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Molecular phylogeny of Cicadomorpha (Insecta: Hemiptera: Cicadoidea, Cercopoidea and Membracoidea): adding evidence to the controversy

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Abstract. The hemipteran infraorder Cicadomorpha comprises the superfamilies Cicadoidea (cicadas), Cercopoidea (spittlebugs or froghoppers) and Membracoidea (leafhoppers and treehoppers). Earlier attempts to determine relationships among these three monophyletic lineages using either morphological or molecular data suffered from insufficient sampling (taxonomic and data) and problematic tree rooting, leading to discordant results. Presented here are phylogenetic reconstructions within Cicadomorpha based on DNA nucleotide sequence data from multiple genetic markers (18S rDNA, 28S rDNA, and histone 3) sequenced from representative taxa of Cicadidae, Tettigarctidae, Cercopidae, Aphrophoridae, Clastopteridae, Machaerotidae, Epipygidae, Cicadellidae, Membracidae, Myerslopiidae and Aetalionidae. To test the robustness of the phylogenetic signal, these sequence data were analysed separately and in combination under various alignment parameters using both manual alignment (of both attenuated and full sequences) and alignment via CLUSTAL X. The results demonstrate clearly that, despite the alignment method used, basing a phylogeny on a single gene region is often misleading. Analyses of the combination of datasets support the major relationships within Cicadomorpha as (Membracoidea (Cicadoidea, Cercopoidea)). Internal relationships recovered within each superfamily shows evidence for: (1) the placement of Myerslopiidae as the sister group of the remaining Membracoidea; (2) the paraphyly of Cicadellidae; (3) the sister-group relationship between Machaerotidae and Clastopteridae; (4) the monophyly of Cercopidae; (5) the diversification of Epipygidae from within the possibly paraphyletic Aphrophoridae.

Introduction

Among the most biodiverse lineages of phytophagous insects, the hemipteran infraorder Cicadomorpha comprises the three superfamilies Cicadoidea (cicadas), Cercopoidea (spittlebugs and froghoppers) and Membracoidea (leafhoppers and treehoppers). To date, approximately 30 000 cicadomorphan species have been described (Dietrich, 2002) and currently are classified into twelve extant families: Cicadidae, Tettigarctidae, Cercopidae, Clastopteridae, Machaerotidae, Aphrophoridae, Epipygidae, Membracidae, Aetalionidae, Melizoderidae, Myerslopiidae and Cicadellidae. The monophyly of Cicadomorpha, and of each cicadomorphan superfamily, is well supported by morphological evidence (Evans, 1963; Blocker, 1996; Hamilton, 1999), and is undisputed.

The superfamily Cicadoidea includes approximately 1200 described species classified into two families, Cicadidae and Tettigarctidae (the latter of which has only two extant, relictual species occurring in mainland Australia and Tasmania). Cicadas are associated with xylem feeding on roots, a relatively unusual practice among phytophagous insects. Typically, they have complex sound production (in males only, except for Tettigarctidae) and reception organs, called tymbals and tympana, respectively. Although eggs are laid in branches or stems of host plants, nymphs drop to the ground and burrow into the soil soon after hatching, and

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prolonged nymphal development (lasting from a few years to 17 years) takes place underground.

Members of Cercopoidea (including the families Cercopidae, Aphrophoridae, Clastopteridae, Machaerotidae and Epipygidae) are known commonly as froghoppers or spittlebugs, the latter name due to the nymphal habit of covering themselves with a frothy salivalike mass composed of tiny air bubbles trapped in plant fluids discharged from the insect alimentary system and supplemented by mucopolysaccharides and proteins produced by the specialized Malpighian tubules of the immatures (Rakitov, 2002). Most species of Cercopidae complete nymphal development within spittle masses on roots at or below ground level (similar to cicadas), whereas most nymphs of Aphrophoridae and all nymphs of Clastopteridae create spittle masses on plant structures above ground; nymphs of Machaerotidae are generally found on their host plants immersed in fluid within 'calcareous' tubes formed from excretory products, and produce spittle masses when out of the tubes during moulting. Like cicadas, spittlebugs feed on fluid contained in xylem tissue and many species (excluding Machaerotidae) exhibit a strong preference for nitrogen-fixing plants (Thompson, 1994); furthermore, different cercopoid families seem to favour different nitrogen-fixing plant groups (Thompson, 1999).

With approximately 25 000 described species, Membracoidea (including the treehopper families Membracidae, Aetalionidae and Melizoderidae, and the leafhopper families Cicadellidae and Myerslopiidae) is easily the largest of the cicadomorphan superfamilies (Deitz & Dietrich, 1993; McKamey, 1998; Dietrich *et al.*, 2001b). Although xylem feeding is retained in cicadelline leafhoppers, most extant species of Membracoidea are phloem (or, less commonly, parenchyma) feeders. Membracoid nymphs are free-living and mostly feed on above-ground host plant structures (relatively few species feed on roots, but neither deep in the soil nor within spittle masses), and, at least ancestrally, can jump (this behaviour was apparently secondarily lost in several membracoid lineages; Dietrich *et al.*, 2001b). Many treehopper species (but only a relatively few leafhopper species) form aggregations of nymphs or nymphs and adults, probably to facilitate predation avoidance. Dietrich (2002) summarized many of the morphological modifications of Membracoidea that distinguished them from other superfamilies of Cicadomorpha.

Previous studies have used morphological data, molecular data, or both, to examine the relationships within cicadomorphan lineages, including Membracoidea (Dietrich *et al.*, 2001b), Membracidae (Dietrich & Deitz, 1993; Cryan *et al.*, 2000, 2004; Dietrich *et al.*, 2001a), and certain cicada groups (Buckley *et al.*, 2001; Duffels & Turner, 2002). Other studies using either morphological or molecular data have attempted to determine higher 'hemipteran' relationships, and thus have included representatives of the three superfamilies of Cicadomorpha. Nevertheless, confounding issues, such as insufficient sampling (taxonomic and data) and problematic tree rooting, have led to discordance among results. Figure 1 illustrates alternative phylogenetic hypotheses within Cicadomorpha, whether Cicadoidea is sister to Cercopoidea + Membracoidea (Fig. 1A; Hamilton, 1981; Sorensen *et al.*, 1995; von Dohlen & Moran, 1995), Membracoidea is sister to Cicadoidea + Cercopoidea (Fig. 1B; Boulard, 1988; Campbell *et al.*, 1995; Ouvrard *et al.*, 2000; Bourgoin & Campbell, 2002; Dietrich, 2002), or Cercopoidea is sister to Cicadoidea + Membracoidea (Evans, 1963; Hamilton, 1996, 1999). The goals of this study were to elucidate the relationships among the three cicadomorphan superfamilies and their constituent families using DNA sequence data from multiple nuclear genes.

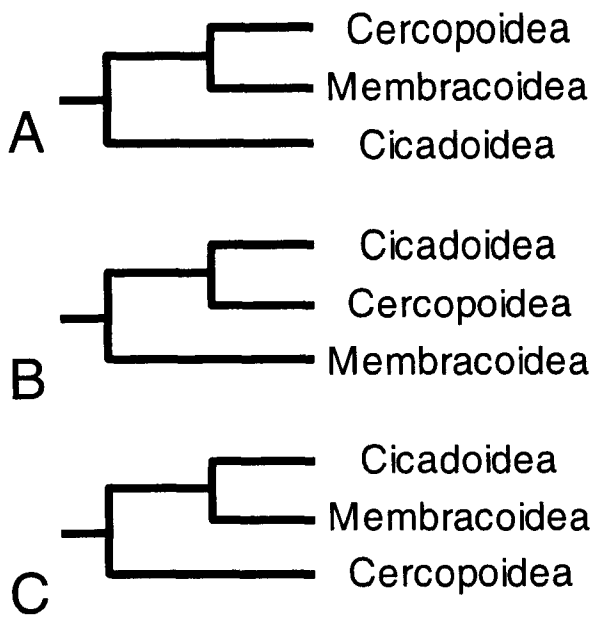

Fig. 1. Alternative hypotheses for the phylogeny of Cicadomorpha. A, (Cicadoidea (Cercopoidea + Membracoidea)) (Hamilton, 1981; Sorensen *et al.*, 1995; von Dohlen & Moran, 1995; Blocker, 1996); B, (Membracoidea (Cicadoidea + Cercopoidea)) (Boulard, 1988; Campbell *et al.*, 1995; Ouvrard *et al.*, 2000; Bourgoin & Campbell, 2002; Dietrich, 2002); C, (Cercopoidea (Cicadoidea + Membracoidea)) (Evans, 1963; Hamilton, 1996, 1999).

Materials and methods

Taxon sampling

Insect specimens, originating from both New World and Old World regions (Table 1), were collected into 95–100% ethanol and stored at −80 °C. Included in these analyses were exemplars of twenty-two Cercopoidea species, ten Membracoidea species and four Cicadoidea species (Table 1), representing all of the currently recognized cicadomorphan families except Melizoderidae. Also included,

Table 1. Taxa included in the 18S, 28S and histone 3 (H3) nucleotide sequence datasets. Classification follows Metcalf (1960, 1961, 1962a, b), McKamey (1998) and Hamilton (2001; for Epipygidae only).

Taxon	Voucher code[a]	Geographical source	GenBank accession number		
			18S	28S	H3
Cercopoidea: Aphrophoridae					
Aphrophora alni (Fallén)	01-07-15-14	U.S.A. (NY)	AY744783	AY744817	AY744855
Aphrophora parallela Say	01-07-15-21	U.S.A. (NY)	AY744785	AY744819	AY744857
Cephisus siccifolius Walker	03-01-15-33	Costa Rica	AY744799	AY744833	AY744871
Lepyronia coleoptrata Linnaeus	01-07-15-11	U.S.A. (NY)	AY744782	AY744816	AY744854
Lepyronia quadrangularis Say	01-07-18-51	U.S.A. (NY)	AY744786	AY744820	AY744858
Liorhina sp.	01-07-15-43	Papua New Guinea	AY744788	AY744822	AY744860
Neophilaenus lineatus Linnaeus	01-07-15-03	U.S.A. (VT)	AY744780	AY744814	AY744852
Philaenus maghresignus Drosopoulos & Remane	01-07-15-42	Spain	AY744793	AY744827	AY744865
Philaenus spumarius Linnaeus	01-07-15-01	U.S.A. (VT)	AY744779	AY744813	AY744851
Cercopoidea: Cercopidae					
Aeneolamia contigua (Walker)	01-07-18-73	Costa Rica	AY744794	AY744828	AY744866
Cosmoscarta sp.	01-07-15-41	Papua New Guinea	AY744787	AY744821	AY744859
Mahanarva costaricensis (Distant)	03-01-15-32	Costa Rica	AY744798	AY744832	AY744870
Prosapia bicincta (Say)	01-07-15-28	U.S.A. (GA)	AY744789	AY744823	AY744861
Zulia vilior (Fowler)	01-07-18-36	Costa Rica	AY744791	AY744825	AY744863
Cercopoidea: Clastopteridae					
Clastoptera brunnea Ball	01-07-18-58	U.S.A. (UT)	AY744790	AY744824	AY744862
Clastoptera obtusa Say	01-07-15-15	U.S.A. (NY)	AY744784	AY744818	AY744856
Clastoptera proteus Fitch	01-07-15-09	U.S.A. (NY)	AY744781	AY744815	AY744853
Clastoptera testacea Fitch	01-07-18-59	U.S.A. (WV)	AY744792	AY744826	AY744864
Cercopoidea: Epipygidae					
Epipyga **n.sp.** 'a'	NYSM CER89	Peru	AY744795	AY744829	AY744867
Evexus **n.sp.** 'r'	NYSM CER90	Peru	AY744796	AY744830	AY744868
Evexus **n.sp.** 'e'	NYSM CER91	Peru	AY744797	AY744831	AY744869
Cercopoidea: Machaerotidae					
Pectinariophyes reticulata (Spångberg)	CHD AH9	Australia	AY744778	AY744812	AY744850
Cicadoidea: Cicadidae					
Froggattoides typicus Distant	CS 02.QLD.ANT.5	Australia	AY744800	AY744834	AY744872
Pauropsalta corticinus Ewart	CS 02.QLD.BBR.4	Australia	AY744801	AY744835	AY744873
Cicadoidea: Tettigarctidae					
Tettigarcta crinita Distant	CS 97–3	Australia	AY744802	AY744836	AY744874
Tettigarcta tomentosa White	CS 00–09	Australia	AY744803	AY744837	AY744875
Membracoidea: Aetalionidae					
Aetalion reticulatum (Linnaeus)	01-08-09-69	Venezuela	AY744777	AY744809	AY744849
Gerridius fowleri (Haviland)	97-02-19-46[b]	Guyana	AY498432	AY744843	AY744881
Lophyraspis sp.	95-05-12-96[b]	Costa Rica	AY498438	AY744846	AY744884
Membracoidea: Cicadellidae					
Flexamia areolata (Ball)	CHD LH38	U.S.A. (VA)	AY498437	AY744845	AY744883
Putoniessa rivularis (Walker)	CHD AH3	Australia	AY498406	AY744810	–
Paracephaleus brunneus (Waterhouse)	CHD AH6	Australia	AY498407	AY744811	–
Membracoidea: Membracidae					
Deiroderes inermis Ramos	CRB Din	Tortola[c]	AY498425	AY744841	AY744879
Guayaquila gracilicornis (Stål)	NCSU 95-05-12-40	Costa Rica	AY498433	AY744844	AY744882
Ophiderma definita Woodruff	NCSU 95-02-01-60	U.S.A. (MD)	AY498447	AY744847	AY744885
Membracoidea: Myerslopiidae					
Pemmation aspera (Knight)	–	New Zealand	–	AF304575	–
Outgroups					
Fulgoroidea					
Cixiidae: *Pintalia alta* Osborn	02-06-17-11	St. John[d]	AY744804	AY744838	AY744876

Table 1. Continued

Taxon	Voucher code[a]	Geographical source	GenBank accession number 18S	28S	H3
Delphacidae: *Nothodelphax gillettei* (VD)	01-07-24-15	U.S.A. (UT)	AY744805	AY744839	AY744877
Dictyopharidae: *Scolops* sp.	02-06-17-07	U.S.A. (NY)	AY744806	AY744840	AY744878
Fulgoridae: *Neolieftinckana* sp.	02-06-17-38	Papua New Guinea	AY744807	AY744842	AY744880
Tropiduchidae: *Tangia viridis* (Walker)	02-06-17-09	St. John[d]	AY744808	AY744848	AY744886

[a]New York State Museum Genome Bank, −80 °C.
[b]Voucher codes from North Carolina State University Genome Bank.
[c]British Virgin Islands.
[d]U.S. Virgin Islands.

as outgroup taxa, were exemplars of five Fulgoroidea species (Table 1).

Nucleotide sampling and laboratory procedures

Genomic nucleic acids were isolated from preserved tissue using FastDNA extraction kits (Qbiogene Inc., Carlsbad, California, U.S.A.) and were stored at −80 °C in purified water. Intact portions of the specimens were given voucher numbers (Table 1) and are currently stored at −80 °C in 95% ethanol in the Laboratory for Conservation and Evolutionary Genetics at the New York State Museum.

Oligonucleotide primers (Table 2), some newly designed (i.e. Cicadomorpha-specific), were used to amplify desired gene regions via standard polymerase chain reaction (PCR) using either AmpliTaq® DNA polymerase (PE Applied Biosystems, Foster City, California, U.S.A.) or Isis™ DNA polymerase (Qbiogene). Partial regions of the protein-coding gene histone 3 (H3) were amplified and sequenced in single fragments (using primer pair AF–AR, Table 2). The small ribosomal subunit, 18S rDNA (18S), was amplified and sequenced in three contiguous regions (using primer pairs 1F–delR1 or 1F–b3.9, a0.7–bi, and a2.0–9R; Table 2). A fragment of the large ribosomal subunit, 28S rDNA (28S), was also amplified and sequenced in three contiguous regions (using primer pairs EE–MM, Lalt–Galt, and V–X; Table 2). All PCR experiments included negative controls to detect possible contamination.

Double-stranded PCR amplification products were visualized on 1–2% agarose gels, purified using Geneclean III DNA purification kits (Bio 101, Vista, California, U.S.A.), and directly sequenced with Big Dye Version 3 Cycle Sequencing Ready Reaction with AmpliTaqFS DNA polymerase (PE Applied Biosystems). Sequences were fractionated by polymer capillary electrophoresis on either a Prism 3700 DNA analyser or a Prism 3100 genetic analyser (PE Applied Biosystems). Sequence confirmation was accomplished by comparing complimentary DNA strands.

Table 2. Oligonucleotide primer sequences used for the polymerase chain reaction amplification of 18S, 28S and histone 3 (H3) from Cicadomorpha and outgroups.

Primer	Sequence (5′→3′)	Primer source
18S 1F	TACCTGGTTGATCCTGCCAGTAG	Cryan *et al.* (2004)
18S delR1	AATTTGTTCAAAGTAAACGTGCCGG	New; designed for Cicadomorpha and Fulgoromorpha
18S b3.9	TGCTTTRAGCACTCTAA	Whiting *et al.* (1997)
18S a0.7	ATTAAAGTTGTTGCGGTT	Whiting *et al.* (1997)
18S bi	GAGTCTCGTTCGTTATCGGA	Whiting *et al.* (1997)
18S a2.0	ATGGTTGCAAAGCTGAAAC	Whiting *et al.* (1997)
18S 9R	GATCCTTCCGCAGGTTCACCTAC	Whiting (2002a)
28S EE	CCGCTAAGGAGTGTGTAA	Hillis & Dixon (1991); Cryan *et al.* (2000)
28S MM	GAAGTTACGGATCTARTTTG	Hillis & Dixon (1991); Cryan *et al.* (2000)
28S Lalt	CCTCGGACCTTGAAAATCC	Dietrich *et al.* (2001b), as 'fragment IV, forward'
28S Galt	TGTCTCCTTACAGTGCCAGA	Dietrich *et al.* (2001b), as 'fragment IV, reverse'
28S V	GTAGCCAAATGCCTCGTCA	Hillis & Dixon (1991); Cryan *et al.* (2000)
28S X	CACAATGATAGGAAGAGCC	Hillis & Dixon (1991); Cryan *et al.* (2000)
H3 AF	ATGGCTCGTACCAAGCAGACVGC	Colgan *et al.* (1998)
H3 AR	ATATCCTTRGGCATRATRGTGAC	Colgan *et al.* (1998)

Sequence alignment

Editing nucleotide sequences, contiguous sequence assembly, consensus sequence calculation and alignment of consensus sequences were performed using the software programs SEQUENCHER™ 4.0.5 (Gene Codes Corp., Ann Arbor, Michigan, U.S.A.) for PC and CLUSTAL X (Thompson *et al.*, 1997). Complete nucleotide sequences are available in GenBank under the accession numbers listed in Table 1, and aligned datasets are available both on TreeBASE (study accession number S1164 and matrix accession number M2001) and at the following website: http://www.nysm.nysed.gov/lceg.

The alignment of H3 sequences was unambiguous due to the functional codon constraint to which this gene is subject. Manual alignment of ribosomal sequences resulted in the identification of one region of ambiguous alignment for 18S and three regions of ambiguous alignment for 28S (differing in sequence length and base composition across sampled taxa, these 28S regions correspond to the hypervariable portions of the divergent domains D7a–b, D8 and D9–D10 of *Drosophila melanogaster*; Hancock *et al.*, 1988; Dietrich *et al.*, 2001b). Recent studies (Hickson *et al.*, 2000; Ogden & Whiting, 2003) have reiterated the potentially important effects of alignment perturbations on phylogenetic reconstruction, particularly when ribosomal loci are included in the analysis. Therefore, to explore the extent to which these data are sensitive to alignment perturbations, multiple sequence alignments were constructed using different methods: (1) manual alignment of sequences, aligning across divergent domains continuously; (2) manual alignment of sequences, blocking divergent domains across monophyletic superfamilies (as in Whiting, 2002b); (3) aligning divergent domains using CLUSTAL X under five 'gap opening : gap extension' cost ratios (specifically 1 : 1, 50 : 1, 50 : 50, 100 : 1 and 100 : 100).

Parsimony analyses

Phylogenetic analyses using the maximum parsimony criterion were performed using the software program PAUP* 4.0 (Swofford, 2001; currently, beta test version b10; also used to calculate data partition statistics and support for nodes in Fig. 2). Heuristic tree searches were performed using 1000 random addition replications with the tree bisection and reconnection option (TBR), and gaps coded as missing data.

Separate and combined data analyses (including all possible data partition combinations) were conducted with the various multiple sequence alignments described above, under the maximum parsimony criterion. Analyses were performed with gaps treated both as missing data and as a fifth character state; also, heuristic searches were performed both unconstrained and with superfamilies constrained as monophyletic.

Support for individual nodes on the resulting topologies was assessed by calculation of partitioned Bremer support values (= decay index; Baker & DeSalle, 1997; computed using the computer program TREEROT.v2; Sorenson, 1999) and by nonparametric bootstrap analysis (1000 replicates).

Results

DNA sequence editing and alignment resulted in an 18S rDNA partition of approximately 1900 characters (the exact number depended on the alignment methodology used), a 28S rDNA partition of approximately 2200 characters, and a H3 partition of 333 characters (Table 3 lists the descriptive statistics for each data partition). Despite the possible presence of multiple copies of H3 in insect genomes, no ambiguity was detected in these sequencing results, in concordance with the results observed by Colgan *et al.* (1998). Of the 119 parsimony-informative sites in the protein-coding gene H3, the following character distributions were observed: nt1 = 17 (14.3%), nt2 = 0, nt3 = 102 (85.7%).

The use of eight different alignment strategies, analysed with two gap treatments (as missing data and as a fifth character state) and two constraint options (free and with superfamilies constrained as monophyletic), for seven data partitions (18S, 28S, H3, 18S + 28S, 18S + H3, 28S + H3, and 18S + 28S + H3) resulted in 212 individual character matrices. Topological results from unweighted parsimony analyses of these matrices, with regard to relationships among cicadomorphan superfamilies, are summarized in Table 4. Trends in the results suggest that the 18S data partition (although equivocal in many cases) weakly supported Cercopoidea as the sister to Membracoidea + Cicadoidea, whereas the 28S data partition supported, in most analyses, Membracoidea as the sister to Cicadoidea + Cercopoidea. The H3 data partition, analysed separately, was unable to resolve cicadomorphan relationships unequivocally. When all three data partitions were combined for analysis, the results almost always supported Membracoidea as the sister to Cicadoidea + Cercopoidea.

Although all three possible hypotheses of relationships among cicadomorphan superfamilies (Fig. 1A–C) were supported under some analytical parameters, the majority of analyses (including analyses of separate partitions and those of all possible data combinations) supported hypothesis B, that Membracoidea is the sister to Cicadoidea + Cercopoidea. Reflecting this, the single most-parsimonious topology (length = 4093; consistency index = 0.626; retention index = 0.706) resulting from unweighted parsimony analysis of the combined dataset (including all data partitions) is shown in Fig. 2, with bootstrap and Bremer support values corresponding to the nodes of the tree listed in Table 5.

Based on the topology shown in Fig. 2, and of the topologies generated through most analyses performed, the following phylogenetic results are asserted: (1) the three superfamilies within Cicadomorpha are monophyletic, in agreement with morphological evidence; (2) Membracoidea is the sister to Cercopoidea + Cicadoidea, in agreement with

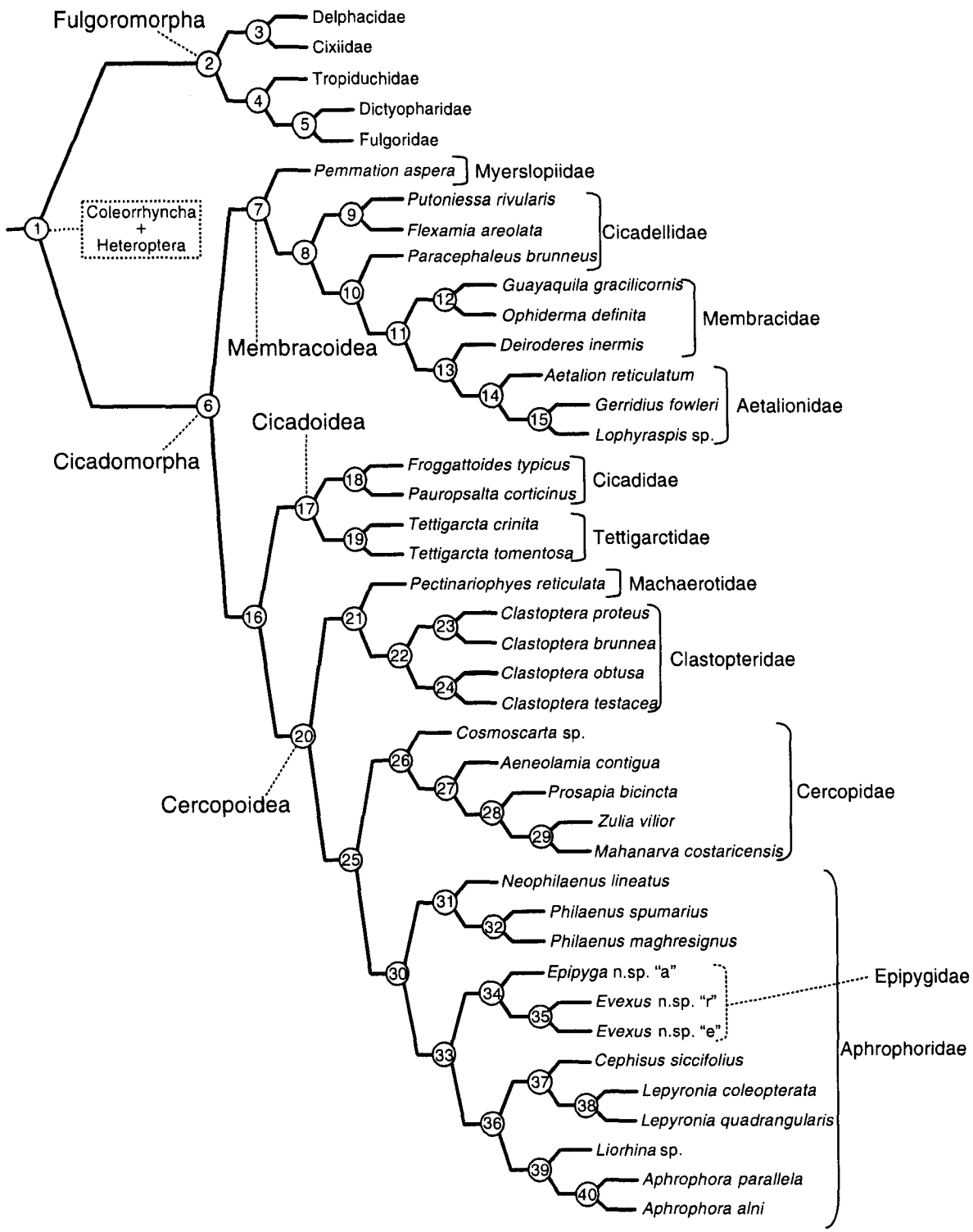

Fig. 2. The single most-parsimonious topology (length = 4093; consistency index = 0.626; retention index = 0.706) resulting from unconstrained and unweighted parsimony analysis of 18S + 28S + histone 3 (H3) using all nucleotide characters in a blocked alignment. The tree is rooted to Fulgoromorpha. However, the unknown position of (Coleorhyncha + Heteroptera) (not included in these analyses) is indicated as a basal polytomy.

Table 3. Descriptive statistics for data partitions.

Data partition	18S	28S	Histone 3	Combined
Alignment length (bp)[a]	1900	2197	333	4430
Number of variable sites/percentage data partition	369/19.4	839/38.2	133/39.9	1342/30.3
Number of informative sites/percentage data partition[b]	300/15.8	661/30.1	119/35.7	1082/24.4
Percentage A	24.3	21.7	23.7	23.0
Percentage C	24.1	25.2	30.7	25.1
Percentage G	27.7	31.8	29.0	29.8
Percentage T	24.0	21.3	16.6	22.1
Percentage sequence divergence	0–9	1–16	1–23	1–14

[a]Based on manual, continuous (unblocked) alignment.
[b]Based on the maximum parsimony criterion.

most recent studies based on morphology (including studies incorporating fossils); (3) Myerslopiidae is the sister to the rest of Membracoidea, in agreement with Hamilton (1999); (4) Cicadellidae is paraphyletic with respect to Membracidae + Aetalionidae, in agreement with Dietrich *et al.* (2001b); (5) Machaerotidae + Clastopteridae is a monophyletic group, in agreement with Hamilton (2001); (6) Epipygidae is derived from Aphrophoridae, in disagreement with Hamilton (2001).

Discussion

The outgroups used for these analyses belong to the hemipteran infraorder Fulgoromorpha. Yoshizawa & Saigusa (2001) examined the forewing base structure of the major paraneopteran lineages, concluding that Fulgoromorpha and Cicadomorpha are sister taxa based on the autapomorphic reduction of the proximal median plate, a character that they claim is prominent and not observed in other insect groups (although they acknowledge that reductive features are not as desirable for inferring relationships as modifications with increased complexity). However, because relationships among Fulgoromorpha, Cicadomorpha and Coleorrhyncha + Heteroptera remain contentious (Campbell *et al.*, 1995; Sorensen *et al.*, 1995; von Dohlen & Moran, 1995; Ouvrard *et al.*, 2000; Bourgoin & Campbell, 2002), the use of this outgroup lineage alone might seem inappropriate. However, parsimony analyses of the present datasets including also nonhemipteran taxa representing the neopteran insect orders Orthoptera and Blattodea (sequences taken from Ogden & Whiting, 2003) resulted in similar topologies (results not shown). Therefore, the polarity of characters in the present results is taken as correct and the use of Fulgoromorpha as the outgroup is justified. The present analyses did not test the hypothesis that Fulgoromorpha and Cicadomorpha form a monophyletic lineage (i.e. Auchenorrhyncha); the unresolved position of Coleorhyncha + Heteroptera is indicated in Fig. 2 with dotted lines. Therefore, it is possible (although seemingly unlikely) that the results of the present research were biased by the lack of outgroups representing the other major lineages of Hemiptera (i.e. Coleorhyncha and Heteroptera).

Not surprisingly, these results highlight that hypothesis testing via phylogenetic reconstruction depends upon sound nucleotide sequence alignment methodology, particularly when noncoding (e.g. ribosomal) genes are included for analysis. These results demonstrate also that phylogenetic signal strength increases with the addition of data from multiple sources. Topological variation resulting from perturbation of alignment methodology (Table 4) was relatively high. Of 212 separate analyses performed, approximately 40% (eighty-one analyses) resulted in topologies recovering (Membracoidea (Cicadoidea + Cercopoidea)) (as in Fig. 1B); those eighty-one analyses comprised mostly separate and combined analyses that included the 28S data partition. Approximately 20% (forty analyses) of the results were unresolved, finding equivocal support for (Membracoidea (Cicadoidea + Cercopoidea)). The results of the remaining ($\approx$ 40%) analyses performed were divided almost evenly in support of the alternative hypotheses of cicadomorphan relationships (Fig. 1A–C). All analyses in which all three datasets (18S, 28S and H3) were combined (except for those in which gap extension costs were greater than one) strongly supported (Membracoidea (Cicadoidea + Cercopoidea)); in most of those cases, a single most-parsimonious topology was recovered (as in Fig. 2).

Interestingly, separate analyses of the 18S dataset never recovered (Membracoidea (Cicadoidea + Cercopoidea)). Rather, analysis of the 18S dataset alone resulted in either unresolved topologies or weakly supported alternative relationships (as in Fig. 1A, C). These results are potentially important in explaining the conclusions of previous studies, also based on 18S nucleotide sequence data. von Dohlen & Moran (1995) analysed partial 18S sequences (544 bp from conservative regions only; 185 bp from variable regions were excluded from their analyses), finding weak support for (Cicadoidea (Cercopoidea + Membracoidea)) (as in Fig. 1A). Similarly, Sorensen *et al.* (1995) used partial 18S sequences (nucleotides from variable regions were successively excluded from their analysis), finding equivocal support for (Cicadoidea (Cercopoidea + Membracoidea)). Moreover, that study used *Tenebrio molitor* Linnaeus (Coleoptera: Tenebrionidae; clearly distal to Hemiptera in the phylogeny of Insecta) as the only outgroup, so character polarity might have been a confounding issue. In their

Table 4. Topological results of the sensitivity analyses. The results are summarized with regard to relationships among cicadomorphan superfamilies.

	Gaps = ?[a]		Gaps = char[b]	
Dataset	Free	Constrained	Free	Constrained
Conserved[c]				
18S	Unres	Unres	Cic	Cic
28S	Mem	Mem	Unres	Mem
H3	Unres	Unres	Unres	Unres
18S + 28S	Unres	Unres	Mem	Mem
18S + H3	Unres	Unres	Cic	Cic
28S + H3	Mem	Mem	Mem	Cer
18S + 28S + H3	Mem	Mem	Mem	Mem
Manual, blocked				
18S	Cer	Cer	–	–
28S	Mem	Mem	–	–
H3	Unres	Cer	Unres	Cer
18S + 28S	Cer	Cer	–	–
18S + H3	Cer	Cer	–	–
28S + H3	Cer	Mem	–	–
18S + 28S + H3	Mem	Mem	–	–
Manual, continuous				
18S	Cer	Cer	Cic	Cic
28S	Mem	Mem	Mem	Mem
H3	Unres	Cer	Unres	Cer
18S + 28S	Mem	Mem	Mem	Mem
18S + H3	Cer	Cer	Unres	Unres
28S + H3	Mem	Mem	Mem	Mem
18S + 28S + H3	Mem	Mem	Mem	Mem
CLUSTAL X [1 : 1][d]				
18S	Cer	Cer	Cic	Cic
28S	Mem	Mem	Mem	Mem
H3	Unres	Cer	Unres	Cer
18S + 28S	Mem	Mem	Mem	Mem
18S + H3	Cer	Cer	Cic	Cic
28S + H3	Mem	Mem	Mem	Mem
18S + 28S + H3	Mem	Mem	Mem	Mem
CLUSTAL X [50 : 1][d]				
18S	Cer	Cer	Unres	Unres
28S	Unres	Cic	Cic	Mem
H3	Unres	Cer	Unres	Cer
18S + 28S	Mem	Cic	Mem	Cic
18S + H3	Cer	Cer	Cic	Cic
28S + H3	Mem	Cic	Cic	Mem
18S + 28S + H3	Mem	Unres	Mem	Mem
CLUSTAL X [50 : 50][d]				
18S	Cer	Cer	Cer	Cer
28S	Unres	Mem	Cic	Cic
H3	Unres	Cer	Unres	Cer
18S + 28S	Mem	Cic	Mem	Cic
18S + H3	Cer	Cer	Cer	Cer
28S + H3	Cic	Unres	Cic	Cic
18S + 28S + H3	Mem	Cic	Cic	Cic
CLUSTAL X [100 : 1][d]				
18S	Cer	Cer	Cer	Cer
28S	Unres	Mem	Unres	Mem
H3	Unres	Cer	Unres	Cer
18S + 28S	Mem	Mem	Mem	Mem
18S + H3	Cer	Cer	Cer	Cer
28S + H3	Mem	Mem	Unres	Mem
18S + 28S + H3	Mem	Mem	Mem	Mem

Table 4. Continued

Dataset	Gaps = ?[a]		Gaps = char[b]	
	Free	Constrained	Free	Constrained
CLUSTAL X [100 : 100][d]				
18S	Unres	Unres	Cic	Cic
28S	Cic	Cic	Mem	Mem
H3	Unres	Cer	Unres	Cer
18S + 28S	Cic	Cic	Cic	Mem
18S + H3	Mem	Mem	Unres	Unres
28S + H3	Cic	Cic	Cic	Mem
18S + 28S + H3	Cic	Cic	Cic	Mem

Free, the analysis was unconstrained; constrained, the analysis was constrained to include only topologies consistent with monophyletic superfamilies (relationships among and within superfamilies, however, were unconstrained); H3, histone 3; Cer, analysis supported (Cercopoidea (Cicadoidea, Membracoidea)); Mem, analysis supported (Membracoidea (Cicadoidea, Cercopoidea)); Cic, analysis supported (Cicadoidea (Cercopoidea, Membracoidea)); Unres, analysis yielded unresolved superfamily relationships; –, treating gaps as discreet characters is not justifiable for a blocked alignment.

[a]Alignment gaps treated as missing data.

[b]Alignment gaps treated as characters (e.g. a fifth base).

[c]Conserved dataset for 18S excluded one region of ambiguously aligned nucleotides (approximately fifty nucleotides); for 28S, three regions of ambiguous alignment were excluded (totalling approximately 400 nucleotides); for H3, third position nucleotides were excluded (111 nucleotides).

[d]CLUSTAL X multiple alignment parameters refer to [gap opening cost : gap extension cost]; in all CLUSTAL X alignments, other settings used were: DNA transition weight = 0; delay divergent sequences = 30%; and DNA weight matrix = CLUSTAL W (1.6).

'most conservative and preferred' analysis (Cicadoidea (Cercopoidea + Membracoidea)) was supported by a single, homoplasious synapomorphy (transition from G→A at their site 263; Sorensen *et al.*, 1995).

Other 18S-based studies, however, supported (Membracoidea (Cicadoidea + Cercopoidea)). Campbell *et al.* (1995) analysed partial 18S sequences, including hypervariable regions, finding some support for (Membracoidea (Cicadoidea + Cercopoidea)).

Table 5. Nodal support for Fig. 2. Columns list bootstrap, Bremer and partitioned Bremer support (the contribution of the specified gene to the total Bremer support at the indicated node) as calculated for the single most-parsimonious topology resulting from unconstrained analysis of 18S + 28S + histone 3 (H3) using all nucleotide characters in a blocked alignment (Fig. 2). Bootstrap support values result from 1000 bootstrap analysis replicates.

Node no.	Bootstrap support	Bremer support	Partitioned Bremer			Node no.	Bootstrap support	Bremer support	Partitioned Bremer		
			18S	28S	H3				18S	28S	H3
1	Root node					21	56	3	−7	4	6
2	100	46	0	46	0	22	100	38	9	17	12
3	96	11	7	4	0	23	100	62	25	30	7
4	100	26	6	20	0	24	54	1	1	4	−4
5	100	20	5	−2	17	25	<50	1	2	0	−1
6	100	46	0	46	0	26	57	1	2	0	−1
7	99	8	1	8	−1	27	99	9	6	1	2
8	100	20	−2	22	0	28	73	3	0	−1	4
9	61	2	0	2	0	29	75	2	−1	0	3
10	71	5	−1	6	0	30	52	1	2	0	−1
11	100	27	6	21	0	31	100	34	12	9	13
12	99	12	1	4	7	32	100	11	4	7	0
13	84	7	0	8	−1	33	55	1	−2	0	3
14	98	16	−1	19	−2	34	100	26	0	17	9
15	100	97	37	40	20	35	100	23	1	8	14
16	65	3	−7	4	6	36	<50	1	−2	0	3
17	100	27	0	27	0	37	83	3	0	2	1
18	100	18	13	0	5	38	92	8	3	3	2
19	60	1	6	1	−6	39	<50	1	−2	0	3
20	97	15	19	7	−11	40	100	35	5	17	13
						Total		671	148	401	122
						Percentage		100	22.1	59.7	18.2

When hypervariable regions were excluded from the analysis, however, relationships among the three superfamilies were equivocal. Ouvrard *et al.* (2000) used complete 18S sequences aligned by secondary structure, finding support for (Membracoidea (Cicadoidea + Cercopoidea)). Nevertheless, as with Campbell *et al.* (1995), when regions of ambiguous alignment were excluded from their analyses, relationships among the superfamilies were equivocal. The implication of the results from those studies, then, is that 18S rDNA sequences alone can be misleading (regarding the phylogeny of Cicadomorpha), especially if hypervariable regions are excluded from the analysis; this effect can seemingly be mitigated, however, by secondary structural alignment of 18S sequences (for example, Ouvrard *et al.*, 2000).

Many relationships were consistent throughout the results of the present analyses, regardless of the alignment methodology and parameters used. For example, the monophyly of each cicadomorphan superfamily (Membracoidea, Cicadoidea and Cercopoidea) was supported strongly in nearly all separate and combined analyses. With few exceptions, each family sampled was found to be monophyletic under most analytical parameters. Notable among those exceptions are: (1) Cicadellidae, which was also shown to be paraphyletic by Dietrich *et al.* (2001b); (2) Membracidae, although because this family was found to be monophyletic in previous analyses (Cryan *et al.*, 2000, 2004; Dietrich *et al.*, 2001a, b), this result may be the result of sampling bias; and (3) Aphrophoridae (found to be monophyletic in approximately half of the present analyses), however, Hamilton (2001) recently hypothesized, based on morphological examination, that this spittlebug family is not monophyletic. Within Membracoidea, the results of these analyses supported the hypothesis (Rakitov, 1998; Dietrich *et al.*, 2001b) that the treehoppers (Membracidae and Aetalionidae) are derived from a lineage of leafhoppers (Cicadellidae) that includes the subfamily Ulopinae, which is represented here by *Paracephaleus brunneus* (Waterhouse).

Morphological evidence has been used previously to support alternative hypotheses of cicadomorphan relationships. Evans (1963) hypothesized that because both Cicadoidea and Membracoidea retain different combinations of plesiomorphic characters that are absent in the Cercopoidea, morphological evidence supports (Cercopoidea (Cicadoidea + Membracoidea)). By contrast, Hamilton (1981) asserted that head structures, wing venation and leg structures show a '... clear phylogenetic progression from Cicadidae through Cercopidae and Cicadellidae to Membracidae', concluding that morphology supported (Cicadoidea (Cercopoidea + Membracoidea)). As evidence, Hamilton cited the successive reduction of anterior tentorial arms, the loss of the median ocellus, and the loss of the division between the frons and the postfrons. Cicadoidea and Cercopoidea usually have a complete tentorium, but these parts are apparently reduced in Membracoidea. Blocker (1996) also pointed to the loss of the median ocellus in Cercopoidea and Membracoidea as evidence supporting (Cicadoidea (Cercopoidea + Membracoidea)).

Boulard (1991a, b) documented potentially synapomorphic characters that support (Membracoidea (Cicadoidea + Cercopoidea)), including structures of the alimentary canal and the larval behaviour of applying excreted liquid (which Boulard called 'urine') to the integument. More recently, two independent morphological studies outlined further evidence for the sister-group relationship of Cicadoidea + Cercopoidea. Liang & Fletcher (2002) documented that Cercopoidea and Cicadoidea have several similar antennal features (imbricated pedicel, numerous coeloconic sensilla on the lateroventral side of the flagellar base, and nymphs with five to seven antennal segments) that support (Membracoidea (Cicadoidea + Cercopoidea)). Rakitov (2002) demonstrated, from anatomical and histological examinations of Malpighian tubules, that Cicadoidea and Cercopoidea share similar glandular modifications (absent in Membracoidea and other non-Cicadomorphan Hemiptera) that indicate common origin. In nonanalytical, 'consensus'-based summaries of previous studies on hemipteran phylogeny, Bourgoin & Campbell (2002) and Dietrich (2002) separately concluded that the preponderance of evidence for cicadomorphan phylogeny supports (Membracoidea (Cicadoidea + Cercopoidea)).

Campbell *et al.* (1995) discussed the hypothesis that nonphloem feeding is a plesiomorphic feeding strategy in Hemiptera; the authors reasoned that the modified, styletiform mouthparts of Hemiptera were probably adapted for plant feeding, but that these adaptations probably occurred prior to the differentiation of plant vasculature in tracheophytes that occurred early in the Triassic. Members of the putative ancestral group of Cicadomorpha, the Hylicelloidea (known largely from Triassic fossils), had an enlarged postclypeus indicative of xylem feeding (Shcherbakov, 2002). Therefore, the 'unusual' behaviour of xylem feeding cannot be considered a synapomorphy of the lineage comprising Cicadoidea and Cercopoidea (Fig. 2, node 16). Because the earliest known Membracoidea (Karajassidae from the lower Jurassic) were also thought to have been xylem feeders (Shcherbakov, 1992; 1996), and xylem feeding persists in some extant leafhopper lineages (subfamily Cicadellinae; Dietrich, 2002), phloem feeding is probably a derived condition within Cicadomorpha.

The current study also presents the first higher-level phylogeny for Cercopoidea (Fig. 2, node 20) based on quantitative cladistic analysis of exemplars representative of all major cercopoid lineages. These analyses indicated support for the monophyly of the families Cercopidae and Aphrophoridae. Support for the monophyly of Aphrophoridae, however, was particularly weak; this result was not unexpected because, as stated above, Hamilton (2001) briefly discussed some morphological characters interpreted as evidence against the monophyly of Aphrophoridae. Indeed, the monophyly of Aphrophoridae was also unsupported in molecular phylogenetic analyses expanded to include additional cercopoid taxa (Cryan, unpublished), and therefore little confidence should be placed on the family's monophyly as recovered here. Epipygidae, the most recently described family of Cercopoidea (Hamilton, 2001), was recovered as a monophyletic group nested within aphrophorid clades, even in

topologies where the monophyly of Aphrophoridae was not supported.

These analyses recovered Clastopteridae + Machaerotidae as a monophyletic clade (Fig. 2, node 21). Hamilton (2001) proposed a reclassification of Cercopoidea that included Machaerotidae (as Machaerotinae) within Clastopteridae, citing as evidence the deep antennal pits observed in both groups and reasoning that a single hindwing vein differentiates Machaerotidae and Clastopteridae taxonomically. Although in the present results statistical support for Clastopteridae + Machaerotidae was relatively weak (bootstrap support = 56% and Bremer support = 3; Table 5), support for the monophyly of Clastopteridae, excluding Machaerotidae, was strong (bootstrap support = 100% and Bremer support = 38; Table 5). Therefore, although data from only one exemplar of Machaerotidae were available for this study, the results suggest that the two families are sister taxa. This, and other hypotheses regarding spittlebug evolution, will be tested rigorously in expanded analyses of Cercopoidea phylogeny already in progress.

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