

# Molecular phylogeny of the Branchiopoda (Crustacea)–Multiple approaches suggest a ‘diplostracan’ ancestry of the Notostraca

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## Abstract

The evolutionary history of Branchiopoda (Crustacea) traditionally has attracted considerable interest due to the diversity of the group. Recently molecular methods have been applied to the study of branchiopod systematics with some success, but central questions, such as the phylogenetic position of Laevicaudata and Notostraca, and the intrinsic cladoceran phylogeny, remain unanswered. We examined the phylogeny of Branchiopoda by using two genes, mitochondrial 16S rRNA and nuclear 28S rRNA, which previously have seen little use for inferring branchiopod phylogeny. The number of ingroup taxa included was 42, representing all eight extant branchiopod orders. The data were analyzed using parsimony, maximum likelihood, and Bayesian Inference of phylogeny. Some higher-level taxa were supported in all analyses of the combined data: Phyllopoda, Cladocera, and Gymnomera. Other higher-level taxa were not supported in any trees: Diplostraca and Conchostraca. A case is made for a possible diplostracan ingroup position of Notostraca based on our data and on previously published molecular and morphological evidence. The recent discovery of a Devonian branchiopod, which is morphologically an intermediate between a notostracan and a ‘conchostracan’, is congruent with a diplostracan ancestry of Notostraca.

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## 1. Introduction

Branchiopoda is a diverse crustacean assemblage comprising about 1180 currently described species (Adamowicz and Purvis, 2005) occurring predominantly in inland aquatic systems (Dumont and Negrea, 2002). On the whole, the group exhibits a ‘curious combination of morphological plasticity and evolutionary stasis’ (Martin, 1992). Some of the branchiopod subtaxa (anostracans, notostracans, ‘conchostracans’) have long been discussed as exhibiting many primitive crustacean features (Calman, 1909; Fryer, 1992; Schram and Hof, 1998), and the discov-

ery of branchiopod-like fossils from the Upper Cambrian ‘Orsten’ fauna has confirmed this view (Müller, 1983; Walossek, 1993; Walossek and Müller, 1998). Paleontologically, the Branchiopoda crown group (Branchiopoda *s. str.*) at least dates back to the Devonian (Scourfield, 1926; Olesen, 2004), while a stem-lineage branchiopod probably was present during the Upper Cambrian (Walossek, 1993). On the other hand, some branchiopods, mainly cladocerans, are highly modified crustaceans, such as the planktonic predatory *Leptodora* and the advanced filter-feeders within Daphniidae. Hence, Branchiopoda, while being most likely monophyletic (Sanders, 1963; Martin, 1992; Walossek, 1993; Spears and Abele, 2000; Olesen, 2004; Giribet et al., 2005), includes a challenging variation in morphology and lifestyles. It is therefore no surprise that in recent years there has been an increased interest in

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branchiopod phylogeny and evolution. Several genes have been sequenced (e.g., Taylor et al., 1999; Spears and Abele, 2000; Braband et al., 2002; Giessler et al., 1999; Swain and Taylor, 2003; DeWaard et al., 2006) and morphological information has also been included to a reasonable extent (Olesen, 1998, 2000; Negrea et al., 1999; Richter, 2004). Some classic groupings of higher taxa have been supported (Cladocera) while others have been shown to be paraphyletic (Conchostraca). The ‘Conchostraca’ has turned out to be a key-taxon in the understanding of the phylogeny and evolution of Branchiopoda (see summary of current view on branchiopod phylogeny in Fig. 1). Linder (1945) recognized the distinctness of the two ‘conchostracan’ subtaxa, Laevicaudata and Spinicaudata, but it was Fryer (1987a) who first expressed serious doubt about the validity of ‘Conchostraca’. Later, based on information provided by Sars (1887), Martin and Cash-Clark (1995) and Olesen et al. (1997) suggested a close relationship between the aberrant *Cyclestheria hislopi* (a former spinicaudatan, see Martin and Davis, 2001) and Cladocera. This has now been confirmed by independent molecular studies (Crease and Taylor, 1998; Taylor et al., 1999; Spears and Abele, 2000; Braband et al., 2002; DeWaard et al., 2006). Ax (1999) suggested the name Cladoceromorpha for *Cyclestheria hislopi* and Cladocera together. The paraphyly of ‘Conchostraca’ with respect to Cladocera is therefore well established. In this paper we explore whether ‘Conchostraca’ is paraphyletic also with respect to Notostraca, yielding a paraphyletic Diplostraca as well.

Recent work has suggested a link between Notostraca and ‘Conchostraca’ in an unexpected way. Notostracans, with their lifestyle as non-filtrating omnivorous animals, for some time have been considered among the most modified branchiopods (Fryer, 1988; Walossek, 1993), but their ancestry has not been explored satisfactorily. A

number of recent morphological discoveries suggest a ‘conchostracan’ origin of Notostraca. For example, Møller et al. (2003) found that the carapace of *Triops cancriformis* has a paired *anlagen*, exactly like that of various spinicaudatans and cladocerans (Olesen, 1999; Olesen et al., 2003; Olesen and Grygier, 2004). Richter (2004), building on information from Linder (1945) and Martin (1989), furthermore showed a detailed similarity in the morphology of the mandibular gnathal edges in Laevicaudata and Notostraca, which were interpreted as indicating a possible close relationship between these two taxa. Finally, Fayers and Trewin (2003) recently described a remarkable new branchiopod fossil, *Castracollis wilsonae*, which in a hitherto unseen way combines notostracan and ‘conchostracan’ features.

The purpose of this paper is twofold. First, to explore the general lines of branchiopod phylogeny based on two genes, a presumed conserved gene (nuclear 28S rRNA) and a presumed less conserved gene (mitochondrial 16S rRNA), in order to contribute to the growing number of genes sequenced for various branchiopods. Secondly, to focus on the status of ‘Conchostraca’ and Diplostraca and discuss the possibility of paraphyly not only of ‘Conchostraca’ but also of Diplostraca with respect to Notostraca.

## 2. Materials and methods

### 2.1. Taxonomic sampling

Table 1 shows the origin and availability of all species sequenced and analyzed in this study. The 42 ingroup species represent all major groups recognized within Branchiopoda. Most material was collected by the authors in Denmark (JTS), in the Moscow region of Russia in 2001 (JTS), in Australia 2001 (JO), in Italy 2001 (JO), or was provided by donations. All materials were preserved in 96% (or higher) ethanol and stored refrigerated or frozen from the time of collection and identification to processing.

### 2.2. Molecular techniques

#### 2.2.1. DNA extraction

Total genomic DNA was extracted from whole single individuals (Cladocera), parts of or whole limbs (larger branchiopods) using the Qiagen DNeasy<sup>®</sup> Tissue Kit following the Qiagen DNeasy Protocol for Animal Tissues 05/2002.

#### 2.2.2. Gene amplification

PCR products were amplified according to standard procedures. The 28S rRNA fragment was amplified using the following primers: 1274 (5'-GAC CCG TCT TGA AAC ACG GA-3') and 1275 (5'-TCG GAA GGA ACC AGC TAC TA-3') for all species (Markmann, 2000). The fragment was extended by amplifying an overlapping fragment using primers designed for this study: 1480ml (5'-TAG GGG CGA AAG ACT AAT CG-3') and Stenderup (5'-GCG TCA

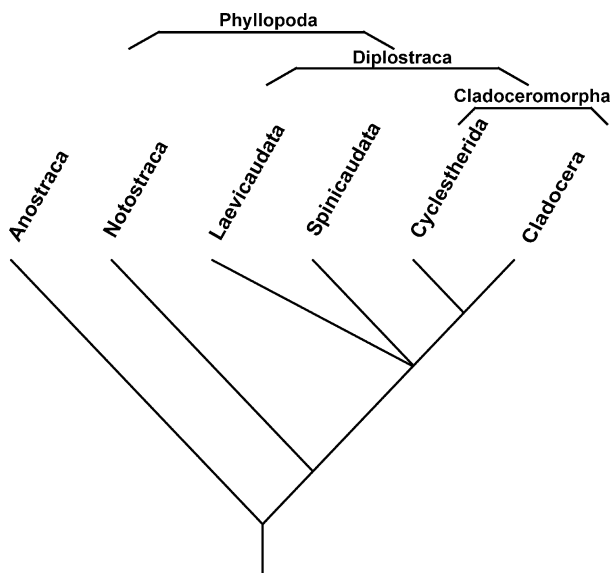


Fig. 1. Traditional view on branchiopod phylogeny based on morphological information (compiled from Walossek, 1993; Olesen et al., 1997; Negrea et al., 1999; Olesen, 1998, 2004).

Table 1

A list of taxa collected and examined for this study. The classification follows Martin and Davies (2001)

	Location site and country, collector and date (ZMUC CRU <sup>d</sup> )	GenBank accession number		
		16S rRNA	28S rRNA	
Branchiopoda (Latreille, 1772)				
Sarsostraca (Tasch, 1969)				
Anostraca (Sars, 1867)				
Branchinectidae (Daday, 1910)				
	<i>Branchinecta paludosa</i> (Müller, 1788)	Disko Island, Greenland. C.Schander, 2001 (4022)	DQ470608	DQ470650
Branchiopodidae (Simon, 1886)				
	<i>Parartemia minuta</i> (Geddes, 1973)	Horseshoe Lake, Paroo, Australia, J.Olesen, 2001 (4085)	DQ470609	DQ470651
Chirocephalidae (Daday, 1910)				
	<i>Eubranchipus grubii</i> (Dubowski, 1860)	Ulvshale, Møn, Denmark. J.T.Stenderup, 2001 (4021)	DQ470610	DQ470652
Thamnocephalidae (Simon, 1886)				
	<i>Thamnocephalus platyurus</i> (Packard, 1877)	Hatched in lab by O.S.Møller, 2002 (4088)	DQ470611	DQ470653
	<i>Branchinella kugenumaensis</i> (Ishikawa, 1895)	Shiga-Ken, Otsu-Shi, Beppo, I-Chôme, Japan. M.J.Grygier, 2001 (4018)	DQ470607	DQ470649
Phyllopoda (Preuss, 1951)				
Notostraca (Sars, 1867)				
Triopsidae (Keilhack, 1909)				
	<i>Triops</i> (Schränk, 1803)			
	<i>Triops cancriformis</i> (Bosc, 1801)	Yamagata-Ken Sakata-Shi Iimoriyama-Nishi. R.Igarashi & M.J.Grygier, 2001 (4009)	DQ470598	DQ470640
	<i>Lepidurus</i> (Leach, 1816)			
	<i>Lepidurus arcticus</i> (Pallas, 1793)	Disko Island, Greenland. C.Schander, 2001 (4005)	DQ470597	DQ470639
	<i>Lepidurus apus</i> (Linné, 1758)	Hatched in lab by O.S.Møller, 2001 (4014)	DQ470599	DQ470641
Diplostraca (Gerstaecker, 1866)				
Laevicaudata (Linder, 1945)				
Lynceidae (Sayce, 1902)				
	<i>Lynceus</i> (O.F.Müller, 1776)			
	<i>Lynceus tatei</i> (Spencer & Hall, 1896)	Box Hole Swamp, Paroo, Australia. J.Olesen, 2001 (4044)	DQ470606	DQ470648
	<i>Lynceus brachyurus</i> (O.F.Müller, 1776)	Ulvshale, Møn, Denmark. J.Damgaard	DQ470604	DQ470646
	<i>Lynceus bififormis</i> (Ishikawa, 1895)	Shiga, Liusatsu, Kataoka-Cho, Japan. M.J.Grygier, 2001 (4020)	DQ470605	DQ470647
Spinicaudata (Linder, 1945)				
Leptestheriidae (Stebbing, 1902)				
	<i>Leptestheria</i> (Sars, 1898)			
	<i>Leptestheria kawachiensis</i> (Véno, 1927)	Kusatsu, Shiga, Kataoua-Cho, Japan. M.J.Grygier, 2001 (4025)	DQ470601	DQ470643
	<i>Leptestheria dahalacensis</i> (Rüpell, 1837)	Hatched in lab by F.L.Alexandrovna, 2001 (4017)	DQ470600	DQ470642
Limnadiidae (Baird, 1849)				
	<i>Eulimnadia</i> (Ishikawa, 1895)			
	<i>Eulimnadia braueriana</i> (Ishikawa, 1895)	Mise, Otsu, Shiga, Japan. M.J.Grygier, 2001 (4024)	DQ470602	DQ470644
Cyzicidae (Stebbing, 1910)				
	<i>Caenestheria lutraria</i> (Brady, 1886)	Bokeen Cane Grass Swamp, Paroo, Australia. J.Olesen, 2001 (4048)	DQ470603	DQ470645
Cyclestherida (Sars, 1899)				
Cyclestheriidae (Sars, 1887)				
	<i>Cyclestheria hislopi</i> (Baird, 1859)	Jabiru, Northern Territory, Australia. S.Richter, 1999 (4049)	DQ470596	DQ470638
Cladocera (Latreille, 1829)				
Ctenopoda (Sars, 1865)				
Sididae (Baird, 1850)				
	<i>Sida crystallina</i> (O.F.Müller, 1776)	Hampen Sø (56°01'N, 09°23'E) Denmark. J.Stenderup, 2001 (4029)	DQ470594	DQ470636
	<i>Diaphanosoma brachyurum</i> (Liéven, 1848)	Lake Glubokoe (55°45'N, 36°30'E), Russia. J.T.Stenderup, 2001 (4060)	DQ470593	DQ470635
	<i>Penilia avirostris</i> (Dana, 1852)	Southwest Monte Argentario, Tuscany, Italy. J.Olesen, 2001 (4059)	DQ470595	DQ470637
Onychopoda (Sars, 1865)				
Polyphemidae (Baird, 1845)				
	<i>Polyphemus pediculus</i> (Linné, 1761)	Frilandsmuseet, Lyngby, Denmark. J.T.Stenderup, 2001 (4137)	DQ470588	DQ470630

Table 1 (continued)

	Location site and country, collector and date (ZMUC CRU <sup>d</sup> )	GenBank accession number	
		16S rRNA	28S rRNA
Cercopagidae (Mordukhai-Boltovskoi, 1968)			
<i>Bythotrephes longimanus</i> (Leydig, 1860)	Tegeler See, Berlin, Germany. J.Olesen and S.Richter, 2000 (4081)	DQ470587	DQ470629
Podonidae (Mordukhai-Boltovskoi, 1968)			
<i>Evadne nordmanni</i> (Loven, 1836)	Sylt Island, Germany. S.Richter, 1997 (4055)	DQ470590	DQ470632
<i>Evadne spinifera</i> (P.E.Müller, 1867)	Helgoland, Germany. S.Richter, 2001 (4056)	DQ470591	DQ470633
<i>Podon leuckartii</i> (Sars, 1862)	Helgoland, Germany. S.Richter, 2001 (4058)	DQ470589	DQ470631
Haplopoda (Sars, 1865)			
Leptodoridae (Lilljeborg, 1861)			
<i>Leptodora kindtii</i> (Focke, 1844)	Lake Glubokoe (55°45'N, 36°30'E), Russia. J.T.Stenderup, 2001 (4130)	DQ470592	DQ470634
Anomopoda (Sars, 1865)			
Daphniidae (Strauss, 1820)			
<i>Daphnia magna</i> (Strauss, 1820)	Ring Sø (55°58'N, 09°36'E), Denmark. J.T.Stenderup, 2001 (4110)	DQ470575	AF346515 <sup>a</sup>
<i>Daphnia pulex</i> (Leydig, 1860)	Ulvshale, Møn, Denmark. J.T.Stenderup, 2001 (4073)	DQ470571 <sup>b</sup>	DQ470613
<i>Ceriodaphnia pulchella</i> (Sars, 1862)	Almind Sø (56°08'N, 09°33'E), Denmark. J.T.Stenderup, 2001 (4089)	DQ470585	DQ470627
<i>Simocephalus vetulus</i> (O.F.Müller, 1776)	Mølleåen, Sealand, Denmark. J.T.Stenderup (4093)	DQ470576	DQ470617
<i>Daphniopsis queenslandensis</i> (Sergeev, 1990)	Blood Wood, Paroo, Australia. J.Olesen, 2001 (4079)	DQ470574	DQ470616
<i>Scapholeberis mucronata</i> (O.F.Müller, 1785)	Almind Sø (56°08'N, 09°33'E), Denmark. J.T.Stenderup, (4033)	DQ470583	DQ470625
Bosminidae (Baird, 1845)			
<i>Bosmina longirostris</i> (O.F.Müller, 1785)	Utterslev Mose, Sealand, Denmark. J.T.Stenderup, 2001 (4097)	DQ470573	DQ470615
<i>Bosmina</i> sp.	Lake Glubokoe (55°45'N, 36°30'E), Russia. J.T.Stenderup, 2001 (4170)	DQ470572	DQ470614
Chydoridae (Stebbing, 1902)			
<i>Pseudochydorus globosus</i> (Baird, 1843)	Lake Glubokoe (55°45'N, 36°30'E), Russia. J.T.Stenderup, 2001	<sup>c</sup>	DQ470620
<i>Alona affinis</i> (Leydig, 1860)	Mølleåen, Sealand, Denmark. J.T.Stenderup (4038)	DQ470586	DQ470628
<i>Rhynchotalona falcata</i> (Sars, 1861)	Lake Glubokoe (55°45'N, 36°30'E), Russia. J.T.Stenderup, 2001	DQ470584	DQ470626
Macrothricidae (Norman & Brady, 1867)			
<i>Macrothrix laticornis</i> (Jurine, 1820)	Pond no. 12 (55°42'N, 36°29'E), Russia. J.T.Stenderup, 2001 (4128)	DQ470579	DQ470621
<i>Lathonura rectirostris</i> (O.F.Müller, 1785)	Lake Glubokoe (55°45'N, 36°30'E), Russia. J.T.Stenderup, 2001 (4135)	DQ470581	DQ470623
<i>Streblocerus serricaudatus</i> (Fischer, 1849)	Lake Poletskoe (55°34'N, 36°33'E), Russia. J.T.Stenderup, 2001 (4099)	DQ470580	DQ470622
<i>Acantholeberis curvirostris</i> (O.F.Müller, 1776)	MSU Biostation, Sima (55°40'N, 36°42'E), J.T.Stenderup, 2001 (4068)	DQ470577	DQ470618
<i>Drepanothrix dentata</i> (Eurén, 1861)	Lake Glubokoe (55°45'N, 36°30'E), Russia. J.T.Stenderup, 2001 (4095)	DQ470582	DQ470624
<i>Ophryoxus gracilis</i> (Sars, 1861)	Biol. Station of MSU, White Sea, Karelia Republic, Russia. 2001 (4067) <sup>c</sup>	DQ470578	DQ470619
Malacostraca			
<i>Tethysbaena argentarii</i> (Stella, 1951)	Monte Argentario, Tuscany, Italy. J.Olesen, 2001.	DQ470612	DQ470654

<sup>a</sup> Position 817–1765.

<sup>b</sup> Corresponds to position 13.106–12.638 on the *Daphnia pulex* mitochondrial genome. GenBank accession No. AF117817.

<sup>c</sup> Sequence provided by Dr. Stefan Richter, Friedrich-Schiller-Universität, Jena, Germany.

<sup>d</sup> ZMUC CRU numbers in parentheses refers to voucher specimens deposited at the Zoological Museum, University of Copenhagen, Copenhagen, Denmark.

<sup>e</sup> Provided by Dr. N. Korovchinsky, AN Severtsov Institute of Ecology and Evolution, Moscow.

GTC CTC CAG GAT C-3') for Anostraca, and 1480 ml and D6br(m2) (5'-CAC ATG AAA CCC TTC TCC GC-3') for the remaining species. To amplify the 16S rRNA fragment primers were found in the literature (Palumbi, 1996); 16Sar (5'-CGC CTG TTT ATC AAA AAC AT-3') and 16Sbr (5'-CCG GTT TGA ACT CAG ATC A-3'). Fifty microliter

double-stranded PCR mix was prepared from 1 (or 2) µl DNA-extraction, 5 µl PCR-buffer, 5 µl each primer at 10 µM, 14 (or 13) µl ddH<sub>2</sub>O, 20 µl (0,5 µM) GATC-mix, and 0,2 µl 5 µM Amplitaq<sup>®</sup> DNA Polymerase. Conditions for all amplifications were two minutes initial denaturing at 94 °C followed by 35 cycles with 94 °C/30s denaturing, 50 °C/30s annealing,



and 72°C/40s extension, with an additional 10 min extension at 72°C. Purification was made using Qiagen QiaQuick Spin Columns following the protocol. The purified PCR products were stored at –20°C.

### 2.2.3. Sequencing

Ten microliters sequencing-mix was made from 3 µl purified PCR products, 1.2 µl of 10 µM primer, 2 µl ABI PRISM™ Big Dye Terminator Cycle Sequencing Ready Reaction Kit, 2 µl 5times buffer and 1.8 µl ddH<sub>2</sub>O, and the reaction were performed using a RoboCycler® or Eppendorf MasterCycler under these conditions: one minute initial denaturing at 96°C followed by 35 cycles with 96°C/10s denaturing, 50°C/10s annealing and 60°C/4 min extension. Four minute termination at 60°C terminated the reactions. Sequencing products were precipitated using Ethanol and were analyzed on an ABI PRISM™ 377 DNA Sequencer for up to 9 h.

### 2.2.4. Sequence analysis

Each sample was sequenced for both heavy and light strands, in order to improve accuracy, and aligned. Forward and reverse sequences were checked for base ambiguity in Sequencher™ 3.1.1 (Gene Codes Corporation) before consensus sequences were compiled and aligned with Clustal X (Thompson et al., 1997) using default parameters and adjusted to accommodate conserved secondary structure (Cannone et al., 2002; Van de Peer et al., 2000; Wuyts et al., 2001). The alignment was optimized by eye by referring to stem-loop regions in the proposed secondary structure. Sections corresponding to the primer sequences at the 3' and 5' end of both aligned gene sequences were eliminated before initiating the analyses.

### 2.3. Phylogenetic analyses

The 16S rRNA and 28S rRNA data were analyzed separately as well as combined. For clarity we have chosen to show only trees based on analyses of the combined genes. The data were analyzed in PAUP\*4b10 (Swofford, 2002) using parsimony and maximum likelihood (ML) methods, and in MrBayes v3.01 (Huelsenbeck and Ronquist, 2001; Huelsenbeck et al., 2002) using Bayesian Inference of Phylogeny. We used the Akaike Information Criterion (AIC) to test for statistically significant differences in model fit for models with increasing complexity. We found the appropriate substitution models using Modeltest version 3.4 (Posada and Crandall, 1998) for the likelihood and Bayesian analyses. The analyses were conducted implementing the models and parameters suggested by Modeltest for both genes separately and combined. The parsimony analyses were conducted using the heuristic search option with tree-bisection reconnection (TBR) branch-swapping (Swofford et al., 1996), and 100 random Wagner trees. Nonparametric bootstrap proportions (nbp) (Felsenstein, 1985) were estimated from 1000 pseudo-replicate datasets. The analyses were executed giving all characters equal weight and by treating gaps as both miss-

ing data or as a fifth character state. Nodes with bootstrap support <50% were collapsed.

Time and computational constraints made bootstrap replicates impractical for the ML analyses. ML tree searches were conducted by performing 100 replicates using the heuristic search option via tree-bisection and reconnection (TBR) branch-swapping, on stepwise-addition trees with random taxon addition and the best-fit model including rate heterogeneity, proportion of invariant sites, and substitutions model with the parameters suggested by the AIC.

Bayesian posterior probabilities (bpp) were estimated as the proportion of trees sampled after burn-in that contained each of the observed bipartitions (Larget and Simon, 1999). We used MrBayes v3.01 (Huelsenbeck and Ronquist, 2001) to perform our analyses using the suggested evolutionary models, and default value of four Markov chains. Multiple chains can assist in more easily traversing tree-space and help avoid entrapment in local topological optima (Wilcox et al., 2002). The Monte Carlo Markov chain (MCMC) length was 4,000,000 generations, and we sampled the chains every 500 generations yielding 8000 trees. Log-likelihood values for sampled trees were visualized and had stabilized after 500,000 generations. Therefore, we used the last 7000 sampled trees to estimate Bayesian posterior probabilities (bpp).

### 2.4. Outgroup selection

The selection of an appropriate outgroup for phylogenetic analysis was difficult since there is no well-accepted sister group for Branchiopoda. Remipedia (*Speleonectes tulumensis*) and Cephalocarida (*Lightiella* sp.) were sequenced and tried as outgroups but we had problems aligning the sequences of these taxa to those of the branchiopods. *Tethysbaena argentarii* (Malacostraca: Thermosbaenacea) proved to be better in this respect and was therefore chosen as the single outgroup. Therefore, monophyly of Branchiopoda was not tested in this study.

## 3. Results

### 3.1. Analyses of combined 16S and 28S rRNA data (Figs. 2–4 and Table 2)

The ML analyses for the combined dataset were performed using the model (TrN+I+G) and parameters suggested by AIC. The best tree score was  $-\text{LogL} = 20,100.47379$ , and the island was hit 91 times. The parsimony analysis retained six equally short trees with a tree score for best tree = 4676. The dataset was analyzed treating gaps as both missing and fifth character, and yielded identical topologies.

When rooting with *Tethysbaena argentarii* all three combined analyses support a monophyletic Anostraca as sister group to a monophyletic Phyllopoda, the latter taxon supported by 97% bootstrap proportions (nbp) in the

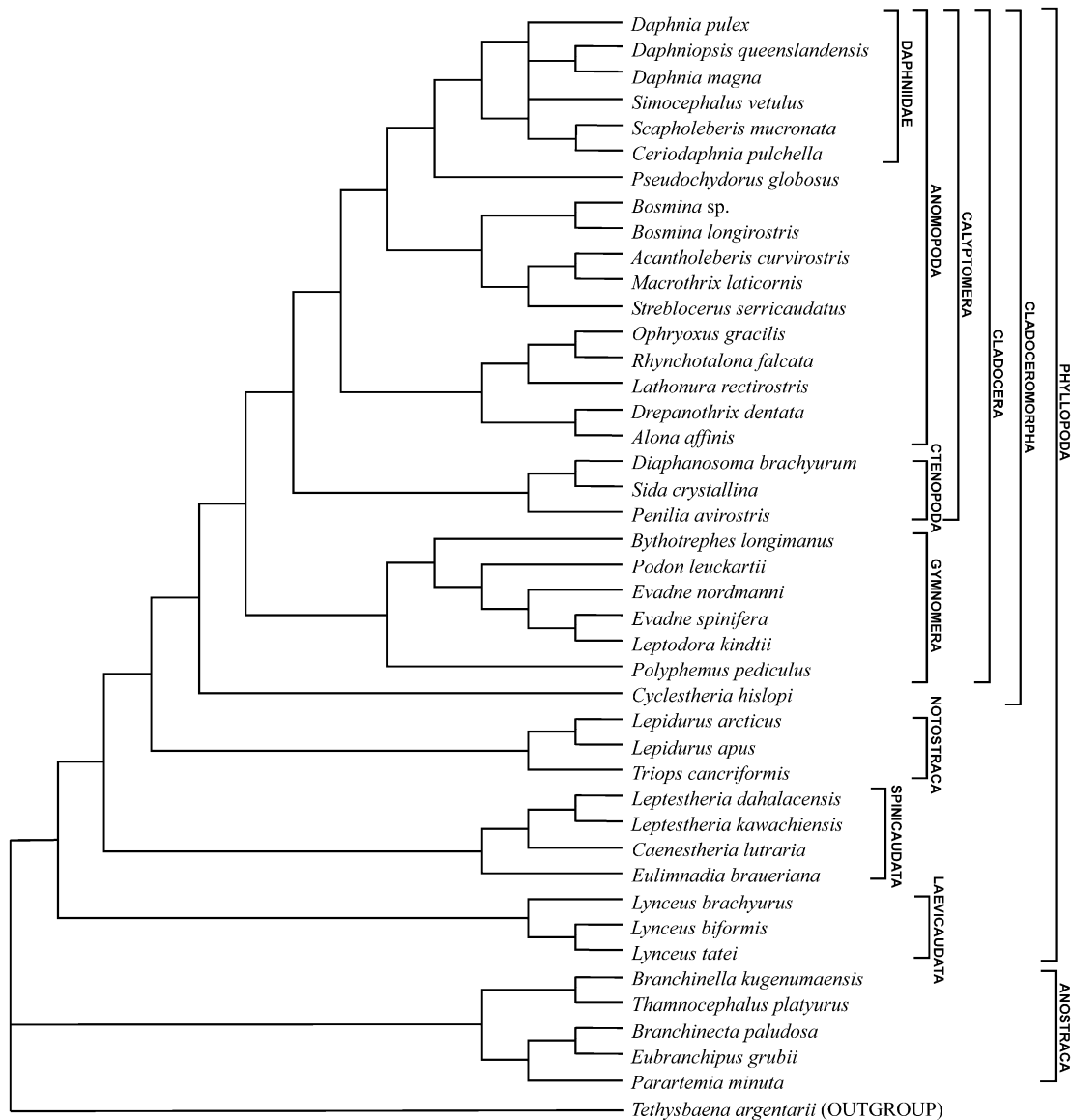


Fig. 2. Branchiopod relationships based on maximum likelihood analyses (ML) of combined 16S and 28S rRNA data. Analysis run under the TrN + I + G model suggested by AIC and 1000 replicates of TBR branch-swapping.  $-\text{LogL} = 20,100.47379$ . The island was hit 91 times. Tree rooted with *Tethysbaena argentarii* (Thermosbaenacea). Names mentioned to the right in figure are supported taxa (same in following figures).

parsimony analysis and a posterior probability of 0.85 (bpp) in the Bayesian Inference (BI) analysis (Figs. 2–4). Within Phyllopoda, all three analyses (Figs. 2–4) also support Laevicaudata, Spinicaudata, and Notostraca with high bootstrap proportions in the parsimony analysis (100%) (Fig. 4) and high posterior probability in the BI analysis (1.00) (Fig. 3). All three combined analyses support Cladoceromorpha (bpp 1.00, nbp 100) and Cladocera (bpp 1.00, nbp 100) (Figs. 2–4). In all three combined analyses, Laevicaudata appears as the sister group to all other Phyllopoda (Figs. 2–4). In both the BI and ML analyses, Notostraca appears as the sister group to Cladoceromorpha and Spinicaudata as a sister group to Cladoceromorpha + Notostraca (bpp 0.85) (Figs. 2 and 3). The parsimony analysis of the combined data shows a slightly different

branching order of the major ‘large’ branchiopod taxa: Spinicaudata is sister group to Cladoceromorpha (nbp 100), Notostraca is sister group to Spinicaudata + Cladoceromorpha (nbp 81) (Fig. 4). ‘Diplostraca’ or ‘Conchostraca’ was not supported in any analysis.

Within Cladocera all combined analyses gave support to Ctenopoda (bpp 1.00, nbp 81), Anomopoda (bpp 1.00, nbp 64), and Gymnomera (bpp 0.57, nbp 100) (Figs. 2–4). Onychopoda was paraphyletic in all analyses with respect to *Leptodora kindtii* (Haplopoda), which appeared as sister group to *Evadne spinifera* (bpp 0.95, nbp 73) (Figs. 2–4). Two different possibilities for the higher-level phylogeny are suggested by the analytical approaches: the BI and ML analyses show Ctenopoda and Anomopoda as sister groups (bpp 0.78) (Figs. 2 and 3), while the parsimony analysis

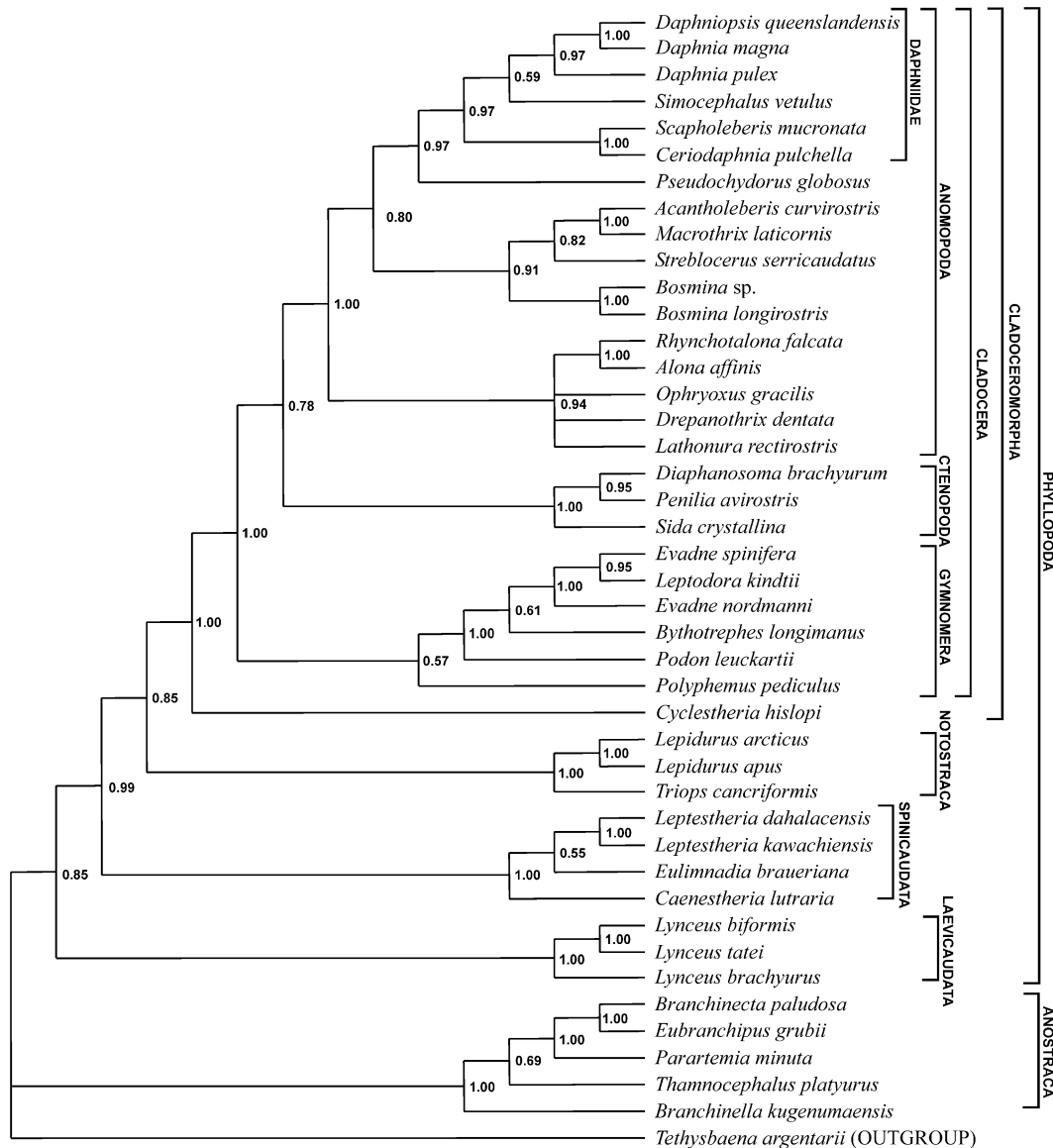


Fig. 3. Branchiopod relationships based on Bayesian Inference analysis (BI) of combined 16S and 28S rRNA data. 50% majority-rule consensus tree.

shows Anomopoda and Gymnomera as sistergroups (nbp 83) (Fig. 4). Within Anomopoda, all analyses show a monophyletic Daphniidae (bpp 0.97, nbp 58) (Figs. 2–4). Neither Macrothricidae nor Chydoridae are supported.

#### 4. Discussion

##### 4.1. Monophyly of phyllopoda and a 'conchostracan' origin of Notostraca

Phyllopoda *sensu* Preuss (1951)—a taxon comprized of Notostraca, the former 'Conchostraca', and Cladocera—is well supported in all types of analyses of the combined data in this study. This is in accordance with morphological evidence provided by Walossek (1993) and Olesen (2004) and with 18S rRNA evidence (Spears and Abele, 2000). The monophyly of Phyllopoda now

appears well established based on at least some genes and on morphological data. In contrast, Diplostraca—a taxon comprized of the former 'Conchostraca' (Laevicaudata, Spinicaudata, Cyclestherida) and Cladocera—is not supported in the present study. The eventual monophyly of Diplostraca based on morphological data has been the subject of recent discussions (see Walossek, 1993; Olesen, 2000, 2002; Fryer, 1999, 2001, 2002). All analyses of the combined data suggest a paraphyletic Diplostraca with respect to Notostraca, and this is also the result of various other studies using molecular data (Taylor et al., 1999; Spears and Abele, 2000; Braband et al., 2002), the significance of which has not been appreciated previously. In the present study, the maximum likelihood and Bayesian analyses of the combined data show Notostraca as sister group to a monophyletic Cladoceromorpha (*Cyclestheria* and Cladocera)

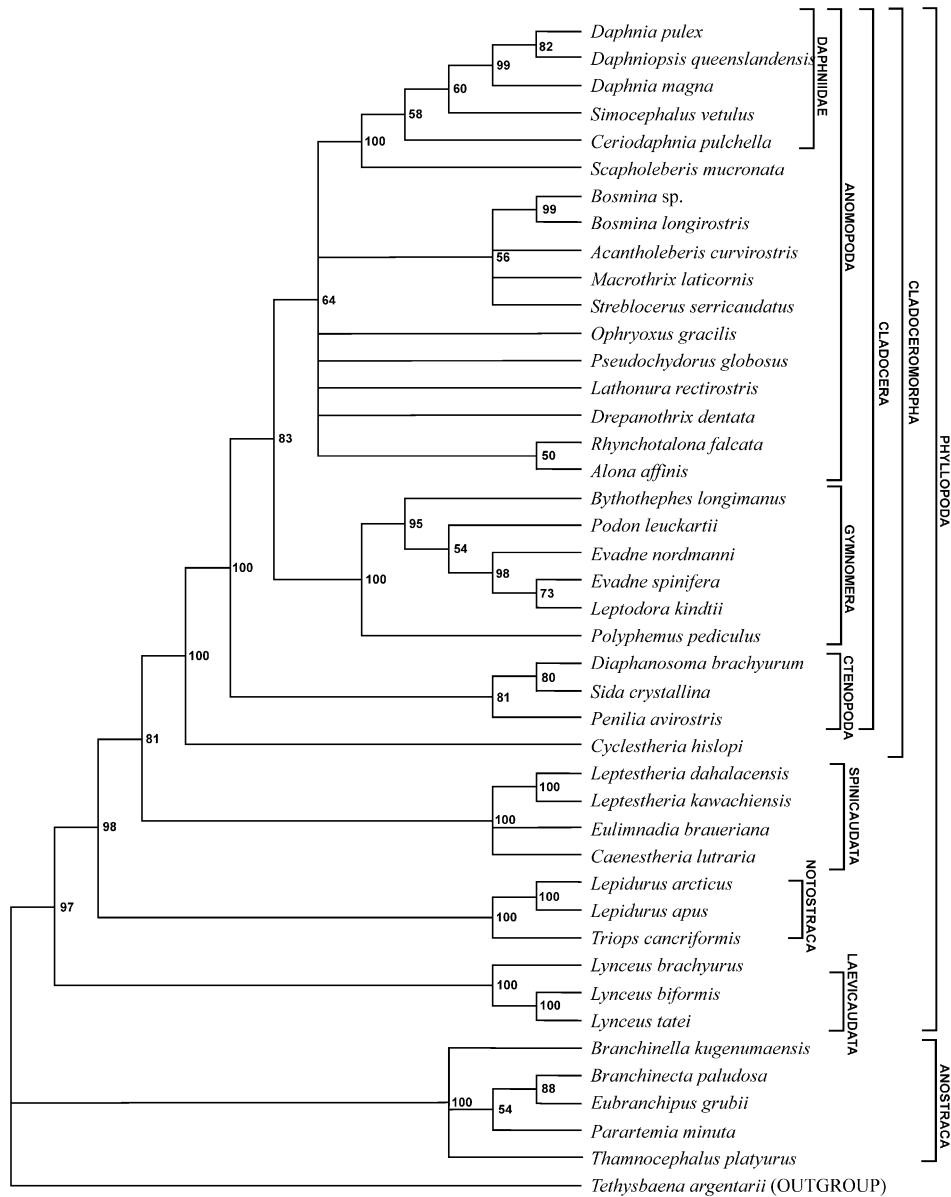


Fig. 4. Branchiopod relationships based on strict consensus of 6 equally short trees Parsimony analysis of combined 16S and 28S rRNA data using heuristic search option in PAUP\*. 100 bootstrap replicates performed, best tree score 4,676. Number of parsimony informative = 668, uninformative = 302, and constant = 604. TBR branch-swapping was performed and gaps treated as fifth characters. 50% consensus tree, only clades with bootstrap values above 50% are shown. RI = 0.492, CI = 0.384, RC = 0.189.

(Figs. 2 and 3). The parsimony analysis of the combined data show a position of Notostraca within Diplostraca as well, but this time one step further down in the cladogram as sister group to a clade consisting of Cladoceromorpha and Spinicaudata (Fig. 4). We conclude that the available molecular evidence clearly point in the direction of a paraphyletic Diplostraca with Notostraca placed somewhere inside. Three different possibilities for an ingroup diplostracan position have been suggested in analyses of various molecular datasets in this and previous works (summarized in Fig. 5): (A) Notostraca as sister group to Laevicaudata, (B) Notostraca as sister group to Spinicaudata and Cladoceromorpha or (C) Notostraca as sister group to Cladoceromorpha alone.

We prefer Notostraca as sister group to Spinicaudata and Cladoceromorpha (position ‘B’ in Fig. 5), since this seemingly is most in accordance with morphological information.

#### 4.2. Morphological and evolutionary implications of a ‘diplostracan’ ingroup position of Notostraca

A paraphyletic Diplostraca with respect to Notostraca conflicts with most published morphological evidence, which suggests monophyly of this taxon (see Walossek, 1993; Olesen, 2004). Basically all similarities between diplostracan taxa, which are not found in Notostraca, and which can be argued to be homologies, will have to



Table 2  
Hypothesized clades listed according to degree of support in the various approaches

Hypothesis	16S rRNA			28S rRNA			Combined			Number of analyses supporting the tested hypothesis
	MP	ML	Bayesian	MP	ML	Bayesian	MP	ML	Bayesian	
<i>Notostraca</i>	100	+	1	100	+	1	100	+	1	9
<i>Laevicaudata</i>	99	+	1	100	+	1	100	+	1	9
<i>Spinicaudata</i>	99	+	1	97	+	0.9	100	+	1	9
<i>Bosminidae</i>	67	+	0.99	100	+	1	99	+	1	9
<i>Lepidurus</i>	100	+	1	79	+	0.98	100	+	1	9
<i>Anostraca</i>	100	+	1	78	+	0.78	100	+	1	9
<i>Phyllopoda</i>	74	+	0.97	52	+	0.56	97	+	0.85	9
<i>Cladoceromorpha</i>	?	+	1	75	+	0.98	100	+	1	8
<i>Cladocera</i>	?	+	0.96	84	–	0.98	100	+	1	7
<i>Ctenopoda</i>	?	–	0.99	55	+	1	81	+	1	7
<i>Leptestheriida</i>	100	+	1	57	–	–	100	+	1	7
<i>Gymnomera (Onychopoda + Haplopoda)</i>	?	–	–	59	+	0.99	100	+	0.57	6
<i>Ctenopoda + Gymnomera</i>	?	–	–	56	+	0.84	–	–	–	2
<i>Notostraca</i> sistergroup to <i>Cladoceromorpha</i>	?	+	0.77	?	–	–	–	+	0.85	4
<i>Notostraca</i> sistergroup to <i>Spinicaudata</i> + <i>Cladoceromorpha</i>	?	–	–	?	+	0.71	98	–	–	3
<i>Gymnomera</i> sistergroup to <i>Calyptomera</i>	?	–	–	–	–	–	–	+	1	2
<i>Anomopoda</i>	?	–	–	?	–	0.96	64	+	1	4
<i>Daphniidae</i>	?	–	0.61	?	–	–	58	+	0.97	4
<i>Chydoridae</i>	?	–	–	?	–	0.96	?	–	–	1
<i>Calyptomera (Ctenopoda + Anomopoda)</i>	?	–	–	–	–	–	–	+	0.78	2
<i>Macrothricidae</i>	?	–	–	–	–	–	–	–	–	0
<i>Conchostraca</i>	?	–	–	–	–	–	–	–	–	0
<i>Diplostraca</i>	?	–	–	–	–	–	–	–	–	0
<i>Onychopoda</i>	–	–	–	–	–	–	–	–	–	0

ML = maximum likelihood. The numbers corresponds to support values: Bootstrap proportions for parsimony analyses, posterior probabilities for Bayesian analyses. “+” indicates supported clade for likelihood analyses. For all analyses “–” indicates that the taxon is not supported in that analysis. For the parsimony analysis “?” indicates that the relevant node is unresolved (soft polytomy).

be explained as having been lost or modified in *Notostraca*. This includes all the specific similarities between laevicaudatan and spinicaudatan (former ‘Conchostraca’) larvae mentioned by Olesen (2004, 2005) and all the general adult ‘conchostracan’ similarities, such as powerful swimming antennae (absent in *Notostraca*), claspers on first pairs of trunk limbs (absent in *Notostraca*), bivalved carapace (not clearly bivalved in *Notostraca*), and dorsal exopodal filaments holding eggs/embryos (absent in *Notostraca*).

Some morphological evidence that is at least congruent with an ingroup diplostracan position of *Notostraca* exists. Møller et al. (2003) found that the larval *anlage* to the univalved dorsal carapace, typical for notostracans, is very similar to the carapace *anlage* in various representatives of *Spinicaudata* and *Cladocera* (see Olesen, 1998, 1999; Olesen et al., 2003; Olesen and Grygier, 2003, 2004). In all of these carapace-bearing branchiopods, the carapace *anlage* is a paired structure, which could indicate a bivalved origin of the dorsal carapace in *Notostraca*, most likely similar to the large bivalved carapace seen in ‘Conchostraca.’ Another recent discovery which suggests a diplostracan (or ‘conchostracan’) origin of *Notostraca* is the description of a remarkably well preserved Devonian branchiopod, *Castracollis wilsonae* Fayers and Trewin, 2003, which looks like a cross between a ‘conchostracan’ and a notostracan. Fayers

and Trewin (2003) argue for a stem-lineage notostracan position of *Castracollis* based on specific similarities to recent notostracans such as a similar external morphology of the abdominal region. Similarities to ‘Conchostraca’ (more specifically *Spinicaudata*) include the presence of powerful swimming antennae and the morphology of the trunk limbs, all of which could be interpreted as symplesiomorphies later lost in the lineage leading to the recent *Notostraca* after *Castracollis* branched off. A third piece of evidence was mentioned by Linder (1945) and Martin (1989), and later explored in more detail by Richter (2004), who described a specific similarity in external morphology of the mandibles in *Notostraca* and *Laevicaudata*, very different from the typical ‘grinding-rolling’ morphology seen in most other branchiopods, and therefore interpreted as support for a sister group relationship by the latter author.

In the light of the morphological evidence presented above, the diplostracan ingroup position of *Notostraca*, suggested by largely all published molecular datasets, is perhaps more feasible. A consequence of this is that the ancestor to *Phyllopoda* also was ‘conchostracan’-like. General ‘conchostracan’ characters such as a bivalved carapace, claspers on the first pairs of trunk limbs of males, and eggs/embryos attached to trunk limbs exopods would then have been present already in the common ancestor of *Phyllopoda*.

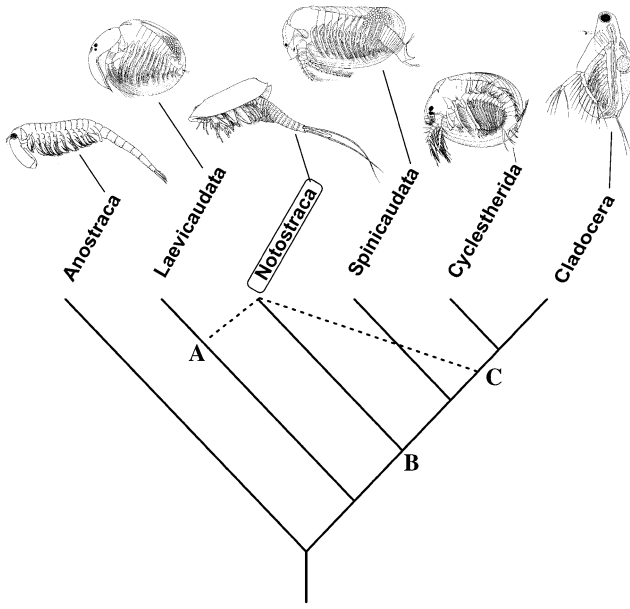


Fig. 5. Summary phylogeny showing three different possible positions of Notostraca within Diplostraca (A, B and C). All three possibilities have been suggested in the present study or in recent papers on branchiopod phylogeny using molecular data. Among the suggested possibilities, a position of Notostraca as sister group to Spinicaudata + Cyclestherida + Cladocera (position B) is preferred here since this position is in least conflict with morphological data. Illustrations are from the following sources: Anostraca and Notostraca, Martin (1992); Laevicaudata, Sars (1896); Spinicaudata, Sars (1898); Cyclestherida, Olesen et al. (1997); Cladocera, Sars (1901).

#### 4.3. Paraphyly of ‘Conchostraca’ and monophyly of Cladoceromorpha

‘Conchostraca’ is not supported in any of the analyses performed in this work. Neither is a sister group relationship between Spinicaudata and Laevicaudata (similar to ‘Conchostraca’, but without *Cyclestheria*). The phylogenetic position of Laevicaudata has caused some trouble in recent accounts based on morphological evidence (Olesen et al., 1997; Olesen, 1998; Richter, 2004). All analyses of the combined data in this study constantly show Laevicaudata placed as the first branch after Anostraca, more precisely as sister group to the remaining phyllopods. This is also the result of the parsimony analysis of 12S rRNA data by Braband et al. (2002, fig 3a), while other available analyses of molecular data in the literature suggest Laevicaudata as sister group to Notostraca (Braband et al., 2002; Spears and Abele, 2000). Cladoceromorpha (*Cyclestheria* and Cladocera) is supported in most analyses in this study, as it has been in a number of previous publications (Taylor et al., 1999; Spears and Abele, 2000; Braband et al., 2002; DeWaard et al., 2006).

#### 4.4. Monophyly of Cladocera

The monophyly of Cladocera is supported in all analyses of the combined data which is also seen in a number of other molecular based papers (Taylor et al., 1999; Spears

and Abele, 2000; Braband et al., 2002; DeWaard et al., 2006). A monophyletic Cladocera is supported by most recent accounts using morphology (Martin and Cash-Clark, 1995; Olesen, 1998; Negrea et al., 1999), but polyphyly has also been advocated (Fryer, 1987a,b). The interrelationship of the higher taxa of the Cladocera (Anomopoda, Ctenopoda, Onychopoda, and Haplopoda) depends on whether these can be considered monophyletic, and will therefore be treated after this subject has been covered.

#### 4.5. Monophyly and phylogeny of Gymnomera

Within Cladocera all analyses of the combined data show support for a monophyletic Gymnomera (Haplopoda and Onychopoda), but with *Leptodora kindtii* within Onychopoda. Gymnomera was supported by Braband et al. (2002) (12S and EF1 $\alpha$ ) and Swain and Taylor (2003) (18S and 28S rRNA), but not by Spears and Abele (2000) (18S rRNA). When aligning the 28S rRNA sequences, we recognized a characteristic motif unique to Gymnomera that should be interpreted as further molecular support for Gymnomera. The motif consists of an insert of 7–8 T’s found only in the Onychopoda and Haplopoda sequences, approximately at position 130, and are similar to those of Swain and Taylor (2003). Martin and Cash-Clark (1995) advocated a sister group relationship between Haplopoda and Onychopoda based on morphological data, a result also found by Olesen (1998) who reintroduced the term Gymnomera (from Sars, 1865). The morphological support for Gymnomera includes the presence of similarly segmented trunk limbs and the reduction in size of the carapace leaving the limbs free. Within Gymnomera all analyses of the combined data suggest paraphyly of Onychopoda with respect to *Leptodora kindtii* (Haplopoda), which appears as sister group to *Evadne spinifera*. In contrast, all analyses of Braband et al. (2002) show Onychopoda as monophyletic with *Leptodora* as sister group. Swain and Taylor (2003) (18S and 28S rRNA) showed two parsimony trees, one with a monophyletic Onychopoda and one with *Leptodora* inside Onychopoda as sister group to a clade consisting of *Podon leuckarti* and *Evadne nordmanni*. In summary, there is good molecular evidence for a monophyletic Gymnomera, possibly with *Leptodora* inside Onychopoda. From a morphological point of view a paraphyletic Onychopoda would be surprising due to the fact that Onychopoda is a very well defined taxon (e.g., Martin and Cash-Clark, 1995; Rivier, 1998; Richter et al., 2001). We do not treat the phylogeny of Onychopoda further here, apart from noting that all combined analyses indicate that *Polyphemus pediculus* is the first offshoot, a result found by at least some other workers (Braband et al., 2002; Swain and Taylor, 2003).

#### 4.6. Monophyly and phylogeny of Anomopoda

Another question that traditionally has attracted interest is whether Anomopoda—the most diverse ‘order’

(following Fryer's (1987a) scheme) within Branchiopoda—is monophyletic. All analyses of the combined data show a monophyletic Anomopoda. Based on 18S rRNA data, Taylor et al. (1999) found a monophyletic Anomopoda, as was also the case for Swain and Taylor (2003) (based on 18S and 28S rRNA), but Braband et al. (2002) found evidence for a paraphyletic Anomopoda with respect to various onychopods, ctenopods, and *Leptodora* (based on 12S rRNA and EF1 $\alpha$ ). Monophyly of Anomopoda based on morphological data has been advocated by Fryer (1995), Olesen (1998) and Elmoor-Loureiro (2004).

An interesting question concerns whether the anomopod families are monophyletic. Daphniidae (no moinids included) is monophyletic in all analyses of the combined data in the present study. This family also appears as monophyletic in most trees in the literature based on molecular data (e.g., Taylor et al., 1999; Braband et al., 2002; Swain and Taylor, 2003). Several authors advocate monophyly of Daphniidae based on morphological evidence (Fryer, 1991; Olesen, 1998; Elmoor-Loureiro, 2004), and a non-monophyletic origin of this family would be highly unexpected seen in a morphological context. Eurycercoidea (= 'old' Chydoridae), or 'chydorids', is not supported in any of the analyses in this study, which is in sharp contrast to the morphological evidence (Fryer, 1968; Olesen, 1998; Dumont and Silva-Briano, 1998; Elmoor-Loureiro, 2004). Sacherová and Hebert (2003) provided a detailed account of the evolutionary history of Chydoridae based on three genes, but the monophyly of the family was not tested. Macrothricoidea (= 'old' Macrothricidae excl. *Ilyocryptus*) was not supported in any analyses in this work. Dumont and Silva-Briano (1998) united Eurycercoidea and Macrothricoidea in the Radopoda but this taxon was not supported in the present study.

#### 4.7. Monophyly of Ctenopoda

The monophyly of Ctenopoda was supported in all analyses of the combined data, but it should be noted that only three species of Ctenopoda were included. A monophyletic Ctenopoda, based on molecular data, was found also by Schwenk et al. (1998), Taylor et al. (1999), Braband et al. (2002) and Swain and Taylor (2003). A monophyletic Ctenopoda is in general agreement with morphological data (Fryer, 1987a; Korovchinsky, 1990; Negrea et al., 1999).

#### 4.8. Higher-level relationships within Cladocera

The question of the relationships among the main groups (the 'orders', following Fryer, 1987a) is ambiguous on the basis of the present dataset. As mentioned above, all analyses of the combined data have a monophyletic Gymnomera (raptorial cladocerans), but the data show no consensus concerning the relationship between this taxon and Anomopoda and Ctenopoda. The ML and Bayesian analyses of the combined data show Anomopoda and Ctenopoda as sister

groups (= Calyptomera Sars, 1865), together being sister group to Gymnomera. The parsimony analysis of the same data shows Anomopoda and Gymnomera as sister groups, together being sister group to Ctenopoda. Morphology-based higher level phylogenies of Cladocera show no consensus in the literature. Olesen et al. (2003) found morphological evidence for a monophyletic Gymnomera, but also pointed out that other data supported a conflicting hypothesis: Onychopoda and Anomopoda as sister groups. It is interesting to note that the parsimony analysis of the combined data in the present account combines these two hypotheses, showing support for Anomopoda and Onychopoda, but with *Leptodora kindtii* (Haplopoda) nested within Onychopoda. More evidence is needed to sort out the higher-level phylogeny of Cladocera with some confidence.

### 5. Summary of main results

- The present study suggests paraphyly of Diplostraca with respect to Notostraca.
- Two different positions of Notostraca within Diplostraca are suggested by this dataset. The position most congruent with morphological data is a sister group relationship to an unnamed clade consisting of Spinicaudata and Cladoceromorpha.
- Some morphological evidence suggests a 'conchostracan'-like ancestor of Notostraca.
- 'Conchostraca' is paraphyletic with respect to *Cyclestheria hislopi* (and with respect to Notostraca, see above).
- Laevicaudata consistently shows up as the first offshoot after Anostraca has branched off, more precisely as sister group to the remaining phyllopods.
- Phyllopoda, Cladoceromorpha, and Cladocera are each supported in all analyses of the combined data.
- Within Cladocera, the clades Gymnomera, Anomopoda, and Ctenopoda are each monophyletic in all analyses of the combined data. Onychopoda always appears paraphyletic with respect to Haplopoda (*Leptodora kindtii*)
- The analyses of the combined data show no consensus regarding the higher-level phylogeny of the data. The ML and Bayesian analyses show Ctenopoda and Anomopoda as sister groups (together they form the sister group to Gymnomera), while the parsimony analysis shows Anomopoda and Gymnomera as sister groups (together they constitute the sister group to Ctenopoda).

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