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Phylogenetic analysis of anostracans (Branchiopoda: Anostraca) inferred from nuclear 18S ribosomal DNA (18S rDNA) sequences

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Abstract

The nuclear small subunit ribosomal DNA (18S rDNA) of 27 anostracans (Branchiopoda: Anostraca) belonging to 14 genera and eight out of nine traditionally recognized families has been sequenced and used for phylogenetic analysis. The 18S rDNA phylogeny shows that the anostracans are monophyletic. The taxa under examination form two clades of subordinal level and eight clades of family level. Two families the Polyartemiidae and Linderiellidae are suppressed and merged with the Chirocephalidae, of which together they form a subfamily. In contrast, the Parartemiinae are removed from the Branchipodidae, raised to family level (Parartemiidae) and cluster as a sister group to the Artemiidae in a clade defined here as the Artemiina (new suborder). A number of morphological traits support this new suborder. The Branchipodidae are separated into two families, the Branchipodidae and Tanymastigidae (new family). The relationship between *Dendrocephalus* and *Thamnocephalus* requires further study and needs the addition of Branchinella sequences to decide whether the Thamnocephalidae are monophyletic. Surprisingly, *Polyartemiella hazeni* and *Polyartemia forcipata* (''Family'' Polyartemiidae), with 17 and 19 thoracic segments and pairs of trunk limb as opposed to all other anostracans with only 11 pairs, do not cluster but are separated by *Linderiella santarosae* (''Family'' Linderiellidae), which has 11 pairs of trunk limbs. All appear to be part of the Chirocephalidae and share one morphological character: double pre-epipodites on at least part of their legs. That *Linderiella* is part of the Polyartemiinae suggests that multiplication of the number of limbs occurred once, but was lost again in *Linderiella*. Within Chirocephalidae, we found two further clades, the *Eubranchipus–Pristicephalus* clade and the *Chirocephalus* clade. *Pristicephalus* is reinstated as a genus.

Keywords: Branchiopoda; Anostraca; Phylogeny; Ribosomal DNA; Nuclear 18S rDNA

1. Introduction

The anostracans, inhabiting temporary rainpools and permanent saltwater worldwide, are branchiopods lacking a carapax and with 19–27 postcephalic segments of which 9–19 carry a pair of similar, foliaceous limbs. They are admittedly the most primitive extant crustaceans. According to a recent classification, anostracans constitute one of the nine extant orders of Branchiopoda (Dumont and Negrea, 2002; Negrea et al., 1999). They have been known, since the Lower Cretaceous (Fryer, 1987). Currently, about 275 species, in 23 genera and nine families, are recognized (Belk and Brtek, 1997). They are noted for their ability to produce encysted embryos that can withstand drought and very high and low temperatures. This characteristic permitted them to survive in extreme environments after bony fish evolved. These conquered both the marine and freshwater realm, preying fairy shrimp to extinction in all but the most inhospitable aquatic environments. Today, anostracans survive in ephemeral pools and in some high mountain lakes and hypersaline environments to which fish have no access.

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Relationships among different groups of Anostraca were first proposed by Daday (1910). Linder (1941) expanded this system to seven families and suggested affinities between them relying heavily on male anatomical features. With the raising to family level of *Artemiopsis* (Artemiopsidae), and the addition of the Linderiellidae, the total number of families was brought to nine (Brtek, 1997). Also, Dodson and Frey (1991) described taxonomic relationships, based on morphological characters only. Remigio and Hebert (2000) adequately visualized and compared these two conflicting hypotheses (viz. Dodson and Frey, 1991; Linder, 1941) of taxonomic relationships.

Information on phylogenetic relationships among anostracans is fragmentary. The Streptocephalidae have been subjected to a morphological cladistic analysis (Maeda-Martínez et al., 1995; Spicer, 1985) and molecular data have served for identifying and studying the taxonomy and evolutionary relationships within *Streptocephalus*, based on the nuclear ribosomal 18S DNA (18S rDNA) (Sanoamuang et al., 2000). The 18S ribosomal gene has been used to study general crustacean phylogeny (Spears and Abele, 1997, 1999; Spears et al., 1994) and to elucidate the relationship between branchiopod orders, including the Anostraca (Crease and Taylor, 1998; Spears and Abele, 2000). Recently, phylogenetic analysis was performed on nine anostracans, representing seven "families," using nuclear (D1–D3 region of the 28S rDNA) and mitochondrial (16S rDNA, COI) gene regions (Remigio and Hebert, 2000). It was a reassessment of anostracan relationships, showing that molecular phylogenetic studies are the best approach. Importantly, the Branchipodidae were shown to be represented by two *Parartemia* species, and not by *Branchipus*, *Branchipodopsis*, or other true branchipodids.

It is generally accepted that phylogenetic hypothesis is most convincing when supported by data from other sources. Here, we analyse the 18S rDNA of 27 anostracan species, representing eight out of Brtek's nine families (only Artemiopsidae are lacking). We combine molecular phylogenetic with morphological data to clarify further the familial relationships in anostracans. The Polyartemiidae, with two genera, having 17 or 19 pairs of trunk limbs instead of the usual 11 pairs, are of special interest because the trunk limb number is traditionally considered a primitive character.

2. Materials and methods

2.1. DNA extraction, PCR amplification, and sequencing reactions

The origin of the samples used in this study is listed in Table 1. Mature animals were collected and washed with sterilized water, followed by a 70% ethanol solution to remove accompanying microorganisms or debris. Muscular tissue was isolated from the thorax and total DNAs were prepared according to the protocol of the Puregene DNA isolation kit type D-5000A (Gentra Systems, BIOzym, Landgraaf, The Netherlands). The complete region of the ribosomal 18S gene was amplified using the polymerase chain reaction (PCR) with Qiagen DNA polymerase (Westburg, Leusden, The Netherlands). Eukaryote-specific primers complementary to the 5′-terminus (5′-TYCCTGGTTGATYYTGCCAG-3′) and the 3′-terminus (5′-TGATCCTTCCGCAGGTTCA CCT-3′) were used to amplify the 18S gene (Weekers et al., 1994). PCR amplifications were done using a total volume of 100 μl, containing 1.5 mM $MgCl_2$, 0.5 μM of each primer, 0.2 mM dNTP mixture, and 10× Taq polymerase reaction buffer, and 2.5 U Taq DNA polymerase (Qiagen) was added to each reaction. The samples were covered with two drops of mineral oil and PCR were performed in a Progene thermal cycler (NBS-Techne). Cycling conditions were 95 °C for 1 min, 55 °C for 1.5 min, and 72 °C for 2 min for 30 cycles.

PCR amplification products were treated with shrimp alkaline phosphatase (1 U/μl; Amersham, E70092Y) and exonuclease I (10 U/μl; Amersham, E70073Z) for 15 min at 37 °C, followed by 15 min at 80 °C to kill the enzymes. Terminal (see above) and internal primers in conserved regions of the 18S rDNA; 373C, 373, 570C, 570, 1262C, 1262, 1200C, and 1200RE (Weekers et al., 1994) were used for sequencing. DNA fragments were sequenced in both directions using the BigDye technology and the protocol of the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). The following program was used for all sequencing reactions: 94 °C for 30 s, 50 °C for 15 s, and 60 °C for 4 min for 25 cycles. The resulting products were precipitated by addition of 50 μl of 95% ethanol and 2 μl of a 3 M sodium acetate solution, pH 4.6 to each tube (20 μl). The pellet was rinsed with 250 μl of 70% ethanol, dried in a Speedvac concentrator, redissolved in loading buffer, and run on 48-cm 4% acrylamide sequencing gels with a Perkin–Elmer ABI Prism 377 DNA sequencer.

2.2. Alignment and phylogenetic analyses

The DNA sequences of the complete 18S gene of all taxa were aligned with CLUSTALW 1.64b (default settings) (Thompson et al., 1994) to create an initial dataset. The alignment of the 18S gene was manually optimized using DCSE 3.4 (Dedicated Comparative Sequence Editor program; De Rijk and De Wachter, 1993) with published 18S rDNA sequences, based on the conservation of both primary sequence data and inferred secondary structure features (Nelles et al., 1984) (the rRNA WWW Server: http://www-rrna.uia.ac.be/ssu/index.html) (The Ribosomal Database Project: http://rdp.cme.msu.edu/download/SSU_rRNA/alignments/).

Table 1
Information on species analysed in this study

Order	Family	Genus/species	Geographical origin	Collector's name	EMBL Accession No.
Anostraca	Artemiidae	*Artemia franciscana*	USA	Commercial sample	AJ238061
		Artemia salina	N.A.	EMBL database	X01723
	Parartemiidae	*Parartemia zietziana*	Australia	M. Geddes	AJ238062
	Polyartemiidae	*Polyartemiella hazeni*[a]	USA	D.J. Taylor	AJ242656
		Polyartemia forcipata	Russia	V. Alekseev	AJ272325
	Linderiellidae	*Linderiella santarosae*	USA	D. Belk	AJ272326
	Branchinectidae	*Branchinecta lindahli*	USA	A. Maeda-Martínez	AJ238063
		Branchinecta mesovallensis	USA	D. Belk	AJ238064
	Tanymastigidae	*Tanymastix stagnalis*	Algeria	G. Mura	AJ238065
		Tanymastigites perrieri	Morocco	E.M. Khattabi	AJ238066
	Branchipodidae	*Branchipodopsis wolffi*	South Africa	M. Hamer	AJ238067
		Branchipus schaefferi	Algeria	L. Beladjal	AJ238068
	Chirocephalidae	*Chirocephalus diaphanus*[b]	Spain	J. Mertens	AJ238069
		Chirocephalus diaphanus	France	A. Thiéry	AJ238070
		Pristicephalus josephinae[c]	Belarus	L.L. Nagorskaja	AJ238071
		Eubranchipus bundyi	USA	D. Belk	AJ293893
		Eubranchipus serratus	USA	D. Belk	AJ293894
	Thamnocephalidae	*Thamnocephalus mexicanus*	Mexico	A. Maeda-Martinez	AJ238072
		Thamnocephalus platyurus	USA	D. Belk	AJ238073
		Dendrocephalus brasiliensis[d]	Brazil	Estacao de Piscicultura de Caico (RN)	AJ238074
	Streptocephalidae	*Streptocephalus proboscideus*	Sudan	A. Jawahar Ali	AJ238075
		Streptocephalus dichotomus	India	N. Munuswamy	AJ238076
		Streptocephalus dorothae	Mexico	A. Maeda-Martinez	AJ238077
		Streptocephalus simplex	Yemen	H.J. Dumont	AJ238078
		Streptocephalus sirindhornae	Thailand	L. Sanoamuang	AJ238079
		Streptocephalus spinifer	India	G. Murugan	AJ238080
		Streptocephalus torvicornis	Spain	K. Dierckens	AJ238081
Notostraca	Lepiduridae	*Lepidurus packardi*	N.A.	EMBL database	L34048
Conchostraca	Limnadiidae	*Limnadia lenticularis*	N.A.	EMBL database	L81934
Decapoda	Raninidae	*Raninoides louisianensis*	N.A.	EMBL database	M91060
Decapoda	Lithodidae	*Oedignathus inermis*	N.A.	EMBL database	Z14062

Collector's institutional affiliations are provided in the acknowledgments.
[a] Sample obtained as genomic DNA (gDNA).
[b] Cysts from the laboratory collection (Cat. No.: Cd-910801) used to raise a culture.
[c] Formerly known as *Chirocephalus josephinae*, named by Brtek (1966).
[d] Sample received from a fish culture station in Brazil.

First, the likelihood ratio test (LRT) and Akaike Information Criteria (AIC) in MODELTEST 3.06 (Possada amd Crandall, 1998) were used to determine the appropriate substitution model of DNA evolution that best fitted the dataset. For the analysis, four representatives of closely related crustaceans viz. *Lepidurus packardi* (Notostraca: Lepiduridae), *Limnadia lenticularis* (Conchostraca: Limnadidae), *Raninoides louisianensis* (Decapoda: Raninidae), and *Oedignathus inermis* (Decapoda: Lithodidae) were used as outgroup. The dataset was analysed with the Bayesian inference algorithm (MrBayes; Huelsenbeck, 2000) and the neighbor-joining (NJ), maximum-parsimony (MP), and the maximum-likelihood (ML) algorithms in PAUP* 4.0b8 (Swofford, 1998) to resolve the phylogenetic relationships. Bootstrap analyses were performed to assess the stability of each branch point in the tree and considered as an index of support for a particular clade and not as a statement about the probability in a statistical sense (Hillis and Bull, 1993). The Bayesian method of phylogenetic inference was used because it provides probability values for each branch and tree, and thus, statistically indicates the reliability of the phylogenetic estimate. Bayesian probabilities and MP and ML bootstrap support values appear to be correlated. Seventy percent bootstrap support corresponds to about 95% confidence (Hillis and Bull, 1993), and for Bayesian probabilities, it is suggested that values above 80% indicate strong branch support (Whittingham et al., 2002). For each dataset, the appropriate DNA evolution model with corresponding nucleotide frequencies, substitution rates and types, and Ti/Tv ratios was determined by MODELTEST 3.06 (Possada amd Crandall, 1998) and used for NJ, MP, and ML algorithms in PAUP*.

The Bayesian analysis consisted of ML comparisons of trees in which tree topology and ML parameters were permuted using a Markov chain Monte Carlo method and sampled periodically. The sampled trees are considered to be drawn from a posterior probability distribution, and thus, the frequency with which they are

sampled indicates their probability. Similarly, the posterior probability of any clade is the sum of posterior probabilities of all trees that contain that clade (Huelsenbeck, 2000). Because examinations of the sequence data with MODELTEST suggested complicated substitution models of DNA evolution, we set the ML parameters in MrBayes according to the specific parameter settings for each dataset. The Markov chain Monte Carlo process was set so that four chains ran simultaneously for 500,000 generations, with trees being sampled every 100 generations for a total of 5000 trees in the initial sample. Variation in the ML scores in the samples was examined by inspecting the MrBayes-logfile and the position (tree number) where the ML scores stopped improving was determined. The portion of trees before the position (tree number) where the ML score stopped improving was discarded and the posterior probability of the phylogeny and its branches was determined for all trees having the same lowest ML scores.

Minimum-evolution analysis was performed with PAUP* by application of the selected ML substitution model to the NJ algorithm. The nonparametric-bootstrap analysis used 1000 replicates to assess the reliability of individual branches in the phylogenetic tree (Felsenstein, 1985).

Equally weighted MP analyses was performed with PAUP*. Heuristic search settings were: stepwise taxon addition, tree bisection-reconnection branch swapping, multiple trees retained, no steepest descent, rearrangements limited to 10,000,000, and accelerated transformation. The nonparametric-bootstrap analysis used 1000 replicates to assess the reliability of individual branches in the phylogenetic tree, obtained by heuristic search with stepwise sequence addition (Felsenstein, 1985). A consistency index (CI) (Klug and Farris, 1969), retention index (RI), and rescaled consistency index (RC) (Farris, 1989) were computed to estimate the amount of phylogenetic signal available for parsimony analysis.

For ML analysis, the substitution model of DNA evolution with corresponding parameters that best fitted the data was determined by the likelihood ratio test (LRT) and the Akaike Information Criterion (AIC), using MODELTEST 3.06 (Possada amd Crandall, 1998). Heuristic search settings were stepwise taxon addition, TBR branch swapping, MulTrees option in effect, no steepest descent, and rearrangements limited to 1,000,000. The nonparametric-bootstrap analysis with 100 replicates was used to assess the reliability of individual branches in the phylogenetic tree, obtained by heuristic search with stepwise sequence addition (Felsenstein, 1985).

Pairwise sequence divergence data between taxa were computed. Absolute distance values and distances based on a maximum-likelihood distance matrix (PAUP*), with appropriate parameters for the correct DNA evolution model (MODELTEST), were calculated for the dataset. The Templeton (1993) and Shimodaira–Hasegawa (1999) tests implemented in PAUP* were carried out to determine which tree(s), generated by Bayesian, NJ, MP, and ML analysis, differed significantly from the best estimate of phylogeny. Trees were displayed with TREEVIEW 1.6.6 (Page, 1996).

3. Results

3.1. Characteristics of the ribosomal DNA sequences and alignment

We obtained the complete, unambiguous ribosomal DNA sequence for the 18S rDNA of 27 anostracans of different geographic origins. These have been deposited in the EMBL database (Table 1). The length of the anostracan 18S rDNA gene was 1806 bp, except for *Branchinecta* (1807 bp), *Artemia franciscana* (1809 bp), and *Artemia cf. salina* (1810 bp). The GC content varies between 49.9% (*Branchinecta mesovallensis*) and 51.3% (*Parartemia zietziana*). The alignment of the 18S rDNA of the in- and outgroup species shows 405 variable sites (21.1%) of which 264 are informative (13.8%). The anostracan ingroup contains 143 variable sites (7.5%), of which 109 are informative (5.7%). The outgroup is less homogeneous and contains 320 variable sites (16.7%) of which 135 are informative (7.0%).

3.2. Genetic distances

Pairwise sequence comparison of 18S sequences using distance measurements by ML with settings corresponding to the general time-reversible substitution model with gamma correction for among site rate variation and a correction for significant invariable sites (GTR + G + I) showed remarkable differences in sequence diversity. The widely distributed genus *Streptocephalus* showed little interspecific genetic variation (0.001–0.006 substitutions per site) and divides into Central American, African/West-Asian, and East Asian taxa (full data matrix not shown). The intraspecific genetic variation between *Chirocephalus*, *Pristicephalus*, and *Eubranchipus* (Chirocephalinae clade) is in the same range (0.001–0.008 substitutions per site) and illustrates the phylogenetic position of the newly reinstated *Pristicephalus* (Table 2). Also, the intraspecific genetic variation in the Polyartemiinae clade is in the same range (0.001–0.007 substitutions per site). Based on genetic distances *Polyartemia*, *Polyartemiella*, and *Linderiella* are closely related to each other (0.001–0.003 substitutions per site) than to *Eubranchipus* (0.006–0.007 substitutions per site) (Table 2). The highest genetic distance between all anostracan taxa is due to extreme variation in the Artemiidae and Parartemiidae

Table 2
Pairwise sequence comparison of selected taxa

	Bsc	Bwo	Tpl	Tme	Dbr	Cd-S	Cd-F	Ebu	Ese	Pjo	Lsa	Pfo	Pha	Bli	Bme
Branchipus schaefferi	—	4	24	23	24	29	26	26	26	25	26	26	22	28	27
Branchipodopsis wolffi	0.22	—	26	25	26	27	24	24	24	23	24	24	20	24	23
Thamnocephalus platyurus	1.40	1.52	—	11	16	33	28	32	32	29	30	32	28	34	34
Thamnocephalus mexicanus	1.33	1.45	0.62	—	14	30	27	30	30	27	28	28	28	34	34
Dendrocephalus brasiliensis	1.40	1.53	0.92	0.80	—	34	29	33	33	29	31	33	28	35	35
Chirocephalus diaphanus (Sp.)	1.71	1.59	1.99	1.78	2.04	—	5	14	14	9	12	14	12	21	22
Chirocephalus diaphanus (Fr.)	1.53	1.40	1.67	1.60	1.73	0.28	—	9	9	4	8	10	7	16	17
Eubranchipus bundyi	1.52	1.39	1.92	1.78	1.98	0.80	0.51	—	0	6	13	11	9	17	18
Eubranchipus serratus	1.52	1.39	1.92	1.78	1.98	0.80	0.51	0.00	—	6	13	11	9	17	18
Pristicephalus josephinae	1.46	1.34	1.73	1.59	1.73	0.51	0.22	0.34	0.34	—	10	8	5	16	17
Linderiella santarosae	1.53	1.41	1.81	1.67	1.87	0.68	0.45	0.74	0.74	0.57	—	2	5	12	13
Polyartemia forcipata	1.53	1.41	1.94	1.67	1.99	0.80	0.57	0.63	0.63	0.45	0.11	—	5	12	13
Polyartemiella hazeni	1.28	1.16	1.68	1.66	1.67	0.68	0.39	0.51	0.51	0.28	0.28	0.28	—	11	12
Branchinecta lindahli	1.65	1.40	2.05	1.97	2.11	1.21	0.92	0.97	0.97	0.91	0.68	0.68	0.62	—	3
Branchinecta mesovallensis	1.58	1.34	2.04	1.96	2.10	1.27	0.97	1.03	1.03	0.97	0.74	0.74	0.68	0.17	—

Note. Above the diagonal are absolute nucleotide differences. Below the diagonal are distances in substitutions per 100 sites. Distance measurements are by maximum-likelihood with setting corresponding to the GTR + G + I model, as determined by MODELTEST. Number of substitution types is 6; substitution rate matrix (0.8964, 2.284, 1.226, 0.7373, and 5.344); assumed nucleotide frequencies ($A = 0.25025$, $C = 0.24576$, $G = 0.27114$, and $T = 0.23284$); among-site rate variation (proportion of invariable sites = 0.548547, distribution of rates at variable sites = gamma (continuous) with shape parameter (α) = 0.657999).

(0.040–0.06 substitutions per site), which is more than twice as high as for the other anostracans (0.0–0.020 substitutions per site).

3.3. Phylogenetic analysis

As outgroup, we used two representatives of the same sub-class but of a different order; *Lepidurus packardi* (Notostraca) and *Limnadia lenticularis* (Conchostraca), together with two representatives of the sub-class Malacostraca, *Raninoides louisianensis*, and *Oedignathus inermis* (both Decapoda). The alignment of in- and outgroup taxa contained 1918 aligned positions; 405 sites were variable, 264 of which were phylogenetically informative.

Bayesian inference analysis (ML comparisons using a Markov chain Monte Carlo method) produced a phylogenetic tree with statistical probabilities. Several major and minor clades are well supported (Fig. 1). The anostracans are a monophyletic group with high support (BPS = 100). The well-separated clade of the Artemiidae and Parartemiidae, with high support (BPS = 100), is the sister group to all other anostracans. The remaining clade is composed of several well-resolved subclades with high Bayesian probability support, each representing an anostracan family (Fig. 1).

The NJ analysis with distance measurement set to maximum likelihood used a gamma correction for among site rate variation and a correction for significant invariable sites (GTR + G + I), with the following values: R = (0.8964, 2.284, 1.226, 0.7373, 5.344), proportion of invariable sites $P_{inv} = 0.548547$, and gamma shape parameter = 0.657999. The analysis showed that the anostracans are a monophyletic group (BS = 97) (Fig. 2). The Artemiidae/Parartemiidae clade (BS = 100) is sister group to all other anostracans. Well-defined clades for Streptocephalidae (BS = 97), Tanymastigidae (BS = 90), Branchipodidae (BS = 95), Thamnocephalidae (BS = 99), Branchinectidae (BS = 100), and Chirocephalidae (BS = 100) are evident. *Dendrocephalus* clusters with *Thamnocephalus*, and *Pristicephalus josephinae* (formerly named *Chirocephalus* by Brtek, 1966) clusters with *Eubranchipus*, suggesting that the taxa in each cluster are related, although well separated. Chirocephalidae are sister to Branchinectidae, showing a deep branching monophyletic clade with high bootstrap support (BS = 100). Chirocephalidae shows two major clades, the Polyartemiinae and the Chirocephalinae, although they are not supported by bootstrap confidence (BS = 55). *P. josephinae* emerges as more closely related to *Eubranchipus serratus* and *E. bundyi*, although weakly supported by bootstrap support (BS = 66). Although full resolution of some genera was not obtained, neighbor-joining clustering is still indicative of the relatedness between anostracan families.

The MP analysis with heuristic search resulted in 169 most parsimonious trees (MPTs) of 646 steps (CI = 0.7663, RI = 0.8124, and RC = 0.6225). The bootstrap 50% majority-rule consensus tree is presented in Fig. 2 and shows an almost identical topology as the NJ tree. Only few minor topological changes occurred. Several major and minor clades within the anostracans are well supported; the group is monophyletic with high MP bootstrap support (BS = 100). The Artemiidae/Parartemiidae clade (BS = 94) is sister group to all other anostracans. Most of the remaining families form well-separated clades of high bootstrap support (BS > 89), except Tanymastigidae (BS = 58). The phylogenetic

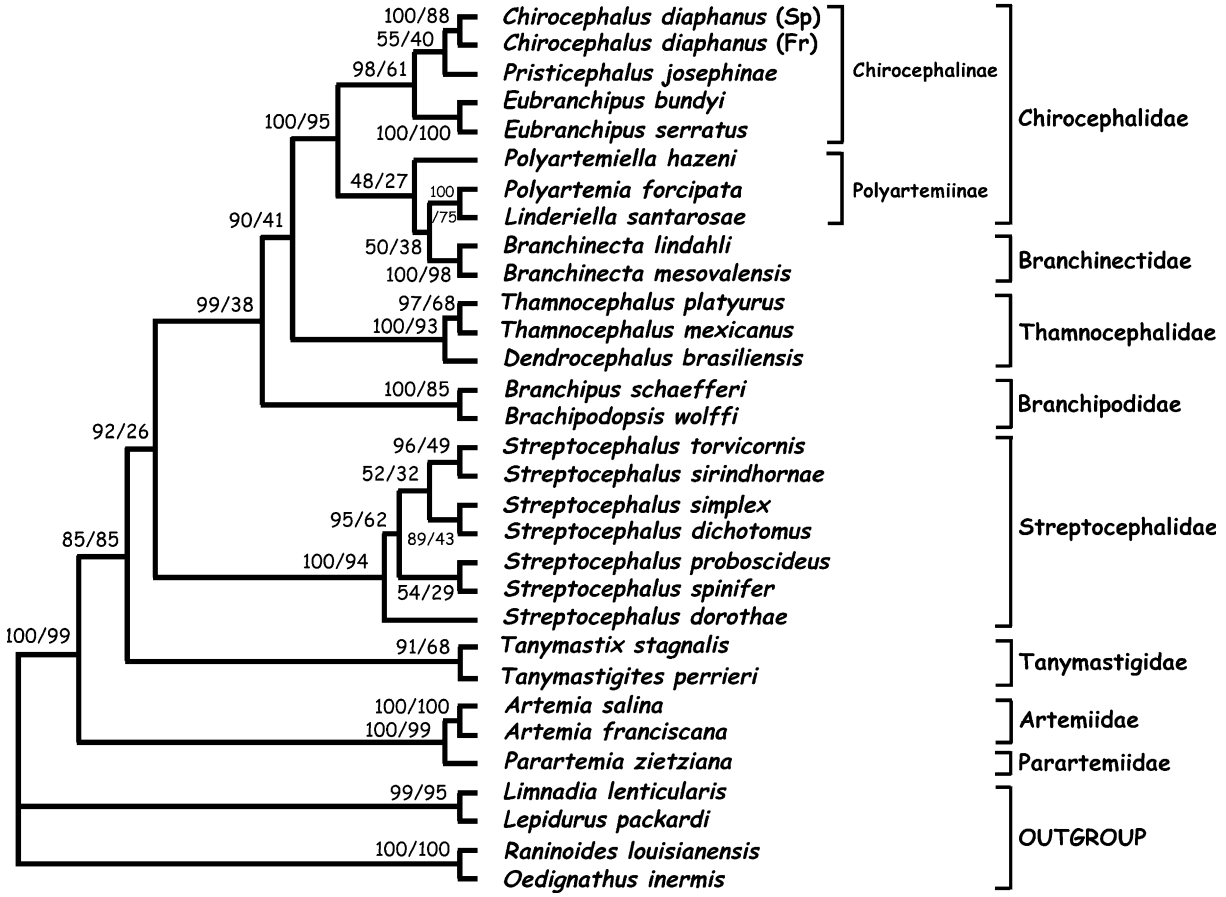

Fig. 1. Estimate of the anostracan phylogeny, based on 18S ribosomal DNA sequences; consensus tree for Bayesian and ML methods. Settings of the ML parameters in MrBayes as determined by MODELTEST are: "lset basefreq = empirical" (empirically determined base frequencies), "nst = 6" (GTR + G + I model), "revmat = estimate" (parameters estimated from the dataset), "rates = gamma" (site-specific rate variation drawn from the gamma distribution), "shape = 0.657999" (gamma shape parameter), and "outgroup = *R. louisianensis*". In the initial sample were 5000 trees and the point of stationary ML scores "burnin") was after 1500 trees. The first 1500 trees were discarded and the posterior probability of the phylogeny was determined from 4500 trees. A 50% majority-rule consensus tree with Bayesian probability values were calculated in PAUP* using the 4500 trees with lowest ML scores, rooted with *Lepidurus packardi*, *Limnadia lenticularis*, *Raninoides louisianensis*, and *Oedignathus inermis* as outgroups. The ML analysis with GTR + G + I, gamma correction, $R = (0.8964, 2.284, 1.226, 0.7373,$ and $5.344)$, Pinv = 0.548547, and gamma shape parameter = 0.657999 generated a tree with a log likelihood of −6124.58138. The numbers along the branches indicate Bayesian support (first number) and ML (second number) bootstrap support, and are expressed as percentage.

position of the latter is not resolved satisfactorily. Although Chirocephalinae seems to be a well-defined clade (BS = 72), the relationship between Chirocephalinae and Polyartemiinae still suffers from poor resolution. However, sister group relationships for taxa within Polyartemiinae and Chirocephalinae, and also between Polyartemiinae and Chirocephalinae, were suggested by all MPTs.

Based on the results of MODELTEST LRT and AIC evaluations, ML analysis was performed with the general time-reversible substitution model, using gamma correction for among site rate variation and a correction for significant invariable sites (GTR + G + I), with the following values: $R = (0.8964, 2.284, 1.226, 0.7373,$ and $5.344)$, proportion of invariable sites $P_{\text{inv}} = 0.548547$, and gamma shape parameter = 0.657999. The analysis resulted in a tree (Ln likelihood = −6124.58138) with an almost identical topology as the Bayesian tree and with high ML bootstrap support (BS > 85) for the clades representing the anostracan families (Fig. 1). Again, the Artemiidae/Parartemiidae clade (BS = 99) is sister group to all other anostracans. Although the relationship between the Streptocephalidae, Branchipodidae, and Thamnocephalidae (BS = 26, 38, 41), and within Chirocephalinae (BS = 61) and Polyartemiinae (BS = 27), suffers from poor resolution, sister group relationships for taxa within Polyartemiinae and Chirocephalinae, between Polyartemiinae and Chirocephalinae, and between Branchinectidae and Chirocephalidae are suggested.

In general, the trees obtained from the Bayesian inference and ML methods showed identical topologies (Fig. 1). The trees from the NJ and MP methods showed almost identical topologies (Fig. 2) and a visual comparison (Figs. 1 and 2) of all methods showed that only

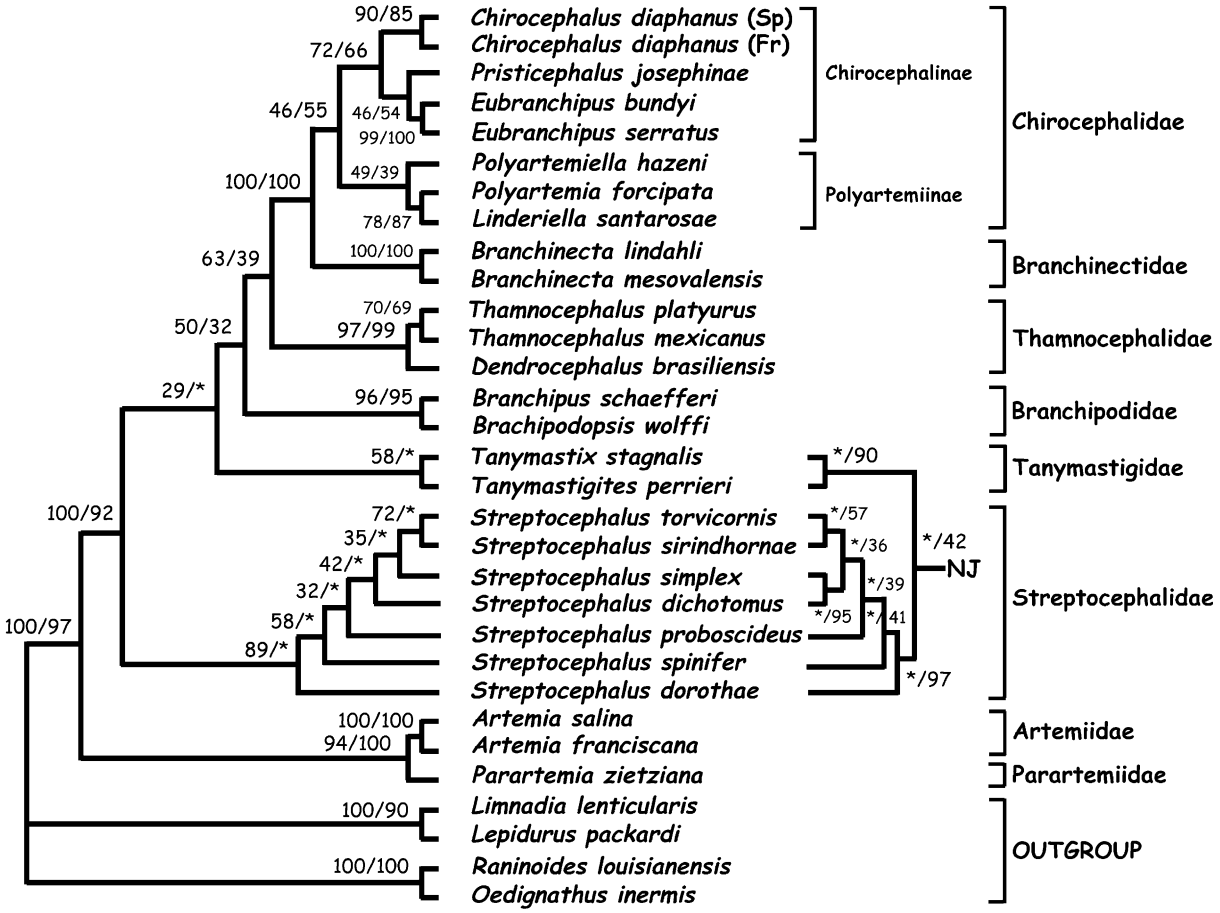

Fig. 2. Estimate of the anostracan phylogeny, based on 18S ribosomal DNA sequences; consensus tree for MP and NJ methods. The MP analysis generated 169 most parsimonious trees (MPTs) of 646 steps (CI = 0.7663, RI = 0.2337, RC = 0.6225). Bootstrap method with heuristic search, stepwise taxon addition, TBR branch swapping, MULTREES option, no steepest descent, rearrangements limited to 10,000,000, and accelerated transformation. The ME analysis used the optimality criterion distance, a bootstrap method with NJ search, ties broken randomly, and distance measure set to maximum likelihood using the data as determined by MODELTEST. The numbers along the branches indicate MP (first number) and ME (second number) bootstrap support, and are expressed as percentage. The different topologies for the Tanymastigidae/Streptocephalidae clade from the NJ analysis are shown as an inset, indicated by NJ.

few minor topological changes occurred. Table 3 presents the results of the Templeton and Shimodaira–Hasewaga tests as an attempt to identify the statistically best topology. The two tests that compared the various estimates of molecular phylogeny were not conclusive. The Templeton test indicated that the MP topologies were the most robust, with ML and Bayesian methods as second best. However, the SH-test indicated that the ML (heuristic) and Bayesian methods were the most robust, followed by ML (bootstrap) and MP methods. In all tests, the NJ methods were significantly worse (Table 3).

Table 3
Summary of the Templeton (1993) and Shimodaira–Hasegawa (1999) tests for different estimates of anostracan phylogeny

Tree method	Templeton test			Shimodaira–Hasewaga test		
	Length	z value	P value	−ln L scores	Δln L	P scores
NJ (heuristic)	647	0.3780	0.7055	6135.67579	11.09441	0.34 (0.33)
NJ (bootstrap)	647	0.4472	0.6547	6130.30720	5.72582	0.60 (0.59)
MP (heuristic)*	646	(Best)		6129.94274 (6127.79850)	5.36136 (3.21417)	0.65 (0.79)
MP (bootstrap)	646	0.0000	1.0000	6133.68903	9.10765	0.42 (0.40)
ML (heuristic)	647	0.3015	0.7630	6124.58138	(Best)	
ML (bootstrap)	647	0.3333	0.7389	6128.20642	3.62504	0.69 (0.68)
MrBayes	647	0.3015	0.7630	6124.58138	0.00000	0.79 (0.80)

Note. *Indicates that there is a difference in log-likelihood scores when the SH-test was performed using parsimony (first number) or likelihood (second number) scores on the best MP topology found by heuristic search.

4. Discussion

4.1. Sequence variation and genetic distances

The 18S rDNA gene varies in length from 1806 to 1810 bp and in GC content from 49.9% to 51.3%. Both values are within the normal range for crustaceans available from the GenBank/EMBL database and literature (Crease and Taylor, 1998; Spears and Abele, 1997; Spears et al., 1994).

It is interesting to compare genetic distances within and among taxa to determine whether a given group of anostracans has diverged, on average, more or less than others. The divergence values among members of the Anostraca vary up to 0.060 substitutions per site (data not shown). The highest genetic distance between all anostracan taxa is caused by extreme variation in the Artemiidae and Parartemiidae (0.040–0.06 substitutions per site), which is more than twice as high as for the other anostracans (0.0–0.020 substitutions per site). They form two basic clades which, based on their difference in genetic distances, should be raised to suborder level. The lowest variation in genetic distance is within Streptocephalidae (0.001–0.006 substitutions per site; data not shown), although they originate from different geographic areas. The genetic distances between taxa belonging to Chirocephalidae (Table 2), combined with a distinctive morphology and ecology, can be helpful in phylogenetically positioning these species.

4.2. Phylogenetic analysis

Previous workers used morphology alone to classify anostracans, starting with Daday (1910). Linder (1941) refined his classification, moving many genera in and out of Daday's families. Brtek (1966) proposed a nine family scheme, mainly by raising one genus (*Artemiopsis*) to family rank, and creating the Linderiellidae for two other genera, but none of these authors have had a clear idea on the phylogenetic relationship between all these groups. Only recently four anostracan 18S rDNA sequences have been included in a branchiopod phylogeny, briefly indicating phylogenetic relationships among anostracans (Spears and Abele, 2000). More recently, Remigio and Hebert (2000) published a phylogeny of anostracans, based on eight species.

All tree reconstruction methods that we used for identifying the relationships between 27 taxa divide the anostracans into two distinct monophyletic groups. The first consists of the Artemiidae and Parartemiidae, representing saline water taxa; the second contains all freshwater taxa. The same division was also found by Remigio and Hebert (2000), but they did not realize the fundamental division between freshwater and saline water taxa, because they treated the *Parartemia* as Branchipodidae.

The methods used in the present study show two slightly different tree topologies (Figs. 1 and 2). However, all methods agree that there are well-separated clades, confirmed by high bootstrap support for Artemiidae, Parartemiidae, Tanymastigidae, Streptocephalidae, Branchipodidae, Thamnocephalidae, Branchinectidae, and Chirocephalidae. The support value for a few nodes connecting families is weak and their true phylogenetic relationship remains unsettled. In contrast, *Parartemia zietziana*, previously considered a subfamily (Parartemiinae) to the Branchipodidae (Belk, 1982; Brtek, 1997), clusters as a sister group to the Artemiidae with high support values. The branch lengths (genetic distances), separating *Artemia* and *Parartemia* (0.040–0.06 substitutions per site) from other anostracans, are equal to or exceed those for interordinal relationships elsewhere in branchiopod phylogeny (Spears and Abele, 2000). These findings suggest that the genus *Parartemia* should be raised to family level (family Parartemiidae) inside a clade of subordinal rank (the Artemiina). These results prompted a re-examination of morphological data and suggests the following characters uniting *Artemia* and *Parartemia*, previously deemed unimportant (Linder, 1941): a tendency towards a fusion of the seventh abdominal segment with the telson (both sexes); females; brood pouch short and tending towards development of two lateral lobes; males: second antennae fused only slightly at their base; median article with two rigid wart- or spine-like outgrowths at its inner side, presence of fleshy processes near the middle of the eversible penis (not in all species of *Artemia*). They define a group of anostracans that vicariate in the saline waters of the world, with the Artemiidae cosmopolitan save in Australia (before the introduction of *Artemia* there), and the Parartemiidae limited to Australia.

The remainder of the order forms the suborder Anostracina, defined here by default as follows: seventh abdominal segment never fused to telson, brood pouch variously shaped but not bilobed, male antennae variously shaped, fused or not, but not with two small, separate outgrowths on inner side of median article; eversible penis without fleshy process near the middle.

The relationship between Thamnocephalidae and Streptocephalidae as described by Remigio and Hebert (2000), based on a limited number of taxa, is misleading. Our analysis, including a wider range of taxa, shows that the Branchipodidae separate the Thamnocephalidae from the Streptocephalidae. The position of the Tanymastigidae remains unclear, as shown by the different topologies (Figs. 1 and 2). The Bayesian, NJ, MP, and ML methods seem to cluster the Chirocephalidae, Branchinectidae, Thamnocephalidae, and Branchipodidae in one clade, with the Streptocephalidae and Tanymastigidae as their sister groups. The position of the Tanymastigidae varies between analyses (Fig. 1 versus Fig. 2) and there is low support for each topology.

The families Branchipodidae and Thamnocephalidae fall in two separate clades; each clade is considered here a family and placed on an equal taxonomic level. Because of tree topology we argue for some further changes in the taxonomy of the anostracans. First, the creation of an additional taxon: *Tanymastix stagnalis* and *Tanymastigites perrieri* are placed in a new family, the Tanymastigiidae. Supporting morphological characters (for definitions of terms, see Brendonck, 1995) include proximal 1/3–2/3 of basal joints of antennae fused to form a clypeus from which arise both frontal and ventral clypeal processes. Frontal appendages elaborate and fused medially to form a basal trunk. Second, the suppression of the family Linderiellidae since *Linderiella santarosae* clusters between the genera of the former Polyartemiidae and the three genera form the Subfamily Polyartemiinae of the Chirocephalidae.

Polyartemiella hazeni and *Polyartemia forcipata* are of special interest because they are morphologically different from all other anostracans in having, respectively, 17 and 19 pairs of thoracic segments and trunk limbs instead of the usual 11. Both Linder (1941) and Dodson and Frey (1991) agree that this morphological difference suggests that they represent their most primitive members. Our results contradict with this hypothesis and show that the Polyartemiinae and Chirocephalinae either combine to form a monophyletic clade the Chirocephalidae with low support, which is sister to the Branchinectidae (Fig. 2), or form a paraphyletic clade including the Branchinectidae as sister group to Polyartemiinae (Fig. 1). Further work is needed to clarify these relationships, as Templeton and Shimodaira–Hasewaga tests gave inconclusive results. However, the monophyly of the Chirocephalidae is confirmed by Remigio and Hebert (2000) who suggested a sister group relationship between Branchinectidae and the Chirocephalidae. This is also supported by morphology: all Polyartemiinae share a rigid medial outgrowth, antennal appendage, from the basal segment of the antenna, whereas in Chirocephalinae this outgrowth is flexible and lamelliform or serrate. We presume that the Chirocephalinae and the Polyartemiinae had a common ancestor, since both share a common double pre-epipodite (sometimes partially fused), as does *Artemiopsis*, correctly assigned to Chirocephalidae by Remigio and Hebert (2000). From this ancestor, the Polyartemiinae diverged. We presume that the gain of 6 or 8 thoracic segments, along with the 6 or 8 pairs of legs by *P. hazeni* and *P. forcipata*, occurred late in the anostracan evolution, since variation in thoracic limb numbers occurs not only in anostracans but also in conchostracans and cladocerans (Olesen, 1998). We speculate that this represents mutations in the homeotic genes (Hox or homeobox genes) and that this mutation was secondarily lost again in *Linderiella*. Although Remigio and Hebert (2000) say that there is no mechanistic explanation for this remarkable morphological flexibility, studies on a number of arthropod homeotic (Hox) genes provide growing evidence for the role of these genes in modulating the repression or development of body segments and their associated appendages. Regarding Chirocephalinae, finally, we find two distinct subclades, one containing *Chirocephalus* proper, and the other *Eubranchipus* (the two species analysed had an identical gene sequence, suggesting they are very close to each other) and *P. josephinae*. The latter genus, sunk into the synonomy of *Chirocephalus* by Brtek (1966), must be reinstated. It is closer to *Eubranchipus* than to *Chirocephalus*. The *Eubranchipus Pristicephalus* clade shares double pre-epipodites in an advanced state of fusion and has a single outgrowth from the basal segment of A2 in males. The *Chirocephalus* clade has pre-epipodites fused at the base only and two outgrowths from the basal segment of A2 in males.

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