptorial limbs, but paraphyly of orders with reduced numbers of specialized limbs. These results highlight the utility of complex molecular structural characters in resolving ancient rapid radiations.

**Keywords:** arthropod limb evolution; molecular morphometrics; rRNA secondary structure; phylogeny

## 1. INTRODUCTION

The ecological and evolutionary success of arthropods has been attributed, in part, to their diverse limb morphologies. However, most of the studies of the evolution and development of arthropod limbs have been carried out on insects, a group with very little variation in limb number or specialization (Galant & Carroll 2002; Ronshaugen *et al.* 2002). Branchiopods (such as *Artemia* and *Daphnia*) are arthropods with spectacular limb and body-plan diversity (figure 1). Some branchiopods possess many serially similar limb segments (fairy shrimps, tadpole shrimps, clam shrimps and ctenopod water fleas), whereas others possess marked or complete thoracic-segment specialization (haplopod, onychopod and anomopod water fleas). Shiga *et al.* (2002) showed that limb specialization within a *Daphnia* body plan could result when *Distalless* (*Dll*) expression is repressed by *Antennapedia*. They proposed that the first and last limbs in *Daphnia* lack large filtering structures because of *Dll* repression. Olesen *et al.* (2001) found that in *Leptodora*, a predator with specialized limbs that lack filter-like structures, limbs develop by fusion of the endites into segments forming a long raptorial limb. Nevertheless, the developmental role of *Dll* in most of the predatory cladoceran genera is still unclear. It is quite possible that limb specialization has occurred via different developmental processes in different water fleas. Do these differing limb developmental processes indicate a distant relationship or a recent innovation? Are similar limb developmental processes the result of shared ancestry or of independent evolution?

A more complete understanding of limb development and diversification depends, critically, on knowledge of the evolutionary history of a group. An apparent evolutionary trend in freshwater arthropods is an increasing specialization of limbs (Lankester 1904; Cisne 1974; Williams & Nagy 1995). This trend has also been proposed as an evolutionary pathway for branchiopods (Martin & Cash-Clark 1995). According to this hypothesis, ctenopods such as *Holopedium* and *Sida*, which have five (out of six) similar pairs of thoracic limbs, should be basal to the orders that possess well-differentiated thoracic limbs such as the anomopods (figure 1). There is now strong evidence that the water fleas (Cladocera) are a monophyletic group (Taylor *et al.* 1999; Spears & Abele 2000; Braband *et al.* 2002), but no robust evidence exists for the among-order relations of the water fleas. There have been many efforts to use molecular data to recover relationships (Hanner & Fugate 1997; Schwenk *et al.* 1998; Taylor *et al.* 1999; Spears & Abele 2000; Richter *et al.* 2001; Braband *et al.* 2002), but a lack of reliability in the tree has been found in each case. There are many possible explanations for weak support (Sanderson & Shaffer 2002), but one possible explanation for these difficulties is that the Cladocera were part of an ancient rapid radiation (Kerfoot & Lynch 1987) leaving a weak signal at the ordinal level and much evolutionary time to erode the signal with noise (homoplasy).

The purpose of this study is to use novel genetically based data that are less susceptible to evolutionary noise to test the hypothesis that water flea taxa with non-specialized limbs are basal to water flea orders with specialized limbs. In addition to standard nucleotide-based analysis, we use several unique structural sources of phylogenetic evidence from the nuclear large-subunit and small-subunit rRNA genes. We examined the molecular morphometrics of secondary structures where the phylogenetic characters are the number of base pairs in a double-stranded helix or single-stranded loop or bulge (Billoud *et al.* 2000). This technique enables recovery of informative sites in length-variable regions that are non-alignable using nucleotide sequences alone. We sequenced a new region of the large

*Author for correspondence (djtaylor@acsu.buffalo.edu).
† Present address: Department of Biological Sciences, Florida State University, Tallahassee, FL 32306, USA.

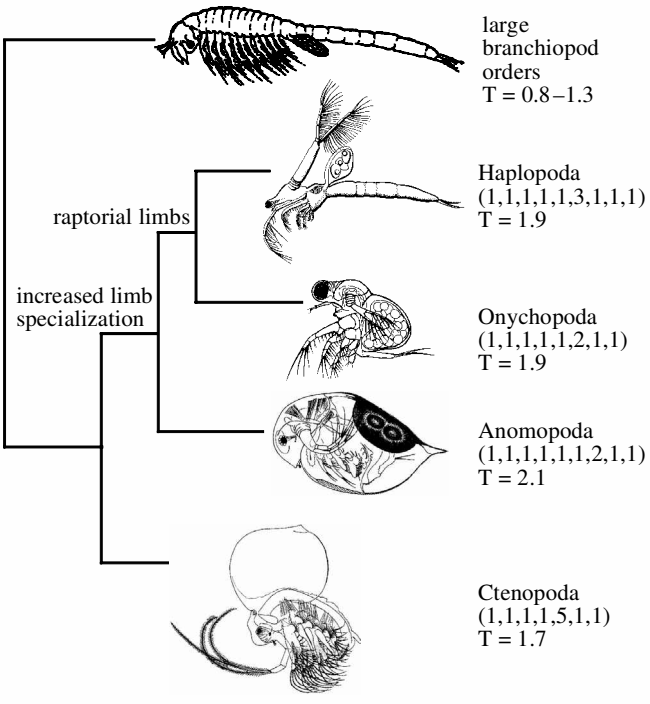


Figure 1. One hypothesis for the relationships of cladoceran orders (Martin & Cash-Clark 1995) based partly on increased limb specialization. Numbers in brackets indicate differentiated segments from anterior to posterior separated by commas (Cisne 1974). The tagmosis value (T) was calculated according to Cisne (1974) with increasing values indicating increased tagmosis. Orders are represented by lateral views of female specimens (not drawn to scale). The anomopod and ctenopod were redrawn from Alonso (1996).

subunit (V5–V7), which showed considerable length variation in daphniids, in an attempt to increase structural phylogenetic resolution. Finally, we identified complex signature sequence inserts for major groups. These characters are potentially less susceptible to evolutionary noise than single nucleotide characters that have less than four possible character states.

## 2. MATERIAL AND METHODS

### (a) *Sampling strategy*

We analysed sequences of the V5–V7 28S rDNA hypervariable regions (De Rijk *et al.* 2000) from 34 taxa representing all eight extant orders of branchiopod crustaceans (see electronic Appendix A, available on The Royal Society's Publications Web site, for sample sites and GenBank accession numbers). Twenty-four of these sequences were from this study and ten were culled from Omilian & Taylor (2001). For the V1–V3 28S rDNA hypervariable regions we used 21 taxa out of which four were sequenced in this study. For the V4 and V7 18S rDNA hypervariable regions, 21 taxa were examined with six taxa newly sequenced in this study. We used Spinicaudata clam shrimps for outgroups in the phylogenetic analysis because there is independent evidence that these are appropriate outgroups for Cladocera (Braband *et al.* 2002).

### (b) *Amplification and sequencing*

Total nucleic acid was extracted from single individuals using a cetyl-trimethylammonium bromide buffer extraction technique (Doyle & Doyle 1987) or the Epicentre QuickExtractTM kit. For *Ceriodaphnia rotunda* different individuals from the same population were used for amplifying 28S rDNA and 18S rDNA regions. We PCR amplified, gel purified and directly sequenced the products (for protocols see Omilian & Taylor (2001)). The primers used to amplify the 18S V4 and V7 regions were from Crease & Colbourne (1998), but additional specific primers were used for *Ceriodaphnia rotunda*, *Simocephalus serrulatus* and *Cercopagis pengoi*: 18S V4 downstream 5′-ATTGGAGGGCAAGTCTGGTGCCAGC-3′ and upstream 5′-GACCGAGGTCCTATTCCATC-3′. Likewise, the primers for the 28S V1–V3 regions were from Taylor *et al.* (1999), but a specific primer pair was used for *Diaphanosoma*: downstream 5′-ATCAGTAAGCGGAGGAAAAGAAAC-3′ and upstream 5′-TCGATTTGCACGTCAGAATCGCT-3′. The 28S V5–V7 region primers were 28ee and D7r from Omilian & Taylor (2001).

PCR products yielding ambiguous direct sequences were cloned using the Invitrogen TOPO TA Cloning Kit. All cloned DNA fragments were amplified using the original PCR primers, except for *Diaphanosoma* 18S V1–V3, which was amplified from the clone using the M13 primers included in the cloning kit. Clones were screened by comparing sequences from multiple clones and selecting the majority consensus. We applied the International Union of Pure and Applied Chemistry (IUPAC) ambiguity codes in cases where no clear consensus was reached.

### (c) *Alignment and secondary-structure analysis*

Forward and reverse sequences were edited and assembled using SEQUENCHER 4.0 (Gene Codes Co., Ann Arbor, MI, USA). A global alignment of all sequences (Treebase.org, SN839) was made using CLUSTALX 1.8 (Thompson *et al.* 1997) with default settings, and adjusted manually in BIOEDIT 4.8.9 (Hall 1999).

Preliminary modelling of secondary structures by energy minimization for the 28S V1, V2, V3, V5, V6, V7 and 18S V4 and V7 were carried out using MFOLD 3.1 (Mathews *et al.* 1999; Zuker *et al.* 1999). The complete variable region (with boundaries defined by De Rijk *et al.* (2000)) was entered into MFOLD and folded with the energy dot-plot off and all other parameters set to default. Energetically stable structures were compared with the eukaryotic rRNA database models (De Rijk *et al.* 2000; Van de Peer *et al.* 2000) and modified in RnaViz 2.0 (De Rijk & De Wachter 1997) to ensure retention of conserved eukaryotic core elements.

Putatively homologous secondary structures were identified and used as characters. The sequence alignment for each hypervariable region was converted to a simple text file and coded with the DCSE (dedicated comparative sequence editor) secondary-structure notations (De Rijk & De Wachter 1993) using Microsoft WordPad. The substructures were labelled alphabetically using capital letters for each double-stranded helix. A single-stranded bulge or loop was labelled with two lower-case letters, indicating the two helices that surround the single-stranded structure. The structures were coded by the number of nucleotides involved in each double-stranded helix, bulge or loop region (Billoud *et al.* 2000). DCSE-style alignments made critical examination of structures possible and permitted conversion from alignments to structural models by RnaViz (see electronic Appendix A).

### (d) *Phylogenetic analysis*

All phylogenetic analyses were conducted using PAUP 4.0 beta 10 (Swofford 2000) and MRBAYES 2.01 (Huelsenbeck &

Ronquist 2001). Hierarchical model fitting using 56 maximum-likelihood (ML) models in a series of likelihood ratio tests was performed using MODELTEST 3.06 (Posada & Crandall 1998).

Minimum-evolution (ME) and ML searches were performed using a heuristic search algorithm with tree-bisection–reconnection (TBR) branch swapping and 10 random-sequence taxon additions. ML distance was used for the ME criterion, with all parameter values empirically determined from the data. The search for the most parsimonious tree was performed using a heuristic search algorithm with equal weight for all characters and gaps treated as either a fifth character state or as a missing character. Estimates of support were obtained by non-parametric bootstrapping with 1000 pseudoreplicates and a Bayesian statistical method using Markov Chain Monte Carlo (MCMC) sampling (Huelsenbeck & Ronquist 2001). The MCMC analysis was performed using MRBAYES 2.01, with settings corresponding to the empirically determined model of molecular evolution. We sampled every tenth tree during a $10^5$ iteration chain and, after inspection for convergence, removed the first $10^3$ trees as 'burn in' (Huelsenbeck & Ronquist 2001); the remaining trees were loaded into PAUP and a 50% majority rule consensus tree was produced.

A structural character matrix (Treebase submission number SN839) was created using MACCLADE 4.0. Phylogenetic trees were constructed using maximum parsimony (MP) with the heuristic search algorithm. Characters were formatted as ordered and equally weighted. Estimates of support were obtained by non-parametric bootstrap analysis. There were no gaps in the character alignment, because missing structures were coded as 0.

### (e) *Taxon sensitivity analysis*

Taxa that have long terminal branches in phylogenetic reconstructions may introduce a bias and lead to incorrect phylogenetic conclusions or alter bootstrap values (Hillis *et al.* 1992). We evaluated the sensitivity of trees to such long-branched taxa by culling the longest internal branches and carrying out a new set of phylogenetic analyses. In the morphometric data, the deletion of taxa with long terminal branches resulted in dramatic changes in tree topology. To examine whether the topology of these trees was being driven by a few problematic taxa, each taxon was singly deleted from the dataset and a heuristic MP search was performed for the reduced data. If the removal of a single taxon caused a change in the tree topology of the remaining taxa, further pruning was attempted. If there was no change in tree topology, no further pruning was performed.

## 3. RESULTS

### (a) *Signature sequences and secondary structure*

The V7 region of the 28S rDNA contained a 19–24 bp region with apparent signature sequences and structures at the ordinal level (figure 2*a*). A positionally homologous insert was shared across the Cladocera, but absent among the orders of large branchiopods. *Cyclestheria*, the proposed transitional taxon between the Order Spinicaudata (clam shrimps) and Cladocera, contained an insert of 2 bp at the same position as the larger inserts of Cladocera. The cladoceran insert could be folded into the same novel helix structure with a stem length of four (figure 3*a*). The first position of the putative stem showed evidence of two full-compensatory mutations and one semi-compensatory mutation—the other stem sites lacked sequence variation. The insert appeared to divide the cladoceran orders with specialized limbs into two derived groups. The Haplopoda, Onychopoda and Ctenopoda (non-specialized limbs) contained a 22–24 bp insert of strong sequence similarity (the stem region was CGCTGKTAAMAAG-GCGA). This insert type shared a unique GC pair in the first stem position and a unique AA insert in the loop. The Anomopoda contained a second type of insert of 19 bp of near-identical sequences (ATWYRGCTGTCAAAGGC-YA).

The 28S V3 region also contained order-specific signature sequences on the D5 helix (figures 2*b* and 3*b*) that appeared to divide the limb specialists into two derived groups. The Anomopoda had the sequence motif (CTCGARYAGAG), whereas the Ctenopoda (non-limb specialists), Gymnomera (Haplopoda and Onychopoda) had an additional three to nine nucleotides (mostly A or T) inserted at the 5′ end of the loop and an additional A inserted near the 3′ end of the loop. The Ctenopoda shared a unique motif (ATA) within the loop and the Haplopoda and Onychopoda shared a C to T mutation in the stem and an expanded loop with four to seven additional thymine nucleotides.

Although there are no apparent unique stems in V3, the putative loop region secondary structure did vary in an order-specific manner. The Anomopoda had a stem of three and a loop of five nucleotides, whereas the remaining cladoceran orders (Ctenopoda, Haplopoda and Onychopoda) had an enlarged loop of 9–16 nucleotides. The Spinicaudata had a stem loop that is similar in size to that of the Anomopoda (4–6 nucleotides in the stem and 6–4 nucleotides in the loop), however the sequence in this order was very different from any of the cladoceran sequences (figure 3*b*).

### (b) *Nucleotide phylogeny*

The aligned sequences of the 18S V4 and V7 and the 28S V1–V3 and V5–V7 regions from 21 branchiopod taxa consisted of 3531 characters, out of which 1632 were excluded from the analysis because they could not be aligned unambiguously (see electronic Appendix A for exclusion set).

Hierarchical model fitting indicated that the Tamura–Nei model (Tamura & Nei 1993) with invariable sites and the gamma parameter (TrN + I + G) had the best fit to the combined 18S and 28S data. The following parameters were included in the analysis: base frequencies (Base = 0.2450, 0.2319, 0.3060), number of substitution types (Nst = 6), substitution-rate matrix (Rmat = 1.0000, 2.9304, 1.0000, 1.0000, 6.00589), gamma shape (Shape = 0.3652) and the proportion of invariable sites (Pinvar = 0.5407).

A search for the optimal ME tree with ML distances resulted in a single best tree (score = 0.390), which made the orders of limb specialists paraphyletic. Recognized orders were recovered but *Leptodora kindti* (Haplopoda) was an exception in that it grouped within the Onychopoda (figure 4). MP using gaps as missing characters resulted in two optimal tree topologies of 850 steps, whereas MP using gaps as a fifth character state resulted in two best tree topologies of 958 steps. Unlike ME, MP grouped *Ceriodaphnia* and *Scapholeberis* in a clade—all other nodes were the same as the ME tree. The Bayesian

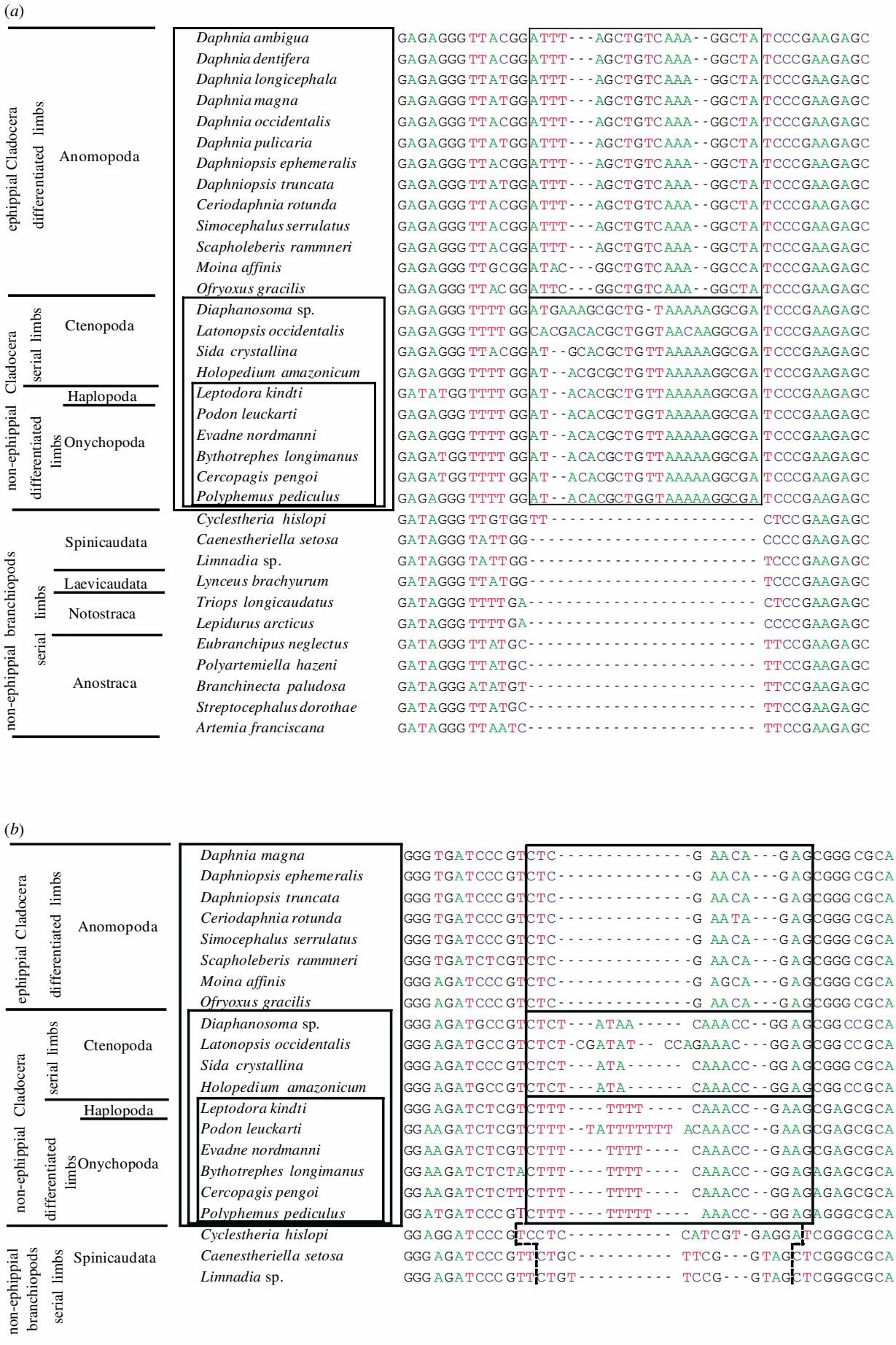


Figure 2. (*Caption opposite*.)

Figure 2. (*a*) Signature sequence region for cladoceran orders in the 28S rDNA V7 region. See figure 3*a* for secondary structure details. Solid-line boxes enclose nucleotides that form a novel insert and stem–loop in Cladocera. There are two apparent signature types for cladoceran orders marked by the boxes: Anomopoda and (Ctenopoda, Haplopoda, Onychopoda). (*b*) Signature sequence region in the 28S rDNA V3 hypervariable region. See figure 3*b* for further secondary structure details. Solid-line boxes enclose the three apparent signature types for cladoceran orders: Anomopoda, Ctenopoda and (Haplopoda, Onychopoda).

analysis produced a consensus tree that differed from the ME tree only in its placing of *Polyphemus* as basal to the other Onychopoda.

Cladocera, Anomopoda and Ctenopoda were strongly supported by all reconstruction methods (figure 4). The paraphyly of the limb specialists (Anomopoda, Haplopoda and Onychopoda) was moderately supported by ME, MP and Bayesian reliability values. The monophyly of a predatory clade (Haplopoda–Onychopoda) was weakly supported.

The removal of the longest ingroup terminal branches (*M. affinis*, *L. occidentalis*, *P. pediculus* and *C. hislopi*) resulted in no change in the best tree topologies for any of the tree-building strategies (ME, MP or Bayesian). Nevertheless, removing long-branched taxa greatly increased the bootstrap support for the paraphyly of the limb-specialist orders and the Haplopoda–Onychopoda clade (figure 4).

### (c) *Phylogeny of rRNA structural characters: molecular morphometrics*

The aligned morphometrics characters for the 18S V4 and the 28S V1–V3, V5 and V7 regions from 21 branchiopod taxa consisted of 350 characters, out of which 238 were parsimony informative and 166 had been omitted from the nucleotide-based analyses.

MP analysis resulted in a single best tree (figure 5) of 2104 steps. This tree was congruent with the nucleotide-based tree and grouped individual taxa in their putative orders. Bootstrap values with the MP and ME criteria supported the monophyly of Cladocera, Anomopoda and Gymnomera. Most other among-order relationships had weak (less than 70%) support.

The removal of ingroup taxa from this tree (regardless of terminal-branch length) during tree-pruning efforts had a drastic effect on both the topology of the best tree (ME or MP) and the bootstrap values, suggesting that the topology of this tree was being driven by a few problematic taxa or clades (orders). To examine this further, each taxon was deleted and a heuristic MP search was performed for the reduced data. The removal of the outgroup Spinicaudata from the tree had a stabilizing effect on the overall tree topology and bootstrap values, such that the continued removal of ingroup taxa had no effect on the tree topology and only a small effect on bootstrap values. After the pruning of Spinicaudata from this tree and midpoint rooting, both the ME and MP bootstrap values strongly supported the paraphyly of limb-specialist orders and a monophyletic Gymnomera (Haplopoda–Onychopoda).

## 4. DISCUSSION

### (a) *Evolution of thoracic limb specialization*

Evolutionary studies are needed to determine whether shared developmental processes are the result of shared ancestry or independent evolution. Our nucleotide, molecular morphometric and rRNA structural evidence strongly supports the hypothesis that cladoceran orders share thoracic limb types because of both independent evolution and shared ancestry. In all analyses we found a monophyletic association of the predatory orders (Haplopoda, Onychopoda) suggesting that the sharing of raptorial limbs is a result of shared ancestry. At a deeper evolutionary level, the orders with specialized thoracic limbs (Haplopoda, Onychopoda, Anomopoda) failed to form a monophyletic group because the ctenopods (with serially similar limbs) strongly grouped with the predatory orders (Haplopoda, Onychopoda). This suggests either that limb segment specialization has independently arisen in anomopods and in the Gymnomera (haplopods, onychopods) or that limb specialization has been lost in the Ctenopoda.

More developmental research is needed to determine whether the limb similarity is parallel or convergent. Olesen *et al.* (2001) found that haplopod (*Leptodora*) limbs develop by fusion of the endites into segments forming a long raptorial limb. It is unknown whether the same developmental process occurs in onychopods, but if so, our results indicate that this sharing would be the result of recent ancestry. The differentiation of the first thoracic limb in anomopods seems to occur when *Antennapedia* represses *Dll* and eliminates the filtering structure (Shiga *et al.* 2002). Much more evolutionary and developmental evidence will be needed from other cladoceran genera to determine how the developmental tools controlling limbs have been used to provide the diversity in cladocerans.

Our results add further support to the monophyly of the Cladocera and its sister-group relationship with the clam shrimp *Cyclestheria*. Prior studies revealed that the 18S contains Cladocera-specific inserts (Crease & Taylor 1998; Spears & Abele 2000). Here, we show that the 28S also contains Cladocera-specific inserts. The sequence similarity, position in the gene and potential to form the same secondary structure are evidence that this complex character is identical by descent. Interestingly, the proposed transitional taxon between clam shrimps and water fleas also has an apparently transitional insert of two nucleotides in the same position as the Cladocera insert. The available genetic evidence seems to support the old hypothesis that the water fleas resulted from a radiation of benthic clam-shrimp ancestors into the plankton and other habitats. Testing the intriguing proposals that the rise of the modern fishes with suction feeding enhanced the radiation of water fleas (Kerfoot & Lynch 1987) or that the initial cladocerans were neotenous clam shrimps requires more evidence about the timing of the radiation and the developmental biology of water fleas.

Surprisingly, our results support an among-orders relationship that has not been proposed by morphological

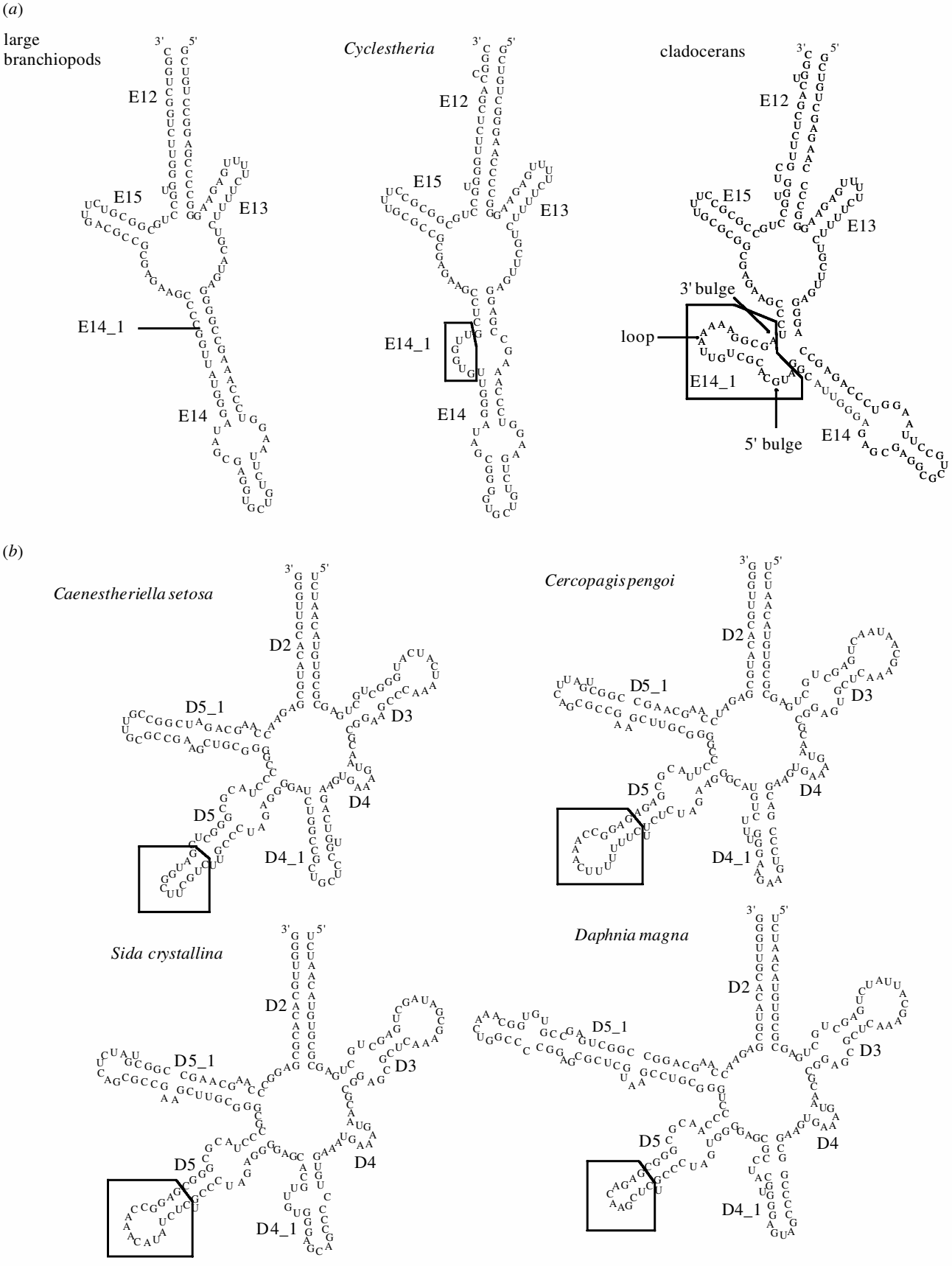


Figure 3. (*a*) Representative putative secondary structures of the 28S rRNA V7 region containing the cladoceran signature inserts. Boxes enclose the Cladocera-specific stem comprising a 5′ bulge, a stem loop and a 3′ bulge. (*b*) Representative putative secondary structures showing the signature regions in the 28S rRNA V3 region of cladocerans. *Caenestheriella setosa* is a large branchiopod in the order Spinicaudata (the branchiopod order most closely related to the cladocerans), *Cercopagis* is an onychopod, *Sida* is a ctenopod and *Daphnia* is an anomopod.

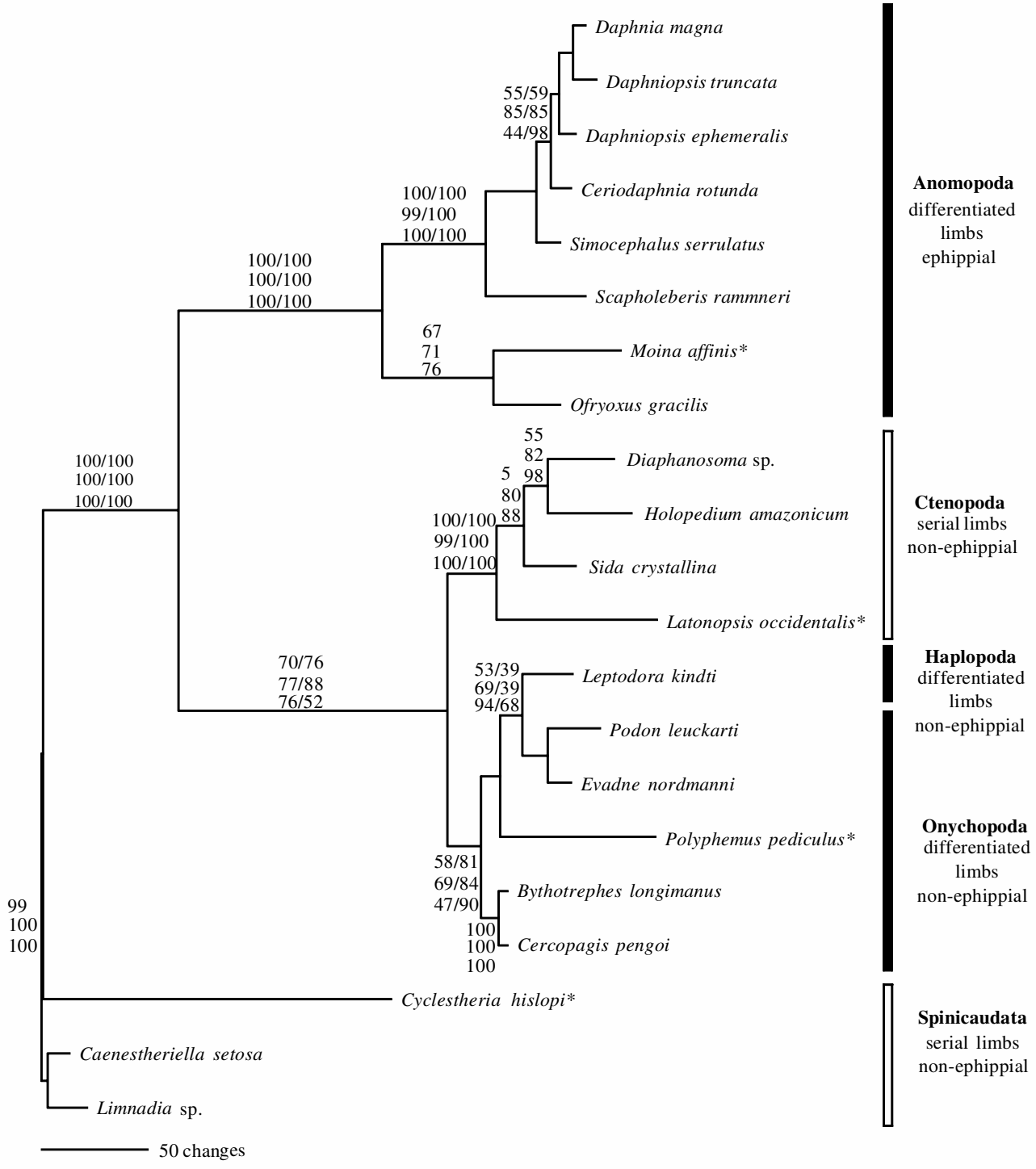


Figure 4. One of two most parsimonious trees with gaps as a fifth character state for 18S V4, V7 and 28S V1-V3, V5-V7 for 21 taxa representing all orders of Cladocera and Spinicaudata. The other MP tree (not shown) has the same topology except that *Ceriodaphnia rotunda* and *Scapholeberis rammneri* group together. Support values are listed vertically from top to bottom: ME (1000 iteration ME using ML DNA distance), MP (1000 iteration MP using gaps as a fifth character state) and Bayesian (10 000 iteration *a posteriori* Bayesian analyses using the empirically determined model of molecular evolution). The support values before the forward slash (/) are values prior to pruning; values after the slash were determined after pruning. Taxa marked with asterisks were pruned from the tree based on taxon sensitivity analysis. Orders with specialized limbs are indicated by solid bars.

analyses despite several hundred years of permutations and proposals. Our evidence suggests a grouping of water flea orders that lack an ephippium or resting egg case (Ctenopoda, Haplopoda, Onychopoda). Some molecular analyses have found modest or weak support for this association (Taylor *et al.* 1999; Spears & Abele 2000; Richter *et al.* 2001; Braband *et al.* 2002), but there has been no shared derived morphological character proposed for this group. The lack of an ephippium is probably a poor character for this group because the state of non-ephippial diapausing eggs is ancestral. The ancestral clam shrimps (cyclestheriids) do have a protective structure

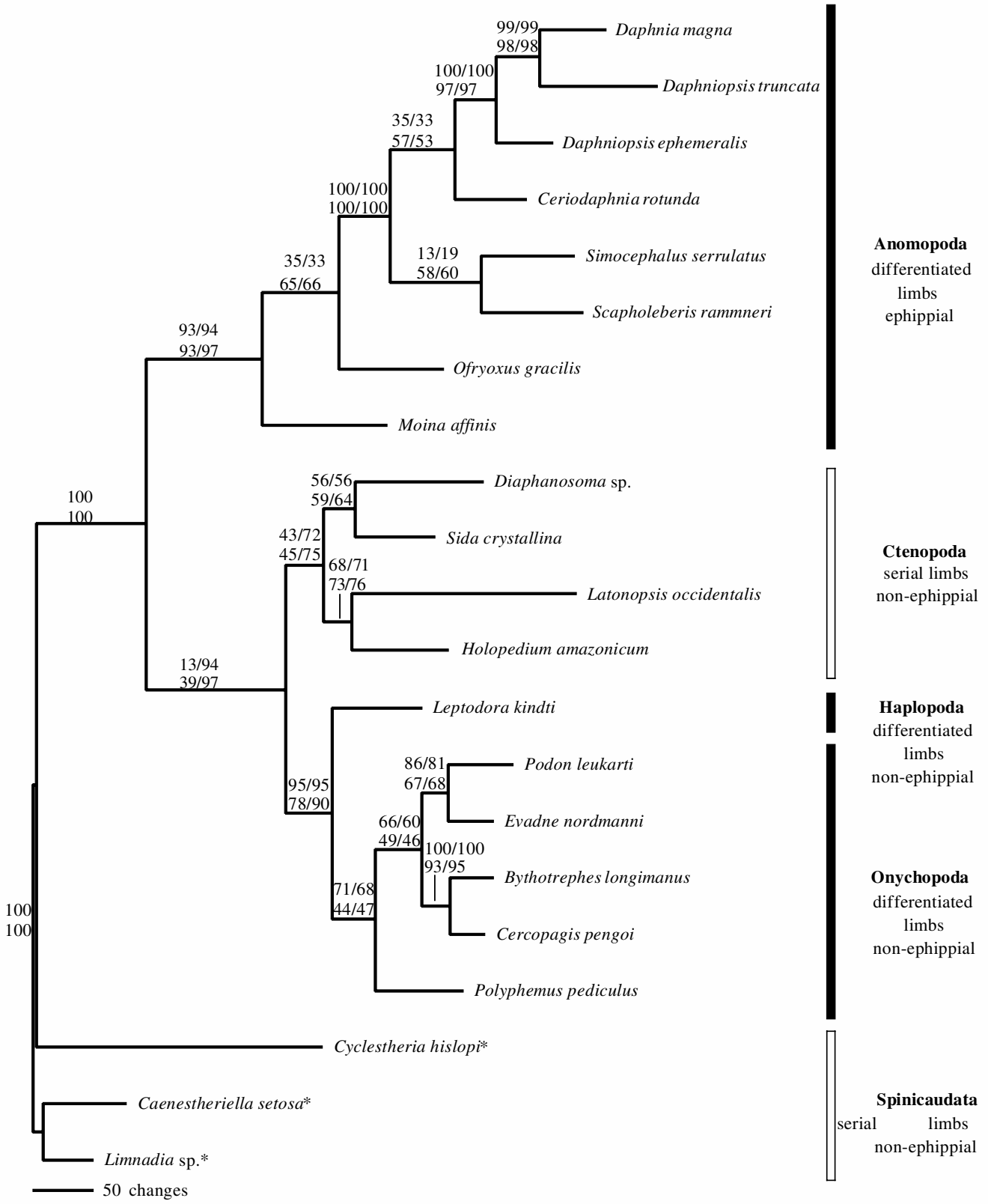


Figure 5. Maximum-parsimony single best tree for 28S V1, V2, V3, V5, V7 and 18S V4 using molecular morphometric characters for 21 taxa representing all orders of Cladocera and Spinicaudata. Bootstrap values are listed vertically from top to bottom: ME (ME using mean character distance as the standard distance) and MP. The bootstrap values before the forward slash (/) are values prior to pruning. The solid bars represent orders with specialized limbs. Taxa marked with asterisks were pruned from the tree based on taxon sampling sensitivity.

derived from the carapace with a similar function to the ephippium, but its homology is ambiguous with respect to the ephippia of anomopods (Roessler 1995). Owing to the paucity of traditional morphological characters for taxa that underwent rapid radiations, such as water fleas, it is quite possible that the future defining structures for ancient groups will be genomic or molecular morphometric in nature.

### (b) *Use of molecular morphometrics to recover phylogenies*

Traditionally, rRNA secondary structure information had been used only indirectly in phylogenetics analyses to improve nucleotide alignments. Our results agree with other recent studies (Billoud *et al.* 2000; Collins *et al.* 2000; Caetano-Anolles 2001, 2002*a*,*b*) that find that phylogenetic analyses based on direct coding of secondary structure can have several advantages. First, molecular morphometrics can add characters that are unavailable or noisy because of alignment ambiguities. In the cladoceran dataset over 70% of the characters in the molecular-morphometrics analysis were excluded from the nucleotide analysis. Character independence may also be improved because our analysis counts stem-based characters only once. In traditional nucleotide analysis stem-coding nucleotides are non-independent because they undergo coordinated evolution with compensatory mutations to maintain secondary structure. Also, structural characters evolve differently from nucleotides and will be less susceptible to some processes that can bias phylogenetic results, such as nucleotide-composition shifts.

Despite the advantages, and the finding that the molecular-morphometrics tree generally agreed with the evolutionary trees estimated based on independent data from rDNA and other genes, we note several limitations to the structural approach. First, although stem characters are more independent than in nucleotide analysis, the length-variable characters in the genome are probably not completely independent. Crease & Taylor (1998) found that length variation among the V2 of the 28S rDNA and the V4 and V7 of the 18S rDNA in cladocerans was correlated. The mechanism of coordinated evolution and the role of selection in molecular length variation remain to be addressed. Second, some areas of the cladoceran rDNA array consist of exceptionally long simple stems that are difficult to code because they lack internal structural homology. The V4 and V7 of 18S and the V6 of 28S in *Daphnia* are among the longest of known rDNAs, and we found here that they possess an 11–524% difference in sequence length. Finally, our taxon sensitivity analysis suggests that molecular morphometrics data are susceptible to long-branch attraction biases.

A commonly perceived limitation to structural analysis is that the structures of most rRNAs are uncertain and therefore useless for phylogenetics. We disagree with this assertion. First, the structures in the comparative databases are quite reliable, as comparisons with crystallography evidence indicate that 97–98% of the base pairings using the two methods are identical (Gutell *et al.* 2002). For regions that lack comparative evidence because of alignment problems, the secondary structure shapes are still very conserved. Even analysis of short structures with the lowest level of confidence (thermodynamically determined) resulted in congruent phylogenetic reconstructions (Caetano-Anolles 2002*a*), suggesting that any bias created by structures folded with minimum-energy predictions is distributed equally across the phylogeny. Thus, inaccurate structure may add some noise but not a systematic bias. Finally, for the complex characters that we found (rDNA inserts), structure is phylogenetically irrelevant—they are clearly homologous by sequence similarity and position.

## 5. CONCLUSIONS

We have provided robust structural DNA evidence for between-order relations in the Cladocera. The results suggest that the specialized raptorial thoracic limbs of the haplopods and onychopods arose once, but that specialization of limb segments either occurred independently in the anomopods or was lost secondarily in the ctenopods. Complex structural characters and molecular morphometrics provided additional characters and resolution for the Cladocera—a group with apparently ancient rapid radiations.

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