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# PHYLOGENETIC RELATIONSHIPS OF PALAEACANTHOCEPHALA (ACANTHOCEPHALA) INFERRED FROM SSU AND LSU rDNA GENE SEQUENCES

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## PHYLOGENETIC RELATIONSHIPS OF PALAEACANTHOCEPHALA (ACANTHOCEPHALA) INFERRED FROM SSU AND LSU rDNA GENE SEQUENCES

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**ABSTRACT:** The Palaeacanthocephala is traditionally represented by 2 orders, Echinorhynchida and Polymorphida, with 10 and 3 families, respectively. To test the monophyly of the class, these 2 orders, and certain families, phylogenies were inferred using nuclear small-subunit (SSU) and large-subunit (LSU) ribosomal DNA sequences obtained for 29 species representing 10 families, 2 other classes of acanthocephalans, and 3 rotifer outgroups. Phylogenetic relationships were inferred by analyzing combined SSU and LSU sequences using maximum parsimony (MP) and maximum likelihood (ML) methods. Parsimony and ML trees inferred from combined analysis of these rDNA data strongly supported monophyly of Palaeacanthocephala and provided good resolution among species. Neither Polymorphida nor Echinorhynchida was monophyletic. *Gorgorhynchoides bullocki* (Echinorhynchida) was nested within the 6 species representing Polymorphida, and this clade was nested within species representing Echinorhynchida. Three of 4 palaeacanthocephalan families that could be evaluated were not monophyletic, and this finding was strongly supported. These results indicate that the family level classification of palaeacanthocephalans, which is mainly based on combinations of shared characters (not shared derived characters), needs to be reevaluated with respect to comprehensively sampled phylogenetic hypotheses.

Acanthocephala (thorny-headed worms) is a small (~1,200 described species) group of endoparasitic pseudocoelomates that use arthropods and vertebrates to complete their life cycles (Schmidt, 1985). The phylum is currently represented by 4 classes: Archiacanthocephala, Palaeacanthocephala, Eoacanthocephala, and Polyacanthocephala (Amin, 1987). These taxonomic groups are distinguished mainly by morphological and ecological features (Bullock, 1969; Amin, 1985, 1987). Phylogenetic hypotheses based on molecular and morphological data sets show that acanthocephalans share most recent common ancestry with members of Rotifera (Lorenzen, 1985; Wallace and Colburn, 1989; Nielsen, 1995; Winnepeninckx et al., 1995; Garey et al., 1996; Melone et al., 1998; Giribet et al., 2000). This clade has been formally named the Syndermata based on the apomorphic condition of the syncytial epidermis (Ahlrichs, 1997).

Most molecular phylogenies based on near-complete 18S (SSU) sequences have shown that Acanthocephala is a monophyletic group with subclades reflecting classes in the current classification (Near et al., 1998; García-Varela et al., 2000; Near, 2002). Cladistic analysis of morphological characters (Monks, 2001) also yielded hypotheses supporting monophyly of the Palaeacanthocephala and Eoacanthocephala, but not the Archiacanthocephala. In contrast to these results, a recent molecular analysis by Herlyn et al. (2003) of partial SSU sequences representing 5 rotifer and 15 acanthocephalan species yielded the unexpected finding of a paraphyletic Palaeacanthocephala. A shortcoming of all these studies is relatively limited taxon sampling, and for the molecular investigations, reliance on sequences from a single gene, SSU ribosomal DNA (rDNA).

The Palaeacanthocephala have the most diverse life histories and structural features of the acanthocephalans and, therefore, the suggestion that this class is paraphyletic (Herlyn et al., 2003) is of particular interest. Palaeacanthocephala includes 2 orders, Echinorhynchida and Polymorphida, with 10 and 3 families, respectively. These families are distinguished by unique combinations of features, including armature of the proboscis, proboscis shape, the presence and arrangement of trunk spines, the number and shape of cement glands, egg shape, and defini-

itive and intermediate host type (Bullock, 1969; Amin, 1985, 1987). Palaeacanthocephalans parasitize a diverse range of vertebrate definitive hosts (mammals, birds, reptiles, amphibians, and fishes), and use various malacostracan (crustacean) intermediate hosts. Consumption of infected invertebrates completes the life cycle, although some palaeacanthocephalans also use vertebrates as paratenic hosts to reach appropriate definitive hosts via the food chain (Nickol and Crompton, 1985).

Palaeacanthocephalan diversity has not been well-sampled in published phylogenetic studies; however, broader taxonomic sampling is essential to understanding patterns of evolutionary diversification in this group. The main objective of the present research was to test palaeacanthocephalan monophyly and relationships more rigorously by sampling additional taxa, and sequences in addition to SSU rDNA. This was accomplished by sequencing the near-complete 18S (SSU) and 28S (LSU) rDNA genes from 19 species representing 10 of 13 palaeacanthocephalan families (Amin, 1987; Pichelin and Cribb, 2001). To provide a context for assessing Palaeacanthocephala monophyly, we also sequenced 7 species of acanthocephalans from the Archiacanthocephala and Eoacanthocephala, plus 3 species of rotifers representing outgroups for the analyses.

### MATERIALS AND METHODS

#### Specimens and DNA isolation

Acanthocephalans used for this study were collected from their naturally infected vertebrate or invertebrate hosts (Table I). Worms were washed 3 times in normal saline solution, preserved in absolute ethanol, and stored at 4 C. Representative specimens were stained with Mayer's paracarmine, mounted in Canada balsam, and identified by microscopy. Rotifers were grown using standard culture methods, washed thoroughly in sterile distilled water, and pelleted by centrifugation before DNA extraction.

Specimens were digested overnight at 56 C in a solution containing 10 mM Tris-HCl (pH 7.6), 20 mM NaCl, 100 mM Na<sub>2</sub>EDTA (pH 8.0), 1% Sarkosyl, and 0.1 mg/ml proteinase K. Following digestion, DNA was extracted from the supernatant using the DNazol reagent (Molecular Research Center, Cincinnati, Ohio) according to the manufacturer's instructions. Some tissues were extracted using the DNeasy Tissue Kit (Qiagen, Valencia, California).

#### Amplification and sequencing of DNA

Two regions of nuclear rDNA were amplified using the polymerase chain reaction (PCR). The near-complete SSU rDNA (~1,800 bp) was

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TABLE I. Specimen information and GenBank accession numbers for species studied. Sequences marked with an asterisk were determined in this study.

	Family	Host	18S rDNA	28S rDNA
<b>Acanthocephala</b>				
<i>Acanthocephalus dirus</i>	Echinorhynchidae	<i>Asellus aquaticus</i>	*AY830151	*AY829106
<i>Acanthocephalus lucii</i>	Echinorhynchidae	<i>Perca fluviatilis</i>	*AY830152	*AY829101
<i>Acanthocephaloides propinquus</i>	Arhythmacanthidae	<i>Gobius bucchichii</i>	*AY830149	AY829100
<i>Centrorhynchus</i> sp.	Centrorhynchidae	<i>Falco peregrinus</i>	*AY830155	*AY829104
<i>Corynosoma enhydri</i>	Polymorphidae	<i>Enhydra lutris</i>	AF001837	*AY829107
<i>Echinorhynchus truttae</i>	Echinorhynchidae	<i>Thymallus thymallus</i>	*AY830156	*AY829097
<i>Filisoma bucerium</i>	Cavisomidae	<i>Kyphosus elegans</i>	AF064814	*AY829110
<i>Floridosentis mugilis</i>	Neoechinorhynchidae	<i>Mugil cephalus</i>	AF064811	*AY829111
<i>Gorgorhynchoides bullocki</i>	Rhadinorhynchidae	<i>Eugerres plumieri</i>	*AY830154	*AY829103
<i>Illiosentis</i> sp.	Illiosentidae		*AY830158	*AY829092
<i>Koronacantha mexicana</i>	Illiosentidae	<i>Pomadasys leuciscus</i>	*AY830157	*AY829095
<i>Koronacantha pectinaria</i>	Illiosentidae	<i>Microlepidotus brevipinnis</i>	AF092433	*AY829094
<i>Leptorhynchoides thecatus</i>	Rhadinorhynchidae	<i>Lepomis cyanallus</i>	AF001840	*AY829093
<i>Macracanthorhynchus ingens</i>	Oligacanthorhynchidae	<i>Procyon lotor</i>	AF001844	*AY829088
<i>Mediorhynchus</i> sp.	Giganthorhynchidae	<i>Cassidix mexicanus</i>	AF064816	*AY829087
<i>Moniliformis moniliformis</i>	Moniliformidae	<i>Rattus rattus</i>	Z19562	*AY829086
<i>Neoechinorhynchus saginata</i>	Neoechinorhynchidae		*AY830150	*AY829091
<i>Oligacanthorhynchus tortuosa</i>	Oligacanthorhynchidae	<i>Didelphis virginiana</i>	AF064817	*AY829090
<i>Oncicola</i> sp.	Oligacanthorhynchidae	<i>Nasua narica</i>	AF064818	*AY829089
<i>Pomphorhynchus bulbocollis</i>	Pomphorynchidae		AF001841	*AY829096
<i>Plagiorhynchus cylindraceus</i>	Plagiorhynchidae		AF001839	*AY829102
<i>Polymorphus brevis</i>	Polymorphidae	<i>Nycticorax nycticorax</i>	AF064812	*AY829105
<i>Profilicollis altmani</i>	Polymorphidae	<i>Enhydra lutris</i>	AF001838	*AY829108
<i>Polymorphus</i> sp.	Polymorphidae	<i>Anas platyrhynchos</i>	AF064815	*AY829109
<i>Rhadinorhynchus</i> sp.	Rhadinorhynchidae	Fish family (Scianidae)	AY06233	*AY829099
<i>Transvena annulospinosa</i>	Transvenidae	<i>Anampses neoguinaicus</i>	*AY830153	*AY829098
<b>Rotifera</b>				
<i>Asplancha sieboldi</i>	Asplanchnidae	Free-living	AF092434	*AY829085
<i>Brachionus patulus</i>	Brachionidae	Free-living	AF154568	*AY829084
<i>Lecane bulla</i>	Lecanidae	Free-living	AF154566	*AY829083

amplified in 1 fragment using the primers forward 5'-AGATTAAGCC ATGCATGCGT and reverse 5'-GCAGGTTACCTACGGAAA (Garey et al., 1996). The near-complete LSU rDNA (~2,600 bp) was amplified using 4 overlapping PCR fragments of 700–800 bp. Primers for LSU amplicon 1 were forward 5'-CAAGTACCGTGAGGGAAAGTT GC and reverse 5'-CAGCTATCCTGAGGGAAAC; amplicon 2, forward 5'-ACCCGAAAGATGGTGAACATG and reverse 5'-CTTCTC CAAC(T/G)TCAGTCTTCAA; amplicon 3, forward 5'-CTAAGGAG TGTGTAACAACCTACC and reverse 5'-AATGACGAGGCATTTGG CTACCTT; amplicon 4, forward 5'-GATCCGTAACCTCGGAAAA GGAT and reverse 5'-CTTCGCAATGATAGGAAGAGCC.

PCR reactions (25 µl) consisted of 0.5 µM of each primer, 200 µM deoxynucleoside triphosphates, 1.5 mM MgCl<sub>2</sub>, and 0.5 U proofreading polymerase (Finnzymes DNAzyme EXT, MJ Research, Alameda, California). PCR cycling parameters included denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, annealing at 50–58 °C (optimized for each rDNA region) for 1 min, and extension at 72 °C for 1 min, followed by a post-amplification incubation at 72 °C for 7 min. All PCR reactions were performed in a MJ Research thermal cycler using a heated lid to reduce refluxing.

Each PCR product was prepared for direct sequencing using enzymatic treatment with exonuclease I and shrimp alkaline phosphatase (PCR product presequencing kit, USB Corporation, Cleveland, Ohio). When PCR direct sequencing yielded poor results (e.g., due to repeated sequence motifs), PCR products were cloned by ligation into pGEM-T vector (Promega, Madison, Wisconsin) and used to transform competent *Escherichia coli* (JM109). Positive clones were identified by blue/white selection, and target inserts of white colonies were confirmed by PCR of bacterial DNA extracts. Liquid cultures for minipreps were grown in Luria broth containing 50 µg/ml of ampicillin. Plasmids for DNA se-

quencing were prepared using commercial miniprep kits (Qiaprep, Qiagen).

PCR products and plasmids were sequenced for both DNA strands using PCR, internal, and plasmid primers as appropriate to each gene template. Sequencing reactions were performed using ABI BigDye (PE Applied Biosystems, Boston, Massachusetts) terminator-sequencing chemistry, and reaction products were separated and detected using an ABI 3730 capillary DNA sequencer. Contigs were assembled and base-calling differences resolved using Sequencher version 3.1.1 (Gene Codes, Ann Arbor, Michigan). Site polymorphisms were recorded only when both alternative nucleotide peaks were present in all sequence reactions representing both DNA strands. All sequences have been deposited in the GenBank database (accession numbers in Table I).

#### Multiple alignments

Twenty-six acanthocephalan and 3 rotifer LSU rDNA sequences were obtained during this study (Table I). Ten new SSU rDNA sequences from acanthocephalans were also obtained so that SSU and LSU sequences were available from all taxa analyzed. These sequences were combined with published SSU sequences from GenBank, including 16 acanthocephalans and 3 rotifers (Monogononta). These 2 groups of sequences (LSU, SSU) were aligned separately using ProAlign version 0.5 (Löytynoja and Milinkovitch, 2003). For each alignment, a ProAlign guide tree was constructed using corrected (for multiple hits) pairwise distances; this guide tree was used to estimate the hidden Markov model parameters (δ and ε) for progressive multiple alignment. Program (Java) memory and band width were increased as required to complete the alignment. The minimum posterior probability of sites was used as the criterion for detecting and removing (filtering) unreliably aligned se-

TABLE II. Tree statistics for rDNA data sets. Small-subunit or 18S rDNA (SSU), large-subunit or 28S rDNA (LSU), combined rDNA (SSU + LSU) datasets. Number of informative characters, consistency index (C.I.) and tree length refer to parsimony inference. Pinv (proportion of invariable sites), Gd (shape of gamma distribution) and -ln likelihood refer to maximum likelihood inference.

Dataset	Total characters	Uninformative characters	Constant characters	Informative characters	C.I.	Tree length	-ln likelihood	Pinv	Gd
SSU	1.385	144	746	495	0.61	1.554	9475.26	0.2257	0.6571
LSU	2.007	121	1,105	781	0.56	2.546	14541.74	0.3558	0.7465
SSU + LSU	3.392	265	1,851	1,276	0.56	4.212	24432.37	0.3229	0.7318

quences. To reduce the likelihood of excluding correctly aligned sites, the filter threshold was set to 60% minimum posterior probability (Löytynoja and Milinkovitch, 2003). For phylogenetic analysis of SSU sequences, using ProAlign to detect and remove unreliably aligned sites by their posterior probabilities excluded 465 of 1,850 sites. For the LSU data set, 1,065 of 3,072 sites were excluded based on posterior probability filtering. Thus, these rDNA data sets included 3,392 characters in combined analyses. Alignments and tree-files from analyses have been deposited in TreeBASE (Sanderson et al., 1994).

### Phylogenetic analyses

The SSU and LSU rDNA filtered alignments were analyzed independently and also as a combined rDNA data set. Tree searches were conducted with the optimality criteria of maximum parsimony (MP) and maximum likelihood (ML) using the software PAUP\* 4.0b10 (Swofford, 2002). For ML analyses, likelihood models were compared using Modeltest version 3.0 (Posada and Crandall, 1998) to perform a nested likelihood ratio test (LRT) to assess the fit of General Time Reversible (GTR) nucleotide substitution models for these data (Rodríguez et al., 1990). The best-fit ML model for each data set (SSU, LSU, combined rDNA) was used for likelihood analysis (Table II). For each data set a GTR model with invariable sites (+I), and rate heterogeneity (+G; Yang, 1994) was used, but the estimated parameters varied by data set (Table II). Tree searches were performed using 50 (ML) and 1,000 (MP) random addition heuristic searches with tree-bisection-reconnection (TBR) branch-swapping. The relative reliability of clades was assessed by bootstrap resampling, with 10,000 (MP) or 100 (ML) bootstrap pseudoreplicates. To compare topologies representing specific alternative phylogenetic hypotheses, constraints were defined on the combined trees. Differences between trees representing alternative hypotheses were evaluated using the Kishino and Hasegawa likelihood test (Kishino and Hasegawa, 1989) and Templeton's modified parsimony test (Templeton, 1983). These tests were used to compare preconceived hypotheses of monophyly versus nonmonophyly of the Palaeacanthocephala. Trees were drawn using RETREE and DRAWGRAM from PHYLIP (Felsenstein, 1999).

## RESULTS

### Base composition

Nucleotide frequencies for the combined SSU + LSU data set were 0.279 (A), 0.195 (C), 0.272 (G), and 0.251 (T). The heterogeneity of nucleotide frequencies across taxa was tested using the "basefreq" option implemented in PAUP\* ( $X^2 = 67.34$ ,  $P = 0.90$ ). This result indicates that rDNA nucleotide frequencies were not significantly heterogeneous across taxa, which is advantageous because MP and ML inference methods perform optimally when nucleotide frequencies are homogeneous (Omilian and Taylor, 2001). Total lengths of the alignments and number of constant and parsimony-informative characters for the SSU, LSU, and combined data sets are provided in Table II.

### Combined SSU + LSU data set

Maximum parsimony analysis of the combined SSU + LSU rDNA data set (Fig. 1A) yielded 2 trees with a consistency index (C.I.) = 0.56 and length of 4,212 steps (Table II). The difference between these MP trees was within the Archiacanthocephala clade. The first tree had the topology

((((Macracanthorhynchus ingens, (Moniliformis moniliformis, Mediorhynchus sp.)), Oncicola sp.)), Oligacanthorhynchus tortuosa)). The second tree placed (((Macracanthorhynchus ingens, (Oligacanthorhynchus tortuosa, Oncicola sp.)), Moniliformis moniliformis), Mediorhynchus sp.)). Both the strict-consensus MP tree (Fig. 1A) and the ML tree (Fig. 1B) depicted each of the 3 sampled classes (Archiacanthocephala, Eoacanthocephala, and Palaeacanthocephala) as monophyletic, with strong ( $\geq 99\%$ , by MP) to moderate (100%, 100%, 84% for these clades, respectively, by ML) bootstrap support. Within Archiacanthocephala, ML branch lengths were short and bootstrap MP analysis yielded no support for clades. Within Palaeacanthocephala, which included representatives of 10 families, few taxonomic groups were monophyletic in MP or ML trees. For example, neither the Echinorhynchida (7 families represented) nor the Polymorphida (3 families represented) was monophyletic as inferred by MP or ML. In both analyses, a clade of 7 species representing Polymorphida plus *Gorgorhynchoides* was nested within families of Echinorhynchida, and the only nontrivial ( $>1$  representative in analysis) monophyletic palaeacanthocephalan family in MP or ML trees was Polymorphidae; however, within this family, *Polymorphus* was not monophyletic. The absence of clades representing traditional families of Palaeacanthocephala was not an artifact of poor tree resolution. The combined rDNA analyses (MP and ML) produced highly resolved trees with many strongly supported clades, particularly as inferred from the parsimony bootstrap analysis (Fig. 1A). In several instances, members of the same family were located in disparate subclades of the Palaeacanthocephala with strong bootstrap support. For example, genera of the Rhadinorhynchidae were dispersed throughout the Palaeacanthocephala; nested within Illiosentidae (*Leptorhynchoides*), within Polymorphida (*Gorgorhynchoides*), and sister (*Rhadinorhynchus*) to the only genus (*Transvena*) representing Transvenidae. These relationships were recovered by MP and ML, and with strong bootstrap support by both inference methods.

The ML tree inferred from the combined rDNA data set yielded 1 tree with the same general topology as the MP strict consensus. None of the differences between the MP strict consensus and ML trees (Fig. 1A, B) involved clades receiving

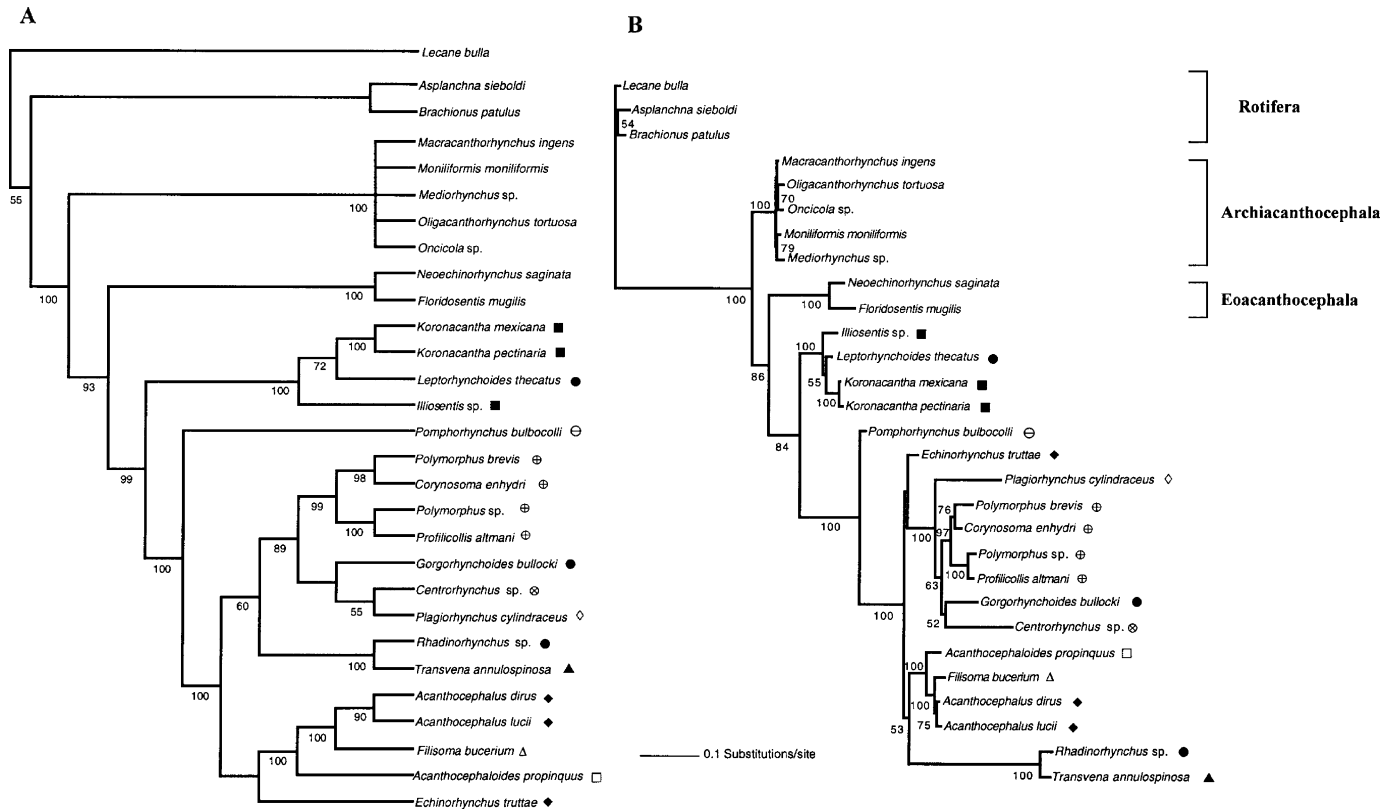


FIGURE 1. Trees recovered from analyses of the combined SSU + LSU rDNA data set. {(A)} Strict consensus of 2 equally parsimonious trees (4,212 steps) inferred from heuristic MP analysis. Numbers below internal nodes show MP bootstrap clade frequencies. {(B)} Maximum likelihood tree (-ln likelihood 24,432.37) obtained from heuristic search with branch lengths scaled to the expected number of substitutions per site. Numbers near internal nodes show ML bootstrap clade frequencies. Palaeacanthocephalan families: Echinorhynchida: (■) Illiosentidae; (●) Rhadinorhynchida; (⊖) Pomphorhynchidae; (▲) Transvenidae; (◆) Echinorhynchidae; (△) Cavisomidae; (□) Arhythmacanthidae. Polymorphida: (◇) Plagiorrhynchidae; (⊗) Centrorhynchidae; (⊕) Polymorphidae.

strong bootstrap support. For example, *Echinorhynchus* has quite different associations in MP and ML trees; however, neither ML nor MP bootstrap trees were resolved for the node involving *Echinorhynchus*. Other taxa with poorly supported and shifting associations between MP and ML trees included the sister-group association of the *Rhadinorhynchus* plus *Transvena* clade, and the positions of *Centrorhynchus*, *Gorgorhynchoides*, and *Plagiorrhynchus*.

A separate MP analysis of the unfiltered (unreliably aligned sites not excluded) combined rDNA data (4,922 characters, 2,260 parsimony informative sites) yielded 1 tree of 10,012 steps (C.I. = 0.50). There were few differences between this MP tree and the strict consensus of MP trees from the filtered data set (Fig. 1A). These differences included increased resolution within the Archiacanthocephala, (*Mediorhynchus* sp., (*O. tortuosa*, (*M. moniliformis*, (*M. ingens*, *Oncicola* sp.))), for the unfiltered MP tree. In addition, part of a clade of palaeacanthocephalans was different in the unfiltered MP tree: (*P. cylindraceus*, ((*Centrorhynchus* sp., *G. bullocki*), ((*P. brevis*, *C. enhydri*), (*Polymorphus* sp., *P. altmani*))). All clades receiving ≥90% support in MP bootstrap analysis of the filtered data set also received ≥90% bootstrap support in analysis of the unfiltered data. One clade that was unique to the MP tree for unfiltered data also received ≥90% bootstrap support: (*O. tortuosa*, *M. moniliformis*, *M. ingens*, *Oncicola* sp.).

SSU data set

Most previous analyses of acanthocephalan relationships have been based exclusively on SSU rDNA. Parsimony analysis of this SSU data set yielded 36 trees with a C.I. = 0.61 and a length of 1,554 steps. The MP strict-consensus tree (Fig. 2A) supported monophyly for all 3 included classes, with the Palaeacanthocephala clade supported with a bootstrap frequency of 81%. Like for combined analysis of rDNA, the SSU trees (Fig. 2A, B) yielded a paraphyletic Echinorhynchida, with Polymorphida plus *Gorgorhynchoides* nested within families representing Echinorhynchida. The MP tree included 2 monophyletic families, Illiosentidae (81% in MP bootstrap) and Polymorphidae (93% in MP bootstrap); none of the other nontrivial families was monophyletic. Resolution for Palaeacanthocephala in the MP strict consensus was slightly reduced relative to the combined rDNA analysis; however, clades that included members from different families received moderate to high bootstrap support. In many cases, group membership for clades containing members of different families was the same as in the combined rDNA analyses. One marked, but well-supported, conflict between the SSU and combined rDNA analyses involved the relationship of *Pomphorhynchus*. This genus was sister to Illiosentidae, plus *Leptorhynchoides* by both MP and ML in the SSU tree. In the combined rDNA tree, *Pomphorhynchus* was

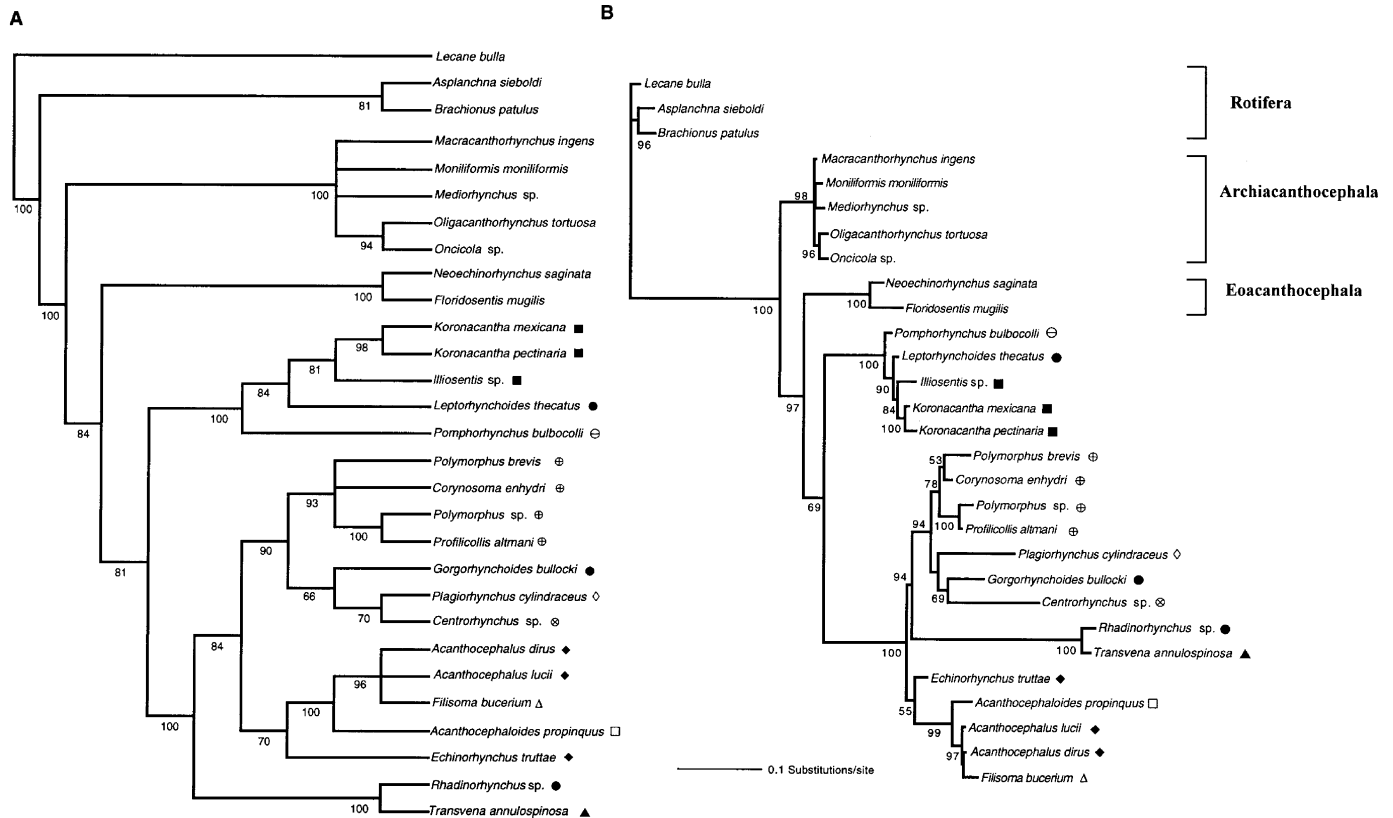


FIGURE 2. Trees recovered from analyses of the SSU rDNA data set. {(A)} Strict consensus of 36 equally parsimonious trees (1,554 steps) inferred from heuristic MP analysis. Numbers below internal nodes show MP bootstrap clade frequencies. {(B)} Maximum likelihood tree ( $-\ln$  likelihood 9,475.26) obtained from heuristic search with branch lengths scaled to the expected number of substitutions per site. Numbers near internal nodes show ML bootstrap clade frequencies. Palaeacanthocephalan families: Echinorhynchida: (■) Illiosentidae; (●) Rhadinorhynchidae; (⊖) Pomphorhynchidae; (▲) Transvenidae; (◆) Echinorhynchidae; (△) Cavisomidae; (□) Arhythmacanthidae. Polymorphida: (◇) Plagiorhynchidae; (⊗) Centrorhynchidae; (⊕) Polymorphidae.

sister to Palaeacanthocephala, excepting Illiosentidae plus *Lep-torhynchoides*. These conflicting topologies were each strongly supported by their respective bootstrap analyses. Similarly, in the SSU MP tree, the position of the clade (*Rhadinorhynchus*, *Transvena*) is moderately well supported, but differs from the combined rDNA topology.

Maximum likelihood analysis of the SSU data set yielded a single tree (Fig. 2B) that has considerable similarity to the MP SSU tree. Clades receiving strong bootstrap support in the MP analysis also usually had high support in the ML bootstrap analysis. The few topological differences between these trees involved species with very short branches as inferred by ML or low bootstrap support, e.g., *Plagiorhynchus*, *Centrorhynchus*, and *Gorgorhynchoides*. One conflict between MP and ML trees was that the clade (*Rhadinorhynchus*, *Transvena*), which was supported by a long branch in the ML tree, was strongly supported (by bootstrap) as the sister group to different sets of taxa in the 2 analyses.

#### LSU data set

Maximum parsimony analysis of the LSU data set yielded a single tree with a C.I. = 0.56 and a length of 2,546 steps (Fig. 3A). The topology of the MP tree inferred from LSU sequences depicts a paraphyletic Palaeacanthocephala, but the resulting clade of Archiacanthocephala plus selected Palaeacanthocephala

has very low MP bootstrap support. Clades with high bootstrap values in the LSU MP tree were often also found in the SSU and combined rDNA analyses. In general, the MP bootstrap consensus tree from the LSU data set had less resolution and lower bootstrap values than did trees inferred for SSU or combined rDNA. Maximum likelihood analysis of the LSU data set yielded a tree with a monophyletic Palaeacanthocephala, but with low bootstrap support (Fig. 3B). In the ML tree, Echinorhynchida was paraphyletic, and Polymorphida plus *Gorgorhynchoides* was monophyletic and nested within families of the Echinorhynchida as found previously for combined and SSU rDNA analyses. Like other rDNA analyses, trees inferred from LSU rDNA did not support monophyly of most palaeacanthocephalan families.

#### DISCUSSION

Previous phylogenetic hypotheses based on SSU rDNA have demonstrated that this gene is informative for inferring acanthocephalan relationships, and most previous molecular systematic studies of acanthocephalans have supported traditional higher-level classifications of the group, recovering clades consistent with the 4 classes (Near et al., 1998; García-Varela et al., 2000, 2002; Near, 2002). However, a more recent analysis of partial SSU sequences depicted Palaeacanthocephala as paraphyletic (Herlyn et al., 2003). This finding is at odds with

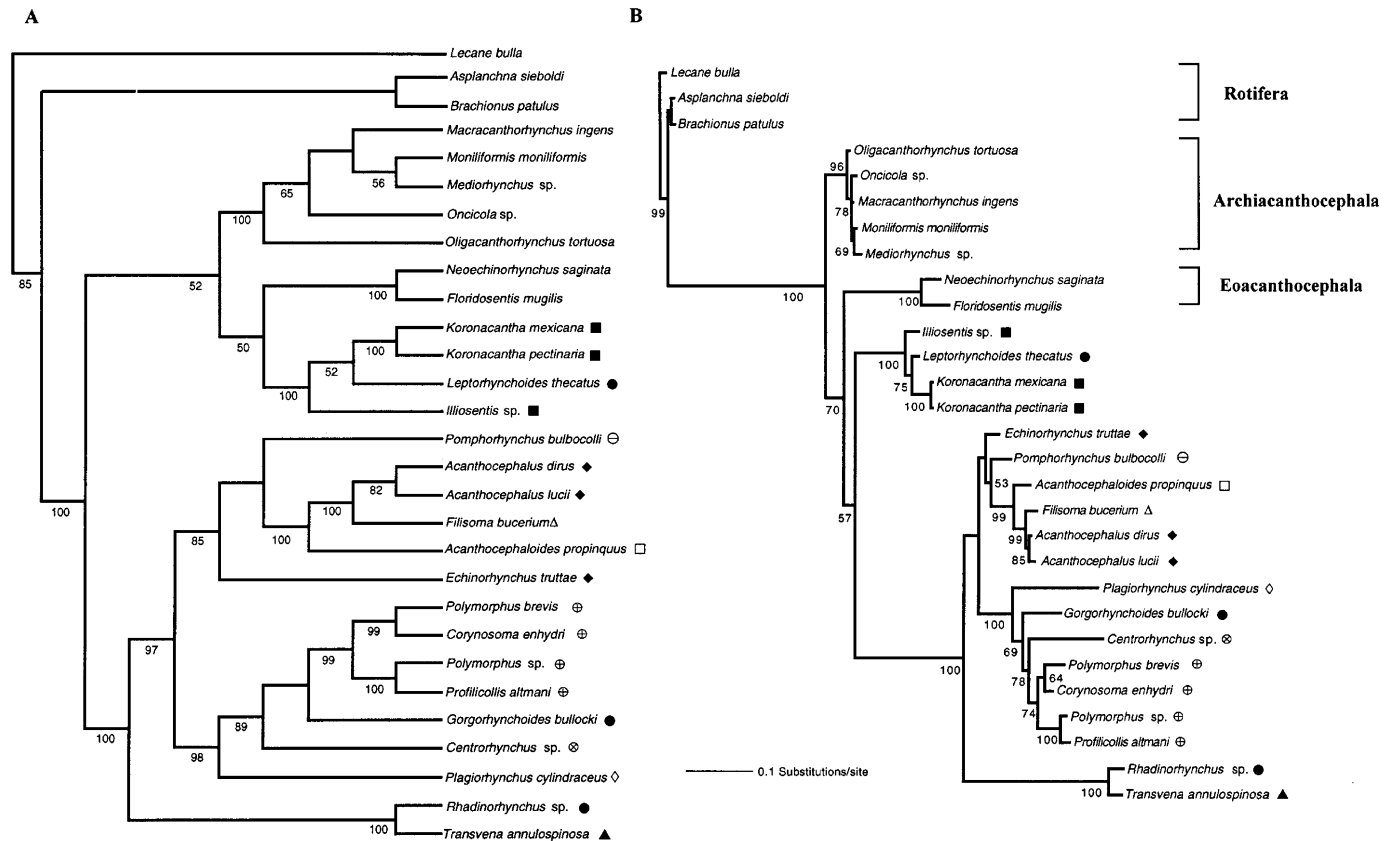


FIGURE 3. Trees recovered from analyses of the LSU rDNA data set. {(A)} Most parsimonious tree (2,546 steps) inferred from heuristic MP analysis. Numbers below internal nodes show MP bootstrap clade frequencies. {(B)} Maximum likelihood tree (-ln likelihood 14,541.74) obtained from heuristic search with branch lengths scaled to the expected number of substitutions per site. Numbers near internal nodes show ML bootstrap clade frequencies. Palaeacanthocephalan families: Echinorhynchida: (■) Illiosentidae; (●) Rhadinorhynchidae; (⊖) Pomphorhynchidae; (▲) Transvenidae; (◆) Echinorhynchidae; (△) Cavisomidae; (□) Arhythmacanthidae. Polymorphida: (◇) Plagiorhynchidae; (⊗) Centrorhynchidae; (⊕) Polymorphidae.

previously published molecular (Near et al., 1998; García-Var-ela et al., 2000, 2002; Near, 2002) and morphological (Monks, 2001) phylogenetic analyses. Nevertheless, the suggestion of a nonmonophyletic Palaeacanthocephala warrants additional investigation because this class includes genera with the most diverse life cycles and morphological features among acanthocephalans (Amin, 1987; Nickol et al., 1999). In addition, previously published molecular phylogenies have been based on few species and 1 gene, offering little opportunity to determine if other taxonomic groups such as families or genera are monophyletic. In the present investigation, the emphasis was on increasing the diversity of palaeacanthocephalan species represented in phylogenetic hypotheses, ending reliance on sequences from a single gene, and testing the monophyly of the class and, to some extent, constituent families.

Although phylogenetic hypotheses were inferred for each gene separately to assess their respective utility, the phylogenetic hypothesis of choice for interpretation of evolutionary history is that based on all available (combined) rDNA data (Fig. 1A, B). This philosophical approach to phylogenetic analysis, termed combined or “total evidence” (Eernisse and Kluge, 1993; Kluge, 1998), argues that the best estimate of evolutionary history is obtained by maximizing the explanatory power of all the available data. Combining data from different genes

without first testing for incongruence can result in decreased phylogenetic resolution if there is substantial conflict between the underlying data sets. However, in the case of SSU and LSU rDNA, these genes represent products of a single transcription unit (locus) with one underlying history that clearly warrants combined analysis. In addition, trees inferred from the combined rDNA were highly resolved and included clades that typically had high bootstrap support. Thus, results from the combined rDNA analysis are emphasized for interpreting the evolutionary history of these palaeacanthocephalan species.

Phylogenetic hypotheses based on combined analysis of rDNA provided strong support for the monophyly of the Palaeacanthocephala, and this result was recovered irrespective of the presence of alignment ambiguous sites in the data set. Monophyly of the Palaeacanthocephala was supported by MP and ML tree inference, and the palaeacanthocephalan clade was strongly supported by bootstrap analyses. Analysis of near-complete SSU sequences alone also provided strong support for the Palaeacanthocephala clade (by both MP and ML); only analysis of LSU sequences provided weak support for monophyly (ML), or yielded a paraphyletic Palaeacanthocephala, but with weak support (MP and MP bootstrap analyses). This combined rDNA phylogeny is the most comprehensive data set (species and sequences) yet available to infer palaeacanthocephalan relation-

ships, and the results of these analyses strongly support monophyly for the group. Previously published molecular (SSU) studies (Near et al., 1998; García-Varela et al., 2000, 2002; Near, 2002) and cladistic analysis of morphological characters (Monks, 2001), also support palaeacanthocephalan monophyly. These findings are in disagreement with the results of Herlyn et al. (2003), suggesting that their study, which was based on partial SSU sequences (842 characters), erroneously represented the Palaeacanthocephala as paraphyletic, presumably because too few characters were analyzed.

To compare the hypothesis of a paraphyletic Palaeacanthocephala as suggested by Herlyn et al. (2003) with alternative hypotheses obtained by analysis of SSU + LSU rDNA, a constraint tree with a paraphyletic Palaeacanthocephala was generated with the combined rDNA data set using MP and ML searches to find the best trees consistent with paraphyly. Maximum likelihood and maximum parsimony analyses showed a tree with a  $-\ln = 24,502.76$  and a length of 4,241 steps respectively. Based on both the Kishino and Hasegawa (1989) likelihood test and Templeton's (1993) parsimony test as executed in PAUP\*, the alternative hypothesis of paraphyly is significantly worse ( $P < 0.05$ ) than the best trees shown in Figure 1A and 1B.

An unexpected feature of these rDNA analyses (combined, SSU, and LSU) was the degree to which species belonging to the same palaeacanthocephalan family were not monophyletic. Of the 4 families that were represented by 2 or more species, 3 were not monophyletic. This limited test of family-level systematics suggests that the current taxonomy of the Palaeacanthocephala may have little congruence with evolutionary history. These results lend support to another study showing that some families of Palaeacanthocephala are paraphyletic (Monks, 2001). The only exception in rDNA trees was Polymorphidae (Meyer, 1931), which was strongly supported as monophyletic (MP and ML trees). Members of this family, which are parasites of aquatic birds and mammals, are considered to be a relatively homogenous group of species characterized by having a trunk with spines arranged in characteristic patterns. However, this inference of a Polymorphidae clade must be tempered by the observation that only 3 of 9 described genera were included in this analysis. For 2 of 3 families of Palaeacanthocephala that are not monophyletic based on analyses of rDNA, the results are congruent with cladistic analysis of morphological characters. For example, Monks (2001) reported that 2 genera from the Rhadinorhynchidae, *Leptorhynchoides* and *Rhadinorhynchus*, were paraphyletic. The rDNA analyses included 3 genera from this family, *Leptorhynchoides*, *Rhadinorhynchus*, and *Gorgorhynchoides*, and the combined rDNA (and separate analyses of SSU and LSU rDNA) strongly supported polyphyly of Rhadinorhynchidae. This speciose family, which includes 20 genera parasitizing marine and freshwater fishes (Amin, 1987), has been subject to some controversial taxonomic decisions. In the diagnosis of the family (Travasso, 1923), the extreme elongation of the proboscis and the presence of 8 cement glands were emphasized. Meyer (1932) added 9 genera to the Rhadinorhynchidae, and in a review of the family, Van Cleave and Lincicome (1940) determined that some genera had 4 cement glands, and transferred these to the Gorgorhynchidae. Golvan (1969) defined the Rhadinorhynchidae as having members with 4 cement glands and a trunk with spines. Subsequent workers

placed species in this family having 6 or 8 cement glands and trunks with or without spines (Cable and Linderoth, 1963; Yamaguti, 1963; Amin, 1985). Examples include *Leptorhynchoides*, *Metacanthocephalus*, and *Pseudoleptorhynchoides*, which have 8 cement glands and no trunk spines (Amin, 1985). For phylogenetic analyses of rDNA, species were included with 8 (*Leptorhynchoides*), 4 (*Rhadinorhynchus*), and 6 (*Gorgorhynchoides*) cement glands. *Leptorhynchoides* was recovered as sister to *Koronacantha* (Illiosentidae), *Rhadinorhynchus* as sister to *Transvena* (with 100% bootstrap support), and *Gorgorhynchoides* was nested within Polymorphida. The unexpected position of *Gorgorhynchoides* is consistent with the suggestion of Pichelin and Cribb (2001) that this genus (plus *Golvanorhynchus*) should be removed from Rhadinorhynchidae. Sampling additional genera and species from the Rhadinorhynchidae is needed to determine if species with the same number of cement glands share common ancestry as reflected by molecular phylogenies.

The Illiosentidae was also not strictly monophyletic in analysis of combined rDNA. Only analysis of SSU rDNA yielded trees (MP and ML) representing the 2 genera of Illiosentidae (*Koronacantha*, *Illiosentis*) as a clade. The combined rDNA data set provided strong support for a clade with *Leptorhynchoides thecatus* (Rhadinorhynchidae) nested within *Koronacantha* plus *Illiosentis*. This result reflects findings from cladistic analysis of morphological data (Monks, 2001), indicating that *Leptorhynchoides* is closely related to Illiosentidae. Interestingly, *Illiosentis* was first placed in Rhadinorhynchidae (Van Cleave and Lincicome, 1939); however, Golvan (1960) suggested that there were a combination of defining features (trunk with spines, genital spines in 1 or both sexes of some species, proboscis cylindrical with numerous longitudinal rows of hooks, male with 8 cement glands) that merited recognition of a new family (Illiosentidae), which currently consists of 10 genera that exclusively parasitize marine fish.

The 4 genera comprising Echinorhynchidae were represented in the rDNA trees by *Echinorhynchus* and *Acanthocephalus*. The position of *Echinorhynchus*, which differed between MP and ML trees, was not well supported as assessed by bootstrap resampling. Trees based on rDNA (combined, SSU, LSU) did not recover a monophyletic Echinorhynchidae, and this result is also congruent with the cladistic study of Monks (2001) that depicts this family as paraphyletic. Petrochenko (1956) suggested that the Echinorhynchidae was derived from the Eoacanthocephala (Neoechinorhynchida), and that this family represented the sister group to other palaeacanthocephalans. This hypothesis was supported by cladistic analysis of morphological characters (Monks, 2001), but was not recovered in analyses of rDNA sequences. Although rDNA hypotheses did not reveal Echinorhynchidae to be monophyletic, *Echinorhynchus* and *Acanthocephalus* were consistently recovered within more derived parts of the palaeacanthocephalan clade, whereas there was strong support for Illiosentidae plus *Leptorhynchoides* as the sister group to all other palaeacanthocephalans.

Phylogenetic trees inferred by MP and ML methods for SSU and combined rDNA data sets showed very similar topologies. For MP analyses, the combined rDNA strict-consensus tree had fewer unresolved clades and generally higher bootstrap support. Tree topologies for MP analyses of filtered and unfiltered data sets for combined rDNA data were very similar, and the re-



sulting clades had similar bootstrap values. In ML trees, SSU and combined rDNA analyses had similar resolution, branch lengths, and bootstrap values. In contrast, several of the clades recovered by MP analysis of LSU rDNA were not recovered in the bootstrap majority-rule consensus tree. Although combined analysis of SSU and LSU rDNA data provided the best-resolved phylogenetic hypothesis, SSU sequences alone recovered much of the same topology, and appear to provide useful resolution at this taxonomic level. In addition, although the LSU data set contained more phylogenetically informative sites (781) than did the SSU data set (495), the C.I. of SSU trees were somewhat higher than for LSU trees. Thus, with limited resources, sequencing SSU rDNA would appear preferable to sequencing LSU rDNA for inferring phylogenies for palaeacanthocephalans, and perhaps other acanthocephalan groups.

Having sampled considerable family-level diversity for palaeacanthocephalans and finding strong support for monophyly, attention needs to be focused on a more comprehensive sampling of genera to assess if the parphyly and polyphyly of families is a general pattern for this class. Such a result would not be unexpected if most families have been diagnosed based on unique combinations of characters, rather than shared derived features. Cladistic analysis of morphological characters has also revealed nonmonophyly of palaeacanthocephalan families (Monks, 2001), and more comprehensive morphological phylogenies have the potential to produce hypotheses that can provide a framework for revising the higher-level classification of acanthocephalans. Similarly, molecular phylogenetic hypotheses with more comprehensive taxon sampling can provide the framework for mapping of morphological features that can be used to redefine higher taxonomic groups (such as families) with traditional characters.

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