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Phylogeny of Ensifera (Hexapoda: Orthoptera) using three ribosomal loci, with implications for the evolution of acoustic communication

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Abstract

Representatives of the Orthopteran suborder Ensifera (crickets, katydids, and related insects) are well known for acoustic signals produced in the contexts of courtship and mate recognition. We present a phylogenetic estimate of Ensifera for a sample of 51 taxonomically diverse exemplars, using sequences from 18S, 28S, and 16S rRNA. The results support a monophyletic Ensifera, monophyly of most ensiferan families, and the superfamily Gryllacridoidea which would include Stenopelmatidae, Anostostomatidae, Gryllacrididae, and *Lezina*. Schizodactylidae was recovered as the sister lineage to Grylloidea, and both Rhaphidophoridae and Tettigoniidae were found to be more closely related to Grylloidea than has been suggested by prior studies. The ambidextrously stridulating haglid *Cyphoderris* was found to be basal (or sister) to a clade that contains both Grylloidea and Tettigoniidae. Tree comparison tests with the concatenated molecular data found our phylogeny to be significantly better at explaining our data than three recent phylogenetic hypotheses based on morphological characters. A high degree of conflict exists between the molecular and morphological data, possibly indicating that much homoplasy is present in Ensifera, particularly in acoustic structures. In contrast to prior evolutionary hypotheses based on most parsimonious ancestral state reconstructions, we propose that tegminal stridulation and tibial tympana are ancestral to Ensifera and were lost multiple times, especially within the Gryllidae.

Keywords: Orthoptera; Ensifera; Phylogeny; Stridulation; Acoustic; Communication; Gryllacridoidea; Crickets

1. Introduction

Orthopteran insects may represent the oldest extant lineage of Neoptera, with numerous fossil taxa already present in the Upper Carboniferous (Carpenter, 1992; Kukalova-Peck, 1991; Zeuner, 1939; Sharov, 1968). The most current catalogue of Orthoptera (Otte and Naskrecki, 1997) lists approximately 20,000 species divided into two suborders of approximately equal size: the Caelifera, or "short-horned" grasshoppers and locusts, and the Ensifera, or "long-horned" grasshoppers, including the crickets, katydids, and related lineages.

Most Orthoptera utilize some form of stridulation (friction between body parts) to produce acoustic signals, usually in the contexts of mate location, courtship, and defense. Ensifera in particular have long been of interest as models for examining acoustic communication, and some taxa have been widely studied for the behavioral, neurological, and evolutionary aspects of their acoustic mating system (reviewed in Huber et al., 1989 and Otte, 1992). In the Ensifera, the Grylloidea (*sensu* Otte, 1997a; including Gryllidae and Gryllotalpidae) and Tettigonioidea (*sensu* Otte, 1997b including Tettigoniidae and Haglidae) produce calling and courtship sounds through friction between specialized veins of the front wings (tegminal stridulation), and these signals are received primarily through auditory tympana on the front tibiae of both sexes. The specialized forewing morphology required for this type of stridulation is absent in most ensiferan families, including

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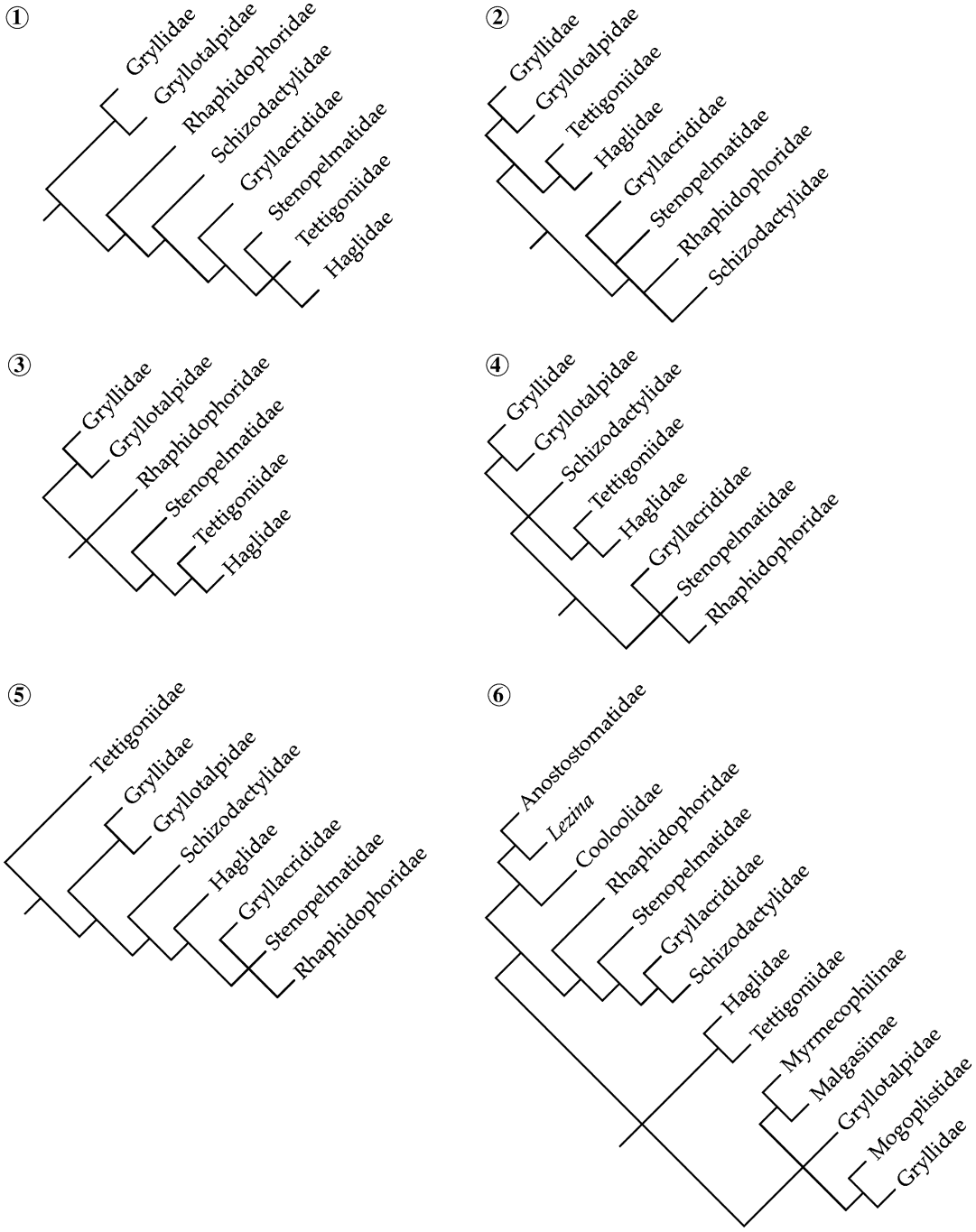
the Rhaphidophoridae, Gryllacrididae, Stenopelmatidae, Anostostomatidae, and some members of the Gryllidae and Tettigoniidae. In addition, the absence of stridulatory structures is usually, but not always, coupled with the absence of auditory organs.

Comparative studies of acoustic communication in Ensifera would benefit greatly from an evolutionary approach that takes phylogeny into consideration. However, the phylogenetic relationships between major ensiferan lineages are still poorly understood, despite many comprehensive efforts to define these lineages and reconstruct relationships using morphological characters (Ander, 1939; DeSutter-Grandcolas, 2003; Gorochov, 1995a; Gwynne, 1995; Judd, 1948; Ragge, 1955; Sharov, 1968; Zeuner, 1939); see Figs. 1–8. A recent numerical phylogenetic analyses of the Ensifera (Gwynne, 1995) reviewed prior studies (Ander, 1939; Judd, 1948; Ragge, 1955; Sharov, 1968; Zeuner, 1939) and compiled 67 morphological and behavioral characters for each of 9 ensiferan families into a single matrix, which was analyzed by an exhaustive search using



Figs. 1–6. Prior phylogenetic hypotheses for Ensifera. (1) Ander (1939); (2) Zeuner (1939); (3) Judd (1948); (4) Ragge (1955); (5) Sharov (1968); (6) Gorochov (1995).

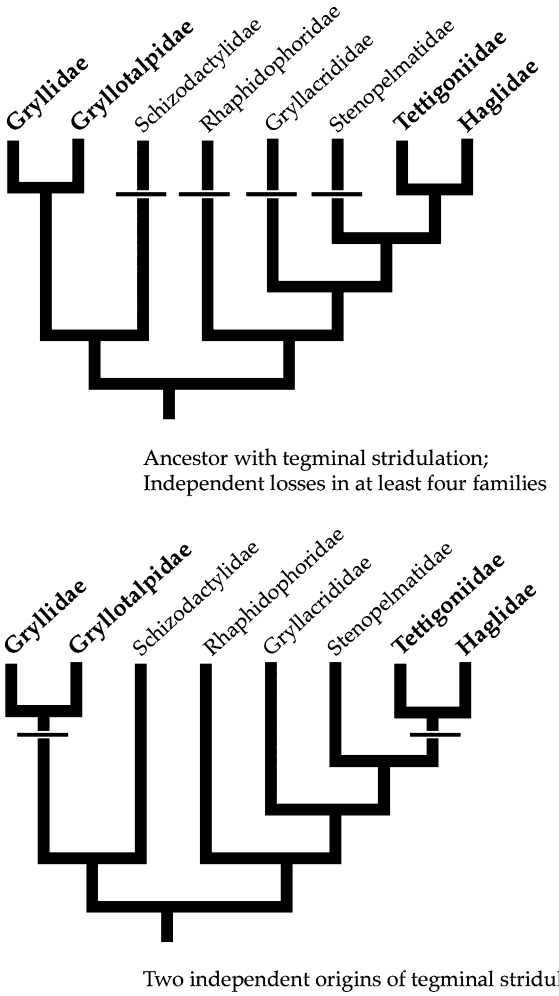


Fig. 7. Most parsimonious phylogenetic hypothesis of Gwynne (1995) from morphological and behavioral characters. Tegminally stridulating families are indicated with boldface font. Gwynne concluded that tegminal stridulation and tibial tympana evolved twice in Ensifera, since this was the most parsimonious character reconstruction on his family-level tree (two changes) under the assumption that gains and losses are equally probable.

the parsimony criterion with unweighted and unordered characters. Gwynne's most parsimonious tree (Fig. 7) divided Ensifera into two major clades, the Grylloidea (Gryllidae + Gryllotalpidae + Schizodactylidae) and the Tettigonioidea (Rhaphidophoridae + Gryllacrididae + Cooloolidae + Stenopelmatidae + Haglidae + Tettigoniidae). However, Gwynne's family-level analysis did not sample the diversity within families, nor did it provide support measures for clade monophyly.

A striking conclusion of Gwynne's analysis was that there were independent origins of tegminal stridulation and tibial tympana in the Grylloidea and Tettigonioidea. This was based on a parsimonious scenario that required fewer changes along a tree (Fig. 7), than the alternative scenario in which these characters would have been lost separately in each non-acoustic family. Although this idea is intriguing, it depends heavily on the phylogenetic analysis of a small data set scored at a high taxonomic level. More

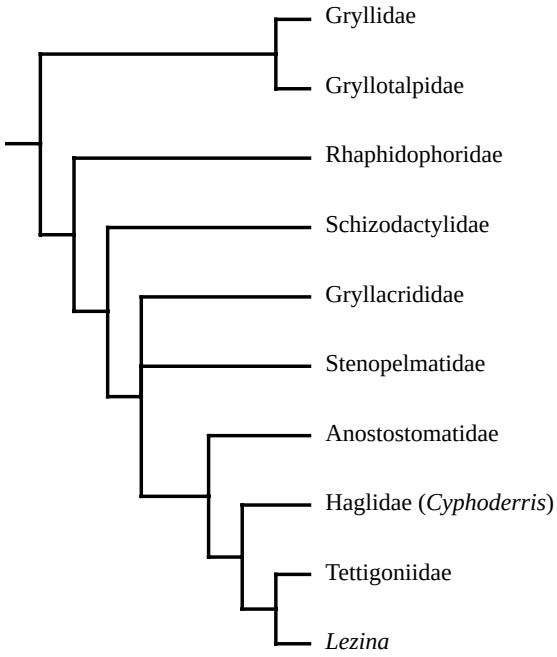


Fig. 8. Phylogenetic estimate from DeSutter-Grandcolas (2003), based on morphological data.

importantly, Gwynne's conclusion of dual origins rested entirely on two debatable assumptions: (1) that parsimonious ancestral state reconstructions on a phylogeny constitute a reliable test for character homology and (2) that independent gains of the multiple, specialized, and closely integrated acoustic characters of Ensifera are equally as probable as independent losses of these traits.

Although a long history of morphological studies of Ensifera has resulted in a literature rich with evolutionary hypotheses and comparative data, the lack of a general consensus on the relationships between families suggests that a new approach may be helpful. Analyses of molecular data are often effective for phylogeny reconstruction, as they benefit from a great number of informative characters without the kinds of biases that can be introduced by the choice and scoring of morphological characters. To date, the only phylogenetic analyses of upper-level Orthopteran relationships using molecular data have been phylogenetic studies of the Caelifera (Flook and Rowell, 1997a,b; Flook et al., 1999; Rowell and Flook, 1998). While these studies provided information about phylogenetic relationships within the Orthoptera and Caelifera, they contributed little to an understanding of relationships within Ensifera.

The goals of the present study are: (1) to estimate phylogeny of the Ensifera using molecular data; (2) to test for monophyly of major recognized lineages, including recently erected groups such as the Anostostomatidae (Johns, 1997); (3) to compare our estimate with phylogenetic hypotheses from recent prior studies; and (4) to ask whether parsimonious character state optimizations under the assumption of equally probable gains and losses is a reliable method of reconstructing the ancestral acoustic

condition in Ensifera. We use molecular sequence data from the nuclear ribosomal genes 18S and 28S, and the mitochondrial ribosomal gene 16S, for a sample of 55 taxa, including 51 ensiferan taxa from 9 of the 10 families recognized by Otte (1997a,b, 2000) as well as 4 outgroup species from the Caelifera. We also review conflicting arguments for single or multiple origins of acoustic characters in Ensifera, and give reasons why even a parsimony-based approach should favor a single origin, multiple-losses hypothesis.

2. Methods

2.1. Taxon sample

The most complete catalogue of Ensifera divides this suborder into three superfamilies that encompass several traditionally recognized families: the Grylloidea, including true crickets (Gryllidae) and mole crickets (Gryllotalpidae); the Tettigonioidea, including sagebrush crickets (Haglidae) and katydids or bush-crickets (Tettigoniidae); and the Gryllacridoidea, including cave and camel crickets (Rhaphidophoridae), Jerusalem crickets (Stenopelmatidae), king crickets and weta (Anostostomatidae *sensu* Johns, 1997), raspy and leaf-rolling crickets (Gryllacrididae), splay-footed crickets (Schizodactylidae) and Cooloola monsters (Cooloolidae). Because many ensiferan clade names are derived from similar roots, the definitions of these potentially confusing names are given in Table 1.

We selected exemplars by sampling from as many of these groups as possible, given the availability of quality tissue or the possibility of collecting it. We collected fresh specimens in North America, South America, southern Africa, the Pacific, and Madagascar, and additional specimens were identified and provided by a number of colleagues (see Acknowledgements). Our data also included six species with sequences available from EMBL and GenBank: *Ceuthophilus carlsbadensis* (Rhaphidophoridae) (EMBL CCZ97563 and EMBL CCZ97613); *Daihinibaenetes giganteus* (Rhaphidophoridae) (GenBank AF212058); *Camptonotus carolinensis* (Gryllacrididae)(GenBank AF212050); *Comicus campestris* (Schizodactylidae) (EMBL CCZ97564 and EMBL CCZ97624); *Batrachideidae* sp. (Caelifera: Tetrigoidea) (GenBank Z97631); *Acrida turrita* (Caelifera: Acridomorpha) (EMBL ATZ97560 and EMBL ATZ97612); and *Trigonopteryx* sp. (Caelifera: Acridomorpha) (GenBank AJ011975). The final taxon sample we used contains representatives from 30 ensiferan subfamilies and from 9 of the 10 families recognized by Otte (1997a,b, 2000). Unfortunately, sequences for Cooloolidae could not be obtained despite many attempts with four older preserved specimens of *Cooloola ziljan*; however, this putative family is small (~5 described spp.) and accounts for less than one tenth of one percent (<0.1%) of ensiferan species diversity. To test a new classification of Stenopelmatidae proposed by Johns (1997), our sample includes two stenopelmatids and four anostostomatids *sensu* Johns (1997). A list of all species used, along with the source of the specimens, is given in Table 2.

2.2. Extractions, amplification, sequencing, and alignment

All tissues were preserved in ethanol ranging from 80 to 100% concentration, and stored at −20 °C upon arrival at the Shaw and Giribet laboratories at Harvard University. Nucleic acid extractions were carried out using leg muscle tissue only, to avoid contamination from gut contents. Muscle tissues were homogenized in a solution of 4 M guanidinium thiocyanate and 0.1 M β-mercaptoethanol, following a modified protocol for RNA extraction from Chirgwin et al. (1979), which included a phenol: chloroform: isoamyl-alcohol extraction series, and a salting-out step with 3 M sodium acetate and ice cold ethanol (95%). Precipitated pellets were resuspended in TE (pH 7.6) and stored at a temperature of 4 °C.

Polymerase chain reactions (PCR) were performed at 50 μL volumes using Perkin–Elmer $MgCl_2$ buffer with 1.25 U of AmpliTaq polymerase, 200 μM dNTPs, and 1 μM of each primer. Primers used for amplifications and sequencing were as follows. For 16S rRNA, primer sequences were 16Sa: (5′-CGC CTG TTT ATC AAA AAC AT-3′) and 16Sb: (5′-CTC CGG TTT GAA CTC AGA TCA-3′) (Xiong and Kocher, 1991). For 28S rRNA, primer sequences (for the d3 expansion fragment) were

Table 1
Definitions for potentially confusing clade names used throughout this study

Clade name	Taxa included in definition
Tettigonioidea sensu Otte, 1997b	Tettigoniidae (katydids) and Haglidae
Tettigonioidea sensu Gwynne, 1995 (used in the present paper, unless noted)	Tettigoniidae, Haglidae, Stenopelmatidae, Cooloolidae, Gryllacrididae, and Rhaphidophoridae
Tettigoniidae	Tettigonioid family, including katydids only
Tettigoniinae	One of several subfamilies of Tettigoniidae
Grylloidea	Gryllidae (true crickets) and Gryllotalpidae (mole crickets)
Gryllidae	Grylloid family, including true crickets only
Gryllinae	One of several subfamilies of Gryllidae
Gryllacridoidea sensu Otte and Naskrecki, 1997	Stenopelmatidae, Anostostomatidae, Gryllacrididae, Cooloolidae, Rhaphidophoridae, and Schizodactylidae
Gryllacrididae	Gryllacridoid family, including raspy and leaf-rolling crickets only

Table 2
Taxon sample, sequence accession numbers, and representative subfamilies following the classification of Otte (1997a,b, 2000)

Taxon, classification, and source (* below indicates data acquired from GenBank or EMBL)	Voucher #	18S (a) accession #	18S (b) accession #	28S accession #	16S accession #	Total bases
Gryllidae (>3500 spp.)						
G *Acheta domesticus* (Gryllinae) [DG]	JSE-02-1	—	AF514506	AF514421	AF514462	1512
G *Allonemobius socius* (Nemobiinae) [DG]	JSE-02-2	AF514546	AF514507	AF514422	AF514463	2091
G *Brachytrupes* sp. (Brachytrupinae) [MCJ]	JSE-02-3	AF514547	AF514508	AF514423	—	2038
G *Gryllus bimaculatus* (Gryllinae) [ST]	JSE-02-4	AF514548	AF514509	AF514424	AF514464	2521
G *Hemiphonus* sp. (Podoscirtinae) [DL]	JSE-02-5	AF514549	AF514510	—	AF514465	1510
G *Homeogryllus* sp. (Homeogryllinae) [MCJ]	JSE-02-6	AF514550	AF514511	—	—	1206
G *Madasumma* sp. (Podoscirtinae) [MCJ]	JSE-02-7	—	AF514512	—	AF514466	1121
G *Malgasia marmorata* (Malgasiinae) [MCJ]	JSE-02-8	—	AF514513	AF514425	AF514467	1487
G *Myrmecophilus* sp. (Myrmecophilinae) [PN]	JSE-02-9	—	—	—	AF514468	378
G *Oecanthus nigricornis* (Oecanthinae) [DG]	JSE-02-10	—	AF514514	AF514426	AF514469	1523
G *Ornebius aperta* (Mogoplistinae) [DG]	JSE-02-11	—	AF514515	AF514427	AF514470	1475
G *Prognathogryllus puna* (Oecanthinae) [KS]	JSE-02-12	AF514551	AF514516	AF514428	AF514471	2072
G *Pteronemobius ohmachii* (Nemobiinae) [ST]	JSE-02-13	AF514552	AF514517	AF514429	AF514472	2624
Gryllotalpidae (~50 spp.)						
M *Gryllotalpa* sp. (Gryllotalpini) [MCJ]	JSE-02-17	AF514555	AF514521	—	AF514475	1643
M *Neocurtilla* sp. (Gryllotalpini) [MCJ]	JSE-02-18	AF514556	AF514522	AF514432	AF514476	2049
M *Scapteriscus* sp. (Scapteriscini) [MCJ]	JSE-02-19	AF514557	AF514523	AF514433	AF514477	2027
Rhaphidophoridae (~300 spp.)						
R *Daihinia* sp. [MCJ]	JSE-02-20	—	—	AF514434	AF514478	523
R *Pristoceuthophilis* sp. [TC]	JSE-02-21	AF514558	AF514524	AF514435	AF514479	2103
R *Tropidischia* sp. [TC]	JSE-02-22	—	—	AF514436	AF514480	866
R **Ceuthophilus carlsbadensis*	—	CCZ97563	—	—	CCZ97613	n/a
R **Daihinibaenetes giganteus*	—	—	—	—	AF212058	n/a
Stenopelmatidae + Anostostomatidae (~200 spp.)						
S *Australostoma* sp. (Anostostomatinae) [GG]	JSE-02-23	AF514559	AF514525	AF514437	AF514481	2066
S *Hemiandrus* sp. (Anostostomatinae) [DG]	JSE-02-24	—	—	—	AF514482	539
S *Hemideina maori* (Deinacridinae) [KS]	JSE-02-25	—	—	AF514438	AF514483	826
S *Hypocophus* sp. (Anostostomatinae) [MCJ]	JSE-02-26	AF514560	AF514526	AF514439	AF514484	2424
S *Sia* sp. (Stenopelmatidae) [MCJ]	JSE-02-27	AF514561	AF514527	AF514440	AF514485	2166
S *Stenopelmatus* sp. (Stenopelmatidae) [TC]	JSE-02-28	AF514562	AF514528	AF514441	AF514486	1840
Tettigoniidae (>6300 spp.)						
T *Acanthoplus* sp. (Heterodinae) [PN]	JSE-02-29	AF514563	AF514529	AF514442	AF514487	2425
T *Ancistrocercus circumdatus* (Pseudophyllinae) [DG]	JSE-02-30	AF514564	AF514530	AF514443	AF514488	1866
T *Atlanticus gibbosus* (Tettigoniinae) [PN]	JSE-02-31	AF514565	AF514531	AF514444	AF514489	2498
T *Austrosalomona* sp. (Conocephalinae, Agraeciini) [PN]	JSE-02-32	AF514566	AF514532	AF514445	AF514490	2434
T *Caulopsis microprora* (Conocephalinae, Copiphorini) [PN]	JSE-02-33	AF514567	AF514533	AF514446	AF514491	2341
T *Conocephalus* sp. (Conocephalinae, Conocephalini) [MCJ]	JSE-02-34	AF514568	AF514534	AF514447	AF514492	2021
T *Copiphora rhinocerous* (Conocephalinae,Copiphorini) [PN]	JSE-02-35	AF514569	AF514535	AF514448	AF514493	1987
T *Coptaspis* sp. 6 (Conocephalinae, Agraeciini) [PN]	JSE-02-36	AF514570	AF514536	AF514449	AF514494	2447
T *Ephippiger ephippiger* (Bradyporinae, Ephippigerini) [PN]	JSE-02-37	—	—	AF514450	AF514495	826
T *Eschatocerus* sp. (Conocephalinae, Agraeciini) [PN]	JSE-02-38	AF514571	AF514537	AF514451	AF514496	2405
T *Kawanaphila nartee* (Zaprochilinae) [GA]	JSE-02-39	—	—	AF514452	AF514497	830
T Meconematine (undescribed Japanese species) [PN]	JSE-02-40	AF514572	AF514538	AF514453	AF514498	2275
T *Orophus* sp. (Phaneropterinae) [PN]	JSE-02-41	AF514573	AF514539	AF514454	AF514499	2034
T *Requena verticalis* (Listroscelidinae) [GA]	JSE-02-42	AF514574	AF514540	AF514455	—	1635
T *Sciarasaga quadrata* (Austrosaginae) [GA]	JSE-02-43	AF514575	—	AF514456	AF514500	1424
T *Scopioricus* sp. (Pseudophyllinae) [PN]	JSE-02-44	AF514576	AF514541	AF514457	AF514501	2361
T *Scudderia furcata* (Phaneropterinae) [MCJ]	JSE-02-45	AF514577	AF514542	AF514458	AF514502	2326
T *Yutjuwalia* sp. (Listroscelidinae) [PN]	JSE-02-46	AF514578	AF514543	AF514459	AF514503	2361
Gryllacrididae (>500 spp.)						
K *Ametrus* sp. (Gryllacridinae) [GA]	JSE-02-15	—	AF514519	—	—	528
K *Hadrogryllacris* sp. (Gryllacridinae) [GA]	JSE-02-16	AF514554	AF514520	AF514431	AF514474	2042
K **Camptonotus carolinensis* (Gryllacridinae)	—	—	—	—	AF212050	n/a
Haglidae (5 spp.)						
H *Cyphoderris monstrosaus*. [TC]	JSE-02-14	AF514553	AF514518	AF514430	AF514473	2001
Schizodactylidae (14 spp.)						
Z **Comicus* sp.	—	—	CCZ97564	—	CCZ97624	n/a

Table 2 (*continued*)

Taxon, classification, and source (* below indicates data acquired from GenBank or EMBL)	Voucher #	18S (a) accession #	18S (b) accession #	28S accession #	16S accession #	Total bases
"Gryllacridoidea," placement unknown						
U *Lezina* sp. (Lezininae) [PN]	JSE-02-47	AF514579	—	AF514460	AF514504	1256
Caeliferan outgroups						
X *Locusta migratoria*	JSE-02-49	AF514580	AF514544	AF514461	AF514505	2072
X **Batrachideidae* sp. (Tetrigoidea)	—	BSZ97631	—	—	—	n/a
X **Trigonopteryx hopei* (Acridomorpha: Trigonopteryginae)	—	AJ011975	—	—	—	n/a
*X *Acrida turrita* (Acridomorpha: Acrididae)	—	ATZ97560	—	—	ATZ97612	n/a

Initials in [brackets] acknowledge the researcher who collected or provided specimens: MCJ, Manda Clair Jost, Harvard University; KS, Kerry Shaw, University of Maryland, College Park; TC, Theodore Cohn, University of Michigan, Ann Arbor; PN, Piotr Naskrecki, University of Connecticut, Storrs; DG, Darryl Gwynne, University of Toronto; ST, Seiji Tanaka, NISES, Ibaraki Japan; GA, Geoff Allen, University of Western Australia, Perth; GG, Gonzalo Giribet, Harvard University; DL, David Lohman, Harvard University. Sequences indicated with an asterisk "*" were acquired from Genbank. Empty table cells indicate that the locus could not be isolated and sequenced from the available tissue for that taxon.

28Sa: (5′-GAC CCG TCT TGA AAC ACG GA-3′) and 28Sb: (5′-TCG GAA GGA ACC AGC TAC-3′) (Whiting et al., 1997). For 18S rRNA, primer sequences for one fragment were 1F: (TAC CTG GTT GAT CCT GCC AGT AG); 3R: (AGG CTC CCT CTC CGG AAT CGA AC); 4R: (GAA TTA CCG CGG CTG CTG G); and 5R: (CTT GGC AAA TGC TTT CGC); and for a second fragment, 18Sa2.0: (5′-ATG GTT GCA AAG CTG AAA C-3′) and 9R: (5′-GAT CCT TCC GCA GGT TCA CCT AC-3′) (Giribet et al., 1996; Whiting et al., 1997). The PCR program began with an initial denaturing step at 94 °C for 60 s, followed by 35 amplification cycles. The PCR annealing temperature was 49 °C for all 3 loci.

PCR products were visualized using agarose gel electrophoresis and ethidium bromide staining. Successful amplification products were purified using BIO 101 GeneClean II and resuspended in TE. Both strands of purified PCR products were cycle-sequenced in 10 μL volumes using the BigDye terminator sequencing system from Applied Biosystems; each reaction contained 4 μL Reaction Mix, 1 μL of the aforementioned PCR primers diluted to 10 μM, and 10–30 ng of PCR product. The cycle-sequencing program began with an initial step at 94 °C for 3 min, followed by 25 sequencing cycles. Sequenced fragments were precipitated and washed with 75% isopropanol following the BigDye manufacturer protocol, resuspended in formamide, and analyzed using a 16 capillary ABI PRISM 3100 Genetic Analyzer. Chromatograms were automatically analyzed by the DNA Sequencing Analysis Software v.3.7 by ABI PRISM.

Experimental chromatograms for the forward and reverse sequences for each species were checked individually for errors (such as double-calls), and assembled using Sequencher v.4.1 (Gene Codes Corporation). Consensus sequences were first aligned roughly in Clustal X (Thompson et al., 1997) using default parameters, and then improved using manual adjustment guided by ribosomal RNA secondary structure (Flook and Rowell, 1997a,b). All data were then concatenated into a single NEXUS file, with separate data partitions for each locus.

2.3. Phylogenetic analyses

2.3.1. Parsimony

The following analyses were conducted for each gene individually, and for the concatenated data from all three genes. PAUP*4.0b10 (Swofford, 2001) was used to calculate base frequencies for each gene, and to search for most optimal trees. The maximum parsimony criterion was applied to search for the shortest trees for each data set individually and for the concatenated data, using 1000 random addition sequence heuristic replicates with tree-bisection–reconnection (TBR) branch-swapping, and no MaxTrees value specified (auto-increasing). Gaps in the alignments were treated as missing data. Trees were rooted by defining four members of the Caelifera as outgroups. When multiple equally parsimonious trees were found by an analysis, a strict consensus tree was calculated for all equally parsimonious trees. The "tree scores" function in PAUP*4.0b10 was used to count the number of informative characters for each data set, as well as consistency (CI) and retention (RI) indices for the most parsimonious trees found by each search.

Bootstrap resampling of 1000 heuristic replicates each (10 random-addition TBR replicates each) was performed using PAUP*4.0b10 to evaluate clade robustness. Bremer (decay) support indices (Bremer, 1988) were calculated by executing a NEXUS treefile containing the most parsimonious tree or strict consensus tree in the program AutoDecay v.4.0.2 by Torsten Eriksson. This program creates constraint subtrees for each clade in a given tree and writes them into an executable NEXUS file that PAUP* uses to search for the shortest trees that conflict with each constraint subtree. For each constraint subtree, we searched for most parsimonious topologies using 10 heuristic replicates with TBR swapping.

2.3.2. Bayesian inference

Bayesian analyses were conducted for the concatenated data using the program MrBayes 3.0 (Huelsenbeck and Ronquist, 2001). The data were partitioned into three character sets, one for each gene, and we specified a general

time-reversible model with invariable sites and a gamma distribution for variable rate sites (GTR + I + G). This model was selected because it has the most parameters and generally results in the best likelihood score for a given tree and data set. Four Markov chains of 1,000,000 generations were run at the default temperature (0.2), and every tenth tree was saved to a file. The burn-in asymptote was estimated by plotting the number of generations against the log likelihood scores for the saved trees. Trees saved below the burn-in generation were discarded from the set of saved trees, and a majority rule consensus of the remaining trees was calculated in PAUP*4.0b10 to estimate posterior probabilities. Four replicates of these Bayesian runs were conducted as described to insure convergence of the posteriors.

2.4. Tree comparisons

We assessed the degree of conflict between our phylogenetic estimate and those of prior studies by using tree comparison tests, testing whether our most optimal estimates of phylogeny were significantly better at explaining the molecular data than alternative phylogenies proposed by Gorochov (1995a) (Fig. 6), Gwynne (1995) (Fig. 7), and DeSutter-Grandcolas (2003) (Fig. 8). For parsimony comparisons using the concatenated data, we searched for the most parsimonious trees under constraint tree settings that matched the family-level topologies of the three prior hypotheses (1000 heuristic replicates, TBR branch swapping). We compared all trees with Templeton (1983), Winning-sites, and two-tailed Kishino and Hasegawa (1989) tests, as implemented in PAUP*4.0b10. For likelihood-based comparisons, we used PAUP*4.0b10 and our Bayesian estimate to calculate parsimony-based approximations of the rate matrix and gamma, averaged over the concatenated data. These values were then used for Kishino–Hasegawa tests, to compare our Bayesian estimate with alternative trees that were rearranged at deep branches to match the phylogenetic hypotheses of Gwynne (1995), Gorochov (1995a), and DeSutter-Grandcolas (2003). For the three alternative family-level hypotheses, relationships within families were retained as they appeared on our Bayesian estimate. For these Kishino–Hasegawa tests, we used a RELL distribution derived from 1000 bootstrap replicates.

Next, we simplified our Bayesian estimate and the phylogenetic hypotheses of Gorochov (1995a) and DeSutter-Grandcolas (2003) to 8-taxon (family-level) trees, and used Gwynne's (1995) morphological matrix (Table 3) to test whether the most optimal tree for the data (Gwynne's tree) was significantly better than any of these three alternatives. Gwynne's (1995) matrix contains 64 morphological characters and three behavioral characters: (male tegminal stridulation (character 18), male on top in copulation (character 66), and male behind female faces same way in copulation (character 67)). We excluded the Cooloolidae from the trees and the matrix, since we had no molecular data to help place this taxon. All characters were unordered, and multiple states were interpreted as polymorphisms, in accord with Gwynne (1995). For tree comparisons using the morphological data, we used Templeton, Winning-sites, and two-tailed Kishino–Hasegawa tests under the parsimony criterion, to compare Gwynne's phylogeny against our tree, DeSutter-Grandcolas's (2003) tree, and all three possible resolutions of Gorochov's (1995a) polytomy.

3. Results

3.1. Extractions, amplification, sequencing, and alignment

A summary of all sequence data we collected is given in Table 2, with mean base frequencies in Table 4. NBCI BLAST searches matched all of our sequences with previously published insect sequences and most matched published sequences for Orthoptera. Due to the quality of some specimens, we were not successful in amplifying and sequencing all loci for all species; the number of species for which sequencing was successful is given at the beginning of the results for each particular locus (below; also see Table 2). The alignment used in all analyses is available as a NEXUS file from the authors.

3.2. Phylogeny reconstrucion: parsimony

3.2.1. 18S rRNA

PCR and sequencing reactions were successful for 42 taxa (Table 2). Base frequencies were approximately equal in the sequences (23.7% A, 24.1% C, 27.8% G, and 24.4% T). A short region of 76 bases (concatenated alignment positions 1405–1480) was excluded from the 18S analyses due to poor sequence conservation in this area, and great difficulty aligning the nucleotides. Out of the remaining 1692 characters (including inserted gaps) there were 359 parsimony-informative characters (approx. 21%). One thousand random addition sequence heuristic search replicates using TBR swapping resulted in 165 most parsimonious trees ($L = 1479$; CI = 0.5774, RI = 0.6368). A strict consensus of these trees is shown in Fig. 9, and a summary of clades recovered is given in Table 5. The strict consensus supports a monophyletic Ensifera relative to the four caeliferan exemplars (bootstrap = 100%, decay = 17), as well as a monophyletic Tettigoniidae (bootstrap = 70, decay = 3) and Gryllotalpidae (bootstrap = 96%, decay = 3). Although the strict consensus of most parsimonious trees for 18S did not contain a monophyletic Gryllidae, bootstrap resampling provided 76% support for this clade. Other clades represented by exemplar pairs and supported by bootstrap values over 90% were the Rhaphidophoridae (*Pristoceuthophilus* sp. + *C. carlsbadensis*), Gryllacrididae (*Ametrus* sp. + *Hadrogryllacris* sp.), Gryllinae (*A. domesticus* + *G. bimaculatus*), Nemobiinae (*A. socius* + *P. ohmachii*), Oecanthinae (*O. nigricornis* + *P. puna*), and Podoscrirti-

Table 3
Character matrix from Gwynne (1995), used here in tree comparison tests

		HG	TE	ST	GC	RH	SC	GR	GT
1	Antennae much longer than body	1	1	1	1	1	1	1	1
2	Fastigium verticalis	1	1	0,1	1	0,1	1	0	0
3	Double fastigium verticalis	0	0	0	0	0,1	1	?	?
4	Pronotum with lateral lobes	1	1	1	1	1	1	1	1
5	Prothorax with cryptopleuron	1	1	1	1	1	1	1	1
6	Meso and metasternum with sclerotized space	0	0	0,1	0	1	1	0	0
7	1st spiracle single (0) divided (1) or dual (2)	1,2	2	0,1	0	0	0	1	1
8	Forecoxae shorter than broad	0,1	1	1	0	0	0	0	0
9	Tibial tympana present	1	1	0,1	0	0	0	0,1	0,1
10	Tympana with thick and thin parts	1	1	1	?	?	?	0	0
11	Tympanal vesicles present	1	1	0	0	0	0	1	?
12	Hind tibiae with alternate spine sizes	0	0	0	0	0,1	0	1	1
13	4 tarsal segments in middle leg	1	1	1	1	1	1	0	0
14	Tarsal segments 1–3 with pulvillae	1	1	1	1	0,1	1	0	0
15	Pulvillae lobed and broad	0	1	0	1	0	1	?	?
16	Pulvillae with tubules	1	1	1	0	0	0	?	?
17	Femoral-abdominal stridulation	0	0	0,1	1	0,1	1	0	0
18	Male tegminal stridulation	1	1	0	0	0	0	0,1	0,1
19	Tegmen with harp	0	0	0	0	?	0	1	1
20	Tegmina folded at base of R, M, Cu	0	0	0	0	?	1	1	1
21	Tegmen Sm1, R, M, Cu run close together	0	0	0	0	?	0	1	1
22	Cu1/MP fused, especially in tegmina	1	1	1	0	?	0	0	0
23	Costa reduced or absent	0	0	0	0	?	1	1	1
24	Archedictyon present	0	1	0	0	?	1	0,1	1
25	Numerous tegminal anal veins	0	0	1	1	?	0	0	0
26	Tegmina wrap around body	0	0	1	1	?	1	1	1
27	Tegmen with mirror and subcostal branching	1	1	0	0	?	0	0	0
28	Tegmina held roof-like over abdomen	1	1	0	0	?	0	0	0
29	Hindwing (HW) fusion of Rs and M	1	1	0	0	?	0	0	0
30	Extension of fanlike folding in HW	0	0	0	0	?	1	1	1
31	HW: no demarcation of vannus	1	1	1	1	?	1	1	1
32	HW: Cu1 forks onto 2 branches	0	0	0	0	?	1	1	1
33	HW: Cu/1a developed, fused with mp	1	1	0	0	?	0	0	0
34	Anal fan with anterior and posterior cubital areas	1	1	1	1	1	1	1	1
35	Pinch on anal veins 3 and 4	0	0	?	1	?	?	0	0
36	Pseudotympanum on abdomen	0	0	0	0	1	0	1	1
37	Two kinds of cercal hairs	0	0	0	0	0	0	1	1
38	Male 10th abdominal segment with paired organ	1	0	1	1	0	0	0	0
39	Male paraproct with grasping projection	1	0,1	1	1	0	0	0	0
40	Male subgenital plate with styles	1	0,1	1	0,1	0,1	1	0	0
41	Blade-like ovipositor	1	1	1	1	1	?	0	0
42	Ovipositor absent	0	0	0	0	0	1	0	1
43	Ovipositor united with fold	1	1	1	0	0	0	0	0
44	Proventricular teeth complex (1) or simple (2)	1	1	1	1	1	1	2	2
45	Proventricular tooth tip sclerotised	0	0	0	0	1	1	0	0
46	One outlet for malpighian tubes	0	0	0	0	0	0	1	1
47	Dorsal space in male genitalia	1	1	1	0	0	0	0	0
48	Abdominal ganglia 2 + 3 fused	1	1	1	1	1	0	0	0
49	Abdominal ganglia 7–10 fused	0	0	0	0	0,1	1	1	1
50	Additional abdominal sternal plates	1	1	1	1	1	?	0	0
51	Ovaries fascicle-shaped	1	1	1	0	0,1	1	1	?
52	Ovary with both comb and fascicle-shaped parts	1	1	0	0	0	0	0	0
53	Testis with suspensory ligament	0	0	0	0	0	0	1	1
54	Long ejaculatory duct	0	0	0	0	1	0	1	1
55	Very short seminal duct	1	1	1	1	1	0	0	0
56	Genital sacs in male	2	2	1	2	2	1	0	0
57	Spermatophylax present	1	1	0,1	1	0,1	0	0,1	0
58	Ventral median vesicle in tracheae	1	1	1	1	0	1	0	0
59	Large ventral tracheae	1	1	1	0	0	0	0	0
60	Thorax trach with large cephalic stem	0	0	0	0	1	0	1	1
61	Trach: separated cephalic stem	1	1	1	1	0	1	1	1
62	Trach: strong transverse in prothorax	1	1	1	1	0	0	0	0
63	Sperm heads not filiform	?	1	?	?	0,1	?	0	0
64	Flat sperm heads + nucleus	?	1	?	?	0	?	0	0

(*continued on next page*)

Table 3 (*continued*)

		HG	TE	ST	GC	RH	SC	GR	GT
65	Spermat cavities: 1 (0), intermediate (1) or 2 (2)	2	2	0,2	2	1	0	0	0
66	Male on top in copulation	0	0	0	0	0	0	0	0
67	Male behind female faces same way in copulation	1	1	0,1	1	0,1	?	0	0,1

HG, Haglidae; TE, Tettigoniidae; ST, Stenopelmatidae; GC, Gryllacrididae; RH, Rhaphidophoridae; SC, Schizodactylidae; GR, Gryllidae; GT, Gryllotalpidae. Unless otherwise indicated, 0 = absent, 1 = present, ? = missing or unknown.

Table 4
Mean base frequencies in our data for each study locus

	18S rRNA	28S rRNA	16S rRNA
A	25.6	17.6	34.9
C	24.5	33.4	20.6
G	23.7	25.5	11.7
T	26.2	23.5	32.8

nae (*Hemiphonus* sp. + *Madasumma* sp.). In addition, the grouping of *M. marmorata* (Malgasiinae) with *O. aperta* (Mogoplistinae) and the grouping of *B. membraneceous* (Brachytrupinae) close to the Gryllinae is in agreement with many grylloid classification schemes based on morphology (e.g., Chopard, 1967; DeSutter, 1987; Otte and Alexander, 1983). Relationships within the Tettigonioidea showed lower resolution, except for the Pseudophyllinae (*A. circumdatus* + *Scopioricus* sp.) and Conocephalinae (*Austrosalomona* sp. + *C. microprora* + *Eschatocerus* sp. + *C. rhinocerous* + *Conocephalus* sp.) The Conocephaline tribe Copiphorini was also recovered (*C. microprora* + *C. rhinocerous*).

3.2.2. 28S rRNA

PCR and sequencing reactions were successful for 41 taxa (Table 2). Base frequencies in the sequences suggest an elevated rate of A–C transversions (17.6%A, 33.4%C, 25.5%G, and 23.5%T) (Table 4). Two short regions totalling 117 bases (concatenated alignment positions 2628–2682 and 2711–2772) were excluded from the 28S analyses due to poor sequence conservation in this area, and great difficulty aligning the nucleotides. Out of the remaining 345 characters (including gaps) there were 81 parsimony-informative characters (approx. 23%). One thousand random addition sequence heuristic search replicates using TBR swapping resulted in 23 most parsimonious trees ($L = 459$; CI = 0.5664, RI = 0.6128.) A strict consensus of these trees is shown in Fig. 10, and a summary of clades recovered is given in Table 5. The strict consensus found a monophyletic Grylloidea (bootstrap = 69%, decay = 5), Gryllotalpidae (bootstrap = 90%, decay = 6) and Rhaphidophoridae (bootstrap = 67%, decay = 4). Other clades represented by exemplar pairs and supported by bootstrap values over 50% were the Gryllinae (*A. domesticus* + *G. bimaculatus*), Nemobiinae (*A. socius* + *P. ohmachii*), and Phaneropterinae (*Orophus* sp. + *S. furcata*).

3.2.3. 16S rRNA

PCR and sequencing reactions were successful for 41 taxa (Table 2). Base frequencies in the sequences showed an A–T bias: 32.9%A, 10.7%C, 19.9%G, and 36.5%T. (Table 4). A short region of 62 bases (concatenated alignment positions 270–331) was excluded from the 16S analyses due to poor sequence conservation in this area, and great difficulty aligning the nucleotides. Out of an alignment of the remaining 559 nucleotides there were 272 parsimony-informative characters (approx. 49%). One thousand random addition sequence heuristic search replicates using TBR swapping resulted in 21 most parsimonious trees ($L = 2340$; CI = 0.2726; RI = 0.3984). A strict consensus of these trees is shown in Fig. 11, and a summary of clades recovered is given in Table 5. The strict consensus recovers a monophyletic Ensifera relative to the Caeliferan outgroups (bootstrap = 100%, decay = 14), and includes a monophyletic Rhaphidophoridae (bootstrap = 69%, decay = 7) and Gryllotalpidae (bootstrap = 69%, decay = 8). The strict consensus also contains a monophyletic Gryllidae, but with low support measures (boostrap < 50%, decay = 5). Other groupings consistent with the classification of Otte (1997a,b, 2000) include the grylloid subfamilies Gryllinae (*A. domesticus* + *G. bimaculatus*), Nemobiinae (*A. socius* + *P. ohmachii*), and Podoscirtinae (*Hemiphonus* sp., *Madasumma* sp.); the gryllotalpid tribe Gryllotalpini; all conocephalines except *Conocephalus* (*Austrosalomona* sp. + *C. rhinocerous* + *C. microprora* + *Eschatocerus* sp.); and a weakly supported grouping of *Lezina* and the gryllacridids (*C. carolinensis* + *Hadrogryllacris* sp.) with all anostostomatids and the stenopelmatid *Sia*, which could imply a monophyletic Gryllacridoidea.

3.2.4. 16S rRNA, 28S rRNA, and 18S rRNA, concatenated data

The 3 ribosomal loci were then analyzed as a concatenated data matrix, excluding the short unalignable regions described above. One thousand random addition sequence heuristic search replicates using TBR swapping resulted in nine most parsimonious trees ($L = 4349$; CI = 0.4029; RI = 0.4872). A strict consensus of these trees is shown in Fig. 12, and a summary of clades recovered is given in Table 5.

The strict consensus implies a monophyletic Ensifera relative to 4 caeliferan outgroups (bootstrap = 99%, decay = 12). Well supported groups include Gryllidae + Gryllotalpidae (bootstrap = 96%, decay = 13); the Tettigoniidae (bootstrap = 64%, decay = 3); Gryllacrididae (bootstrap = 93%, decay = 5), and Rhaphidophoridae (bootstrap = 87%, decay = 3). Weak support was found for a monophyletic basal Gryllacridoidea including

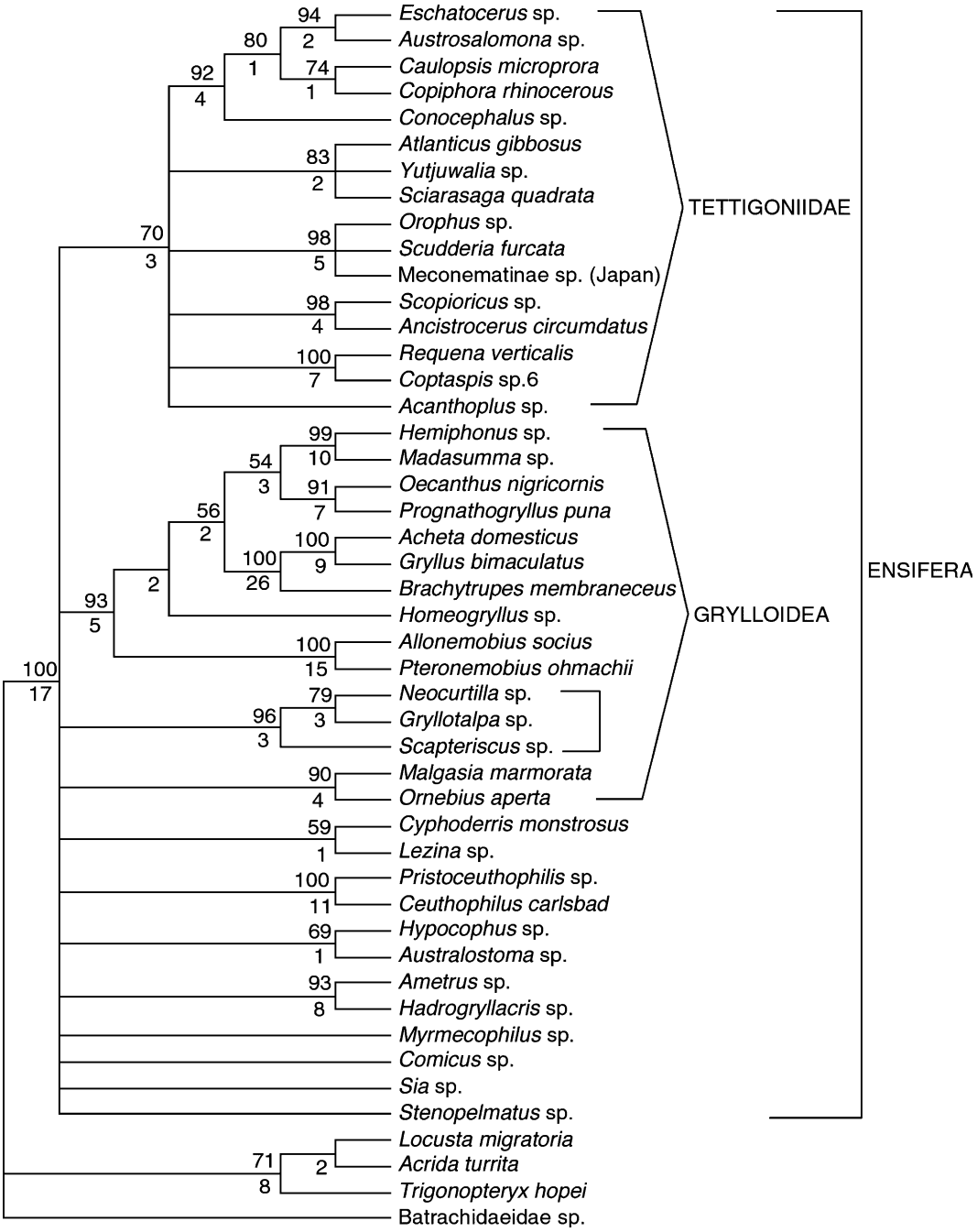


Fig. 9. 18S, strict consensus of 165 most parsimonious trees ($L = 1479$; CI = 0.5774, RI = 0.6368). Bootstrap values above branches, decay indices below.

stenopelmatids, anostostomatids, gryllacrididae, and *Lezina* (in strict consensus, bootstrap < 50%, decay = 3), and for the placement of Schizodactylidae (represented here by *Comicus*) as the sister to Gryllidae+Gryllotalpidae (in strict consensus, bootstrap < 50%, decay = 3). A monophyletic Anostostomatidae *sensu* Johns (1997) was recovered with weak support (bootstrap <50%, decay = 3). The Rhaphidophoridae were recovered as the sister group to Grylloidea + *Comicus* (decay = 3), and *Cyphoderris monstrosus* (Haglidae) was placed as the most basal Ensiferan taxon (decay = 2). Additional clades found were as follows (see Fig. 12 for indices of bootstrap and decay support): Gryllinae (*A. domesticus* + *G. bimaculatus*), Podoscirtinae (*Hemiphonus* sp. + *Madasumma* sp.), Oecanthinae (*O. nigricornis* + *P. puna*), Nemobiinae (*A. socius* + *P. ohmachii*), Gryllotalpini (*Gryllotalpa* sp. + *Neocurtilla* sp.), Conocephalinae (*Austrosalomona* sp. + *C. microprora* + *c. rhinocerous* + *Eschatocerus* sp. + *Conocephalus* sp.), *Pseudophyllinae* (*A. circumdatus* + *Scopioricus* sp.), and Phaneropterinae (*Orophus* sp. + *S. furcata*). The grouping of *Brachytrupes* sp. (Brachytrupinae) with the Gryllinae is consistent with the classifications of Chopard (1967) and Otte and Alexander (1983). The position of the tettigoniid conocephaline tribe Copiphorini (*C. microprora* +

Table 5
Major clades found by separate and combined maximum parsimony analyses, with indices of support

Clade	18S rRNA	28S rRNA	16S rRNA	Concatenated rRNA
Ensifera	In MPT consensus Bootstrap = 100% Decay = 17	No support relative to only 1 outgroup *Locusta*	In MPT consensus Bootstrap = 100% Decay = 14	In MPT consensus Bootstrap = 99%, Decay = 12 BPP = 100
Grylloidea	Not in MPT consensus Bootstrap = 60%	In MPT consensus Bootstrap = 69% Decay = 5		In MPT consensus Bootstrap = 96% Decay = 13, BPP = 100 + *Comicus*: BPP = 93
Grylloidea + Schizodactylidae + Rhaphidophoridae				In MPT consensus Bootstrap <50% Decay = 3, BPP = 98
Grylloidea + Schizodactylidae + Rhaphidophoridae + Tettigoniidae				In MPT consensus Bootstrap <50% Decay = 2, BPP = 100
Gryllacrididae + Stenopelmatidae + Anostostomatidae (''Gryllacridoidea'')			In MPT consensus Decay = 4	In MPT Consensus Bootstrap < 50% Decay = 3, BPP = 100
Gryllotalpidae	In MPT consensus Bootstrap = 96% Decay = 3	In MPT consensus Bootstrap = 90% Decay = 6	In MPT consensus Bootstrap = 69% Decay = 8	In MPT consensus Bootstrap = 100% Decay = 17, BPP = 100
Gryllidae	Did not include *Malgasia*, *Ornebius* or *Myrmecophilus*	Did not include *Ornebius*	In MPT consensus Bootstrap = 50% Decay = 5	Not in MPT consensus BPP = 88
Gryllinae	In MPT consensus Bootstrap = 100% Decay = 9	In MPT Consensus Bootstrap = 62% Decay = 2	In MPT consensus Bootstrap = 84% Decay = 7	In MPT consensus Bootstrap = 99% Decay = 12, BPP = 100
Gryllinae + Brachytrupinae	In MPT consensus Bootstrap = 100% Decay = 26			In MPT consensus Bootstrap = 100% Decay = 38, BPP = 100
Nemobiinae	In MPT consensus Bootstrap = 100% Decay = 15	In MPT Consensus Bootstrap = 96% Decay = 11	In MPT consensus Bootstrap = 90% Decay = 11	In MPT consensus Bootstrap = 100% Decay = 31, BPP = 100
Podoscirtinae	In MPT consensus Bootstrap = 99% Decay = 10		In MPT consensus Bootstrap = 74% Decay = 4	In MPT consensus Bootstrap = 99% Decay = 11, BPP = 100
Oecanthinae	In MPT consensus Bootstrap = 91% Decay = 7			In MPT consensus Bootstrap = 99% Decay = 16, BPP = 100
Tettigoniidae	In MPT consensus Bootstrap = 70% Decay = 3			In MPT consensus Bootstrap = 64% Decay = 3, BPP = 100
Conocephalinae	In MPT consensus Bootstrap = 92% Decay = 4	Did not include *Conocephalus*		Not in MPT consensus BPP = 93
Pseudophyllinae	In MPT consensus Bootstrap = 98% Decay = 4	In MPT consensus Decay = 1		In MPT consensus Bootstrap = 88% Decay = 6, BPP = 100
Phaneropterinae	Grouped with Meconematinae			In MPT consensus Bootstrap = 93% Decay = 8, BPP = 100
Stenopelmatidae		In MPT consensus Decay = 1		
Anostostomatidae				In MPT consensus Bootstrap < 50% Decay = 3, BPP = 99
Gryllacrididae	In MPT consensus Bootstrap = 93% Decay = 8		In MPT consensus Bootstrap = 96% Decay = 12	In MPT consensus Bootstrap = 93% Decay = 5, BPP = 100

Table 5 (*continued*)

Clade	18S rRNA	28S rRNA	16S rRNA	Concatenated rRNA
Rhaphidophoridae	In MPT consensus Bootstrap = 100% Decay = 11	In MPT consensus Bootstrap = 67% Decay = 4	In MPT consensus Bootstrap = 69% Decay = 7	In MPT consensus Bootstrap = 62% Decay = 2, BPP = 100

The initials "BPP" in the right column (concatenated data) indicate that the clade was also supported by a Bayesian analysis for the partitioned data; the number that follows is the posterior probability for the clade (computed in PAUP as a majority rule consensus of post burn-in trees).

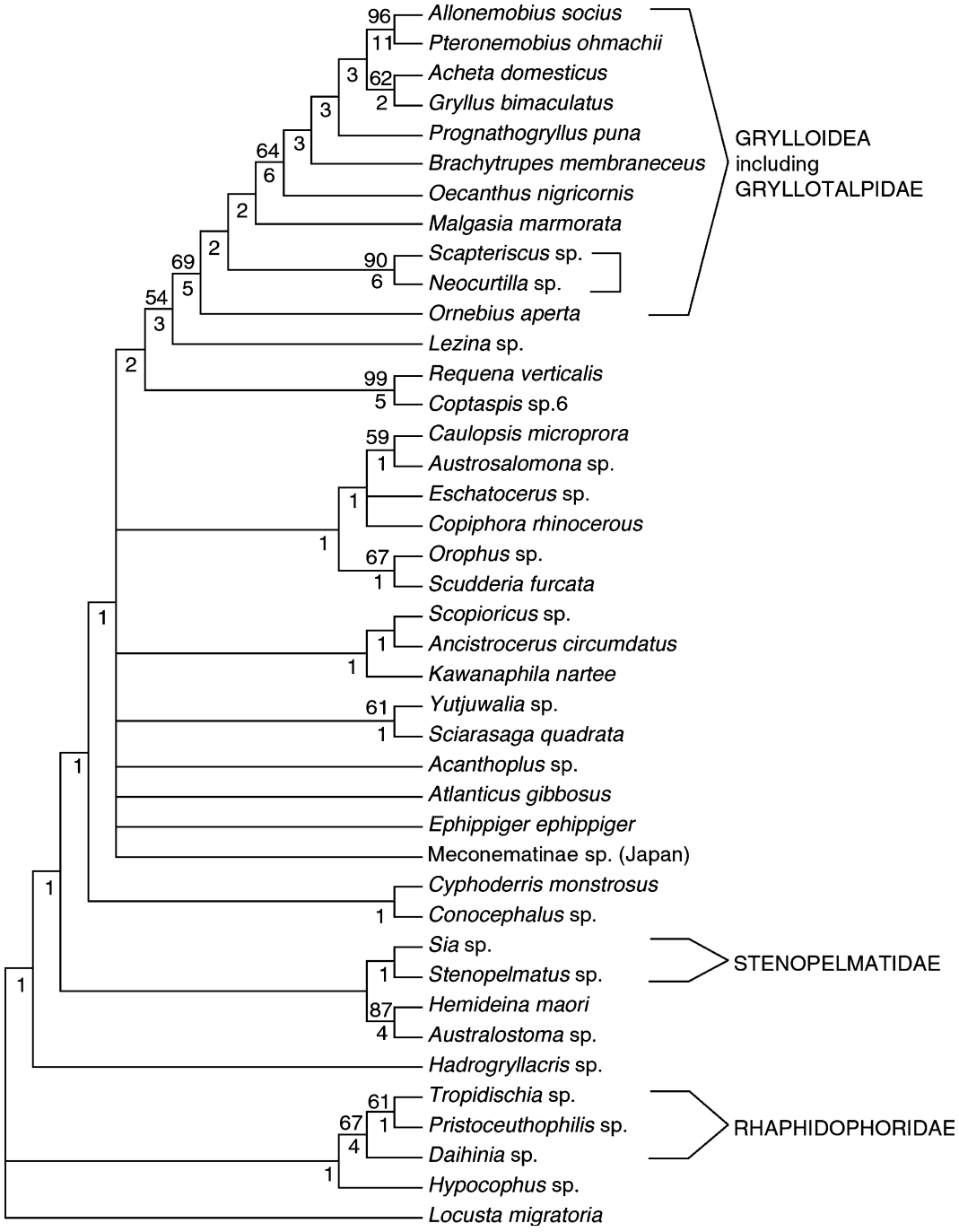


Fig. 10. 28S, strict consensus of 23 most parsimonious trees ($L = 459$; CI = 0.5664, RI = 0.6128.). Bootstrap values above branches, decay indices below.

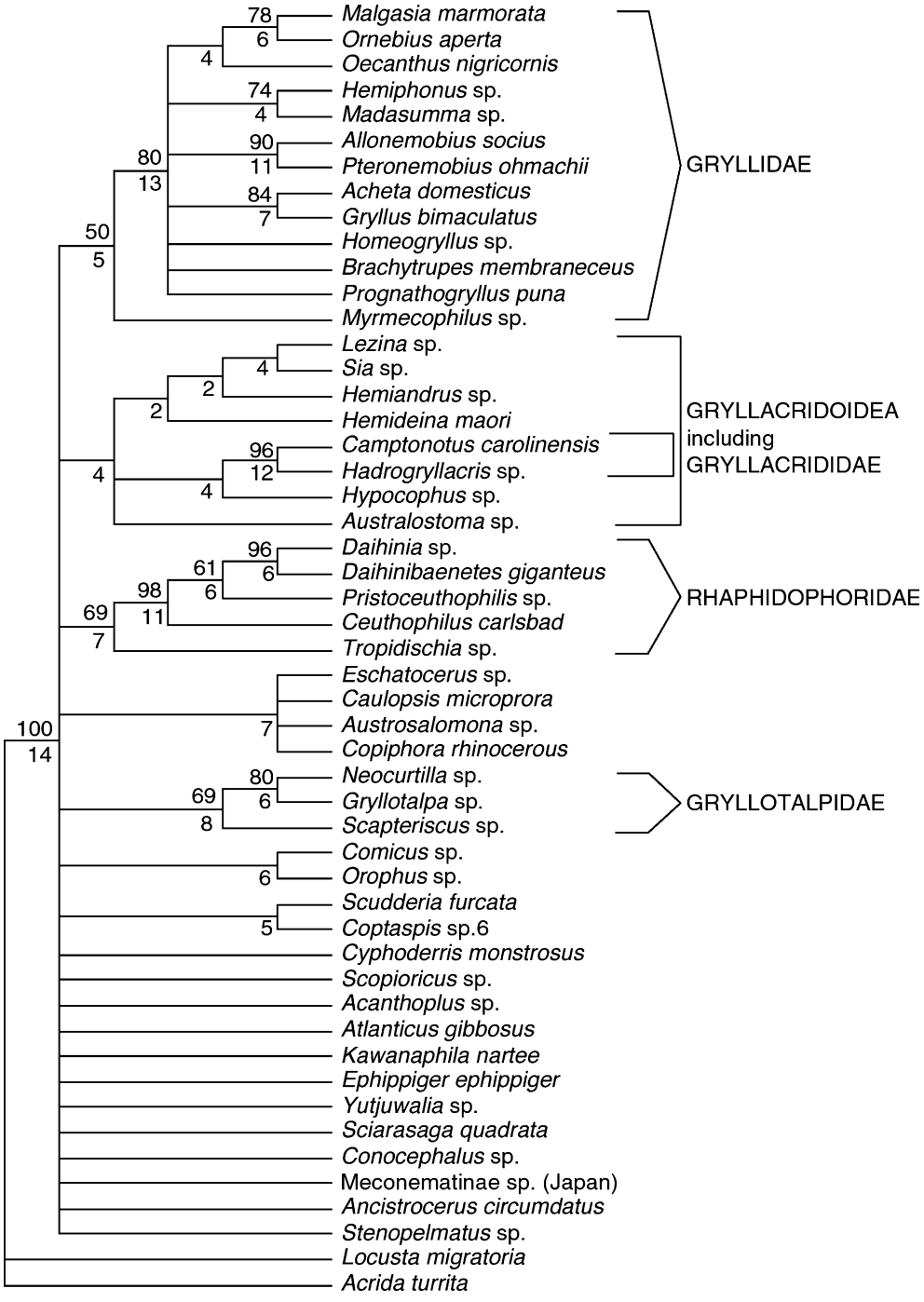


Fig. 11. 16S, strict consensus of 21 most parsimonious trees ($L = 2340$; CI = 0.2726; RI = 0.3984). Bootstrap values above branches, decay indices below.

C. rhinocerous) suggests they may have been derived from within the Agraceiini; other relationships within the Tettigoniidae are consistent with morphological analyses by Naskrecki, 2000 (Ph.D. Thesis).

3.3. Phylogeny reconstruction: Bayesian inference, partitioned data

Four Markov chains of more than 1.5 million generations on the partitioned data reached a log-likelihood asymptote (“burn-in”) at around generation 14,000. A majority rule consensus of the 1,50,273 trees saved to disk after the burn-in is given in Fig. 13 (posterior probabilities above branches). This tree is identical to two other Bayesian replicates of 1.5 million generations each and posterior values from those replicates did not deviate more than 1%. The Bayesian tree is nearly congruent with the strict consensus of the most parsimonious trees found for the concatenated data. The Bayesian tree found the Ensifera to be monophyletic relative to the four caeliferan outgroup taxa (100%), and found a monophyletic Grylloidea *sensu* Gwynne (1995) including Gryllidae, Gryllotalpidae,

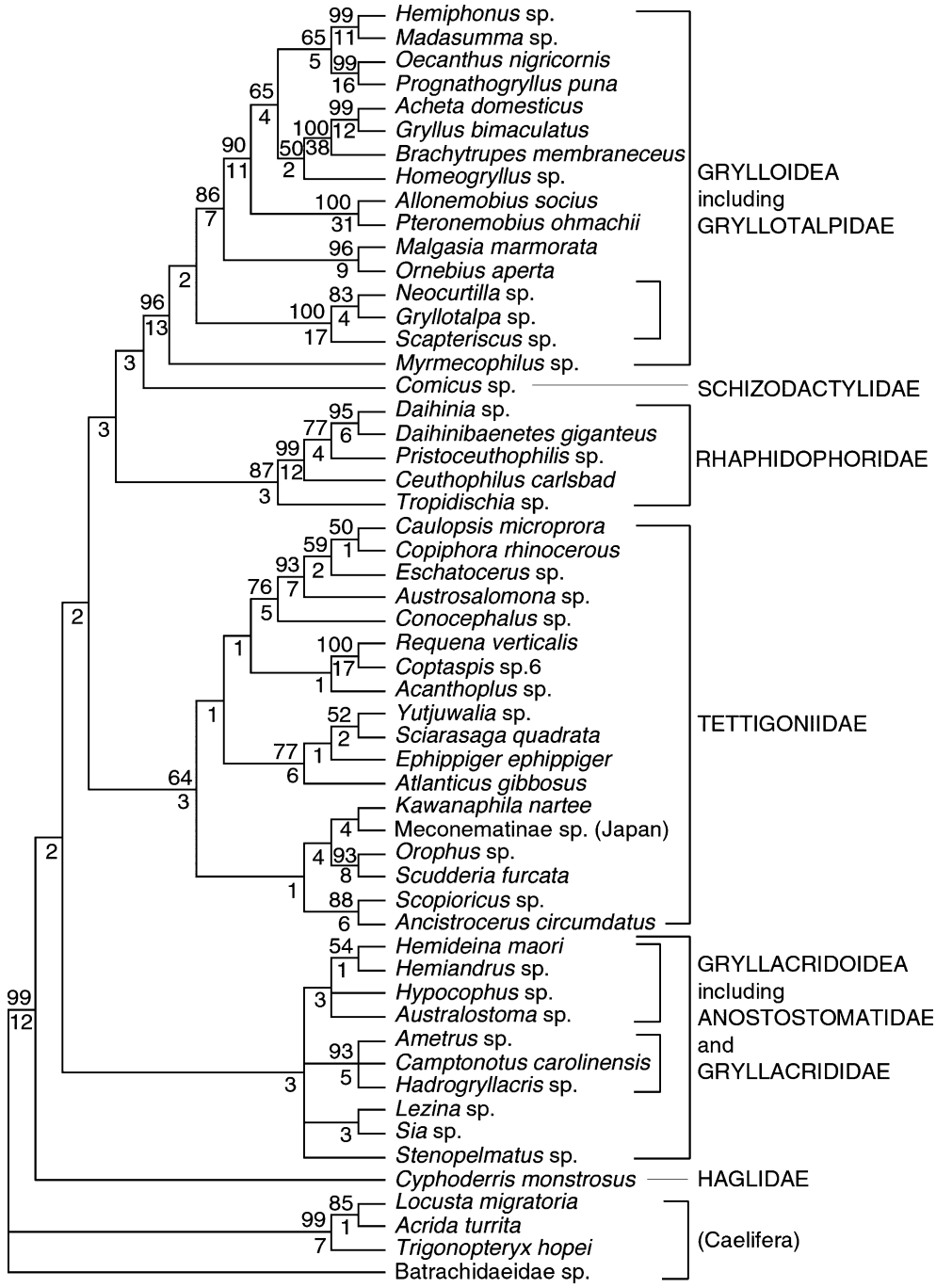


Fig. 12. Concatenated 18S, 28S, and 16S, strict consensus of nine most parsimonious trees ($L = 4349$; CI = 0.4029; RI = 0.4872). Bootstrap values above branches, decay indices below.

and Schizodactylidae (93%). Strong support was also found for a basal Gryllacridoidea (100%) including Stenopelmatidae, a monophyletic Anostostomatidae (99%), Gryllacrididae (100%), and *Lezina*. Additional clades included Tettigoniidae (100%), Rhaphidophoridae (100%), Gryllotalpidae (100%), Gryllotalpini (100%), *Malgasia* + *Ornebius* (100%), Nemobiinae (100%), Podoscirtinae (100%), Oecanthinae (100%), Gryllinae + *Brachytrupes* (100%), Gryllinae (100%), Pseudophyllinae (100%), Phaneropterinae (100%), and Conocephalinae (100%). The Rhaphidophoridae were placed as the sister clade to the Grylloidea + Schizodactylidae (98%). The haglid *Cyphoderris* was recovered as the basal (sister) taxon to all other Ensifera, but with a low posterior value (57%).

A summary of all clades that were found by separate and combined analyses, and are consistent with the most current classification of Otte and Naskrecki (1997) is presented in Table 5. A simplified phylogenetic hypothesis of the Ensifera based on our combined analysis of all 3 genes, collapsing robust clades to their commonly used family or subfamily names, is given in Fig. 14. With the exception of the position of Myrmecophilinae (usually classified within

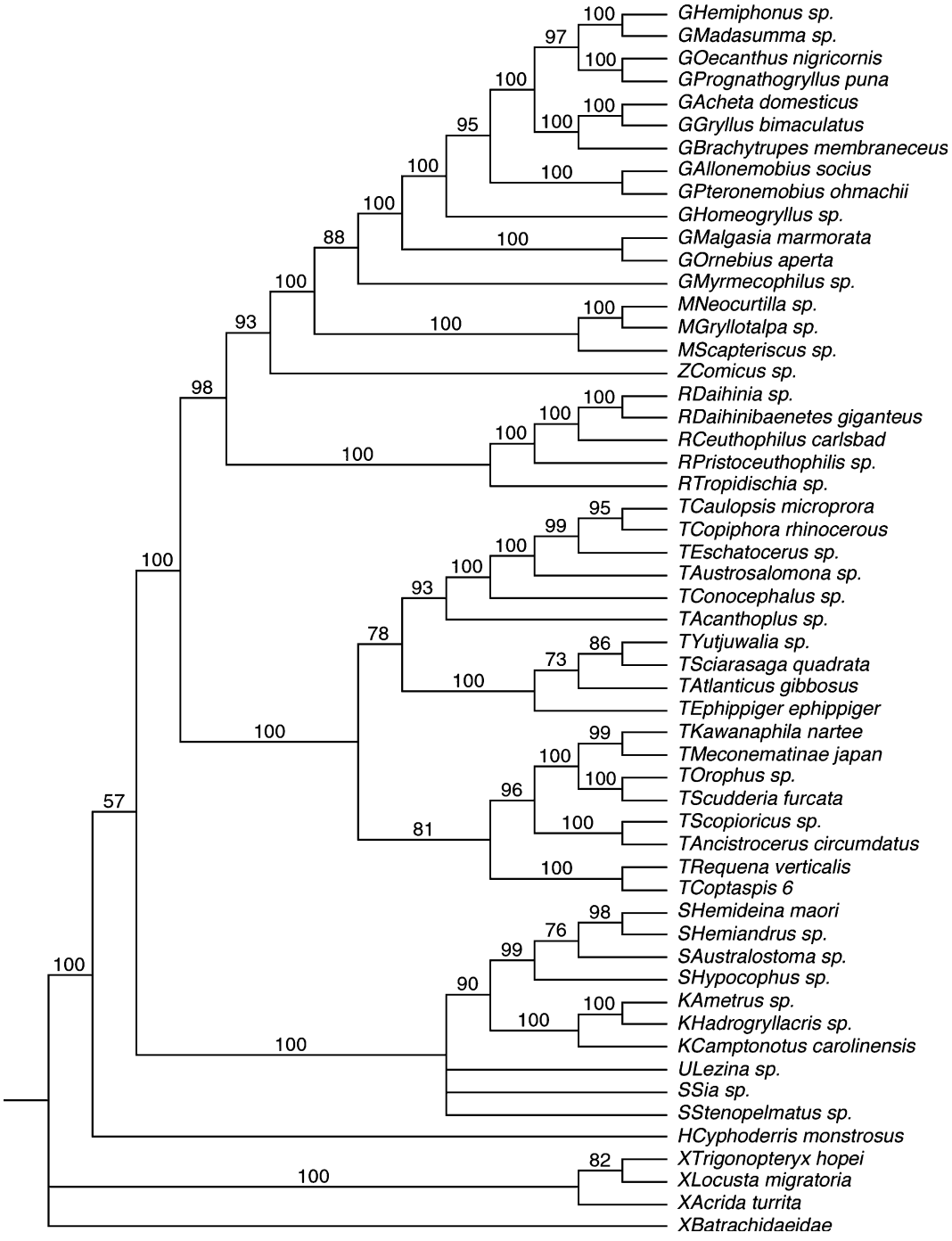


Fig. 13. Partitioned 18S, 28S, and 16S data, Bayesian consensus. Numbers above branches indicate posterior probabilities.

the Gryllidae), this tree is congruent both with the strict consensus of most parsimonious trees for the concatenated data, and with the Bayesian estimate.

3.4. Tree comparisons: molecular data

Separate heuristic searches (1000 replicates with TBR swapping) found 18 most parsimonious trees under family-level structural constraints consistent with Gwynne (1995) ($L = 4574$, CI = 0.412, RI = 0.469), three MPTs under structural constraints consistent with Gorochov (1995a) ($L = 4547$, CI = 0.415, RI = 0.474) and six MPTs under structural constraints consistent with DeSutter (1995) ($L = 4515$, CI = 0.417, RI = 0.481). In all cases, the Templeton, winning-sites, and two tailed Kishino–Hasegawa tests found our most parsimonious estimates (9 trees) to be significantly better at explaining the data than any most parsimonious trees found under constraints consistent with any of the three alternative hypotheses (p values always <0.05). Under the likelihood

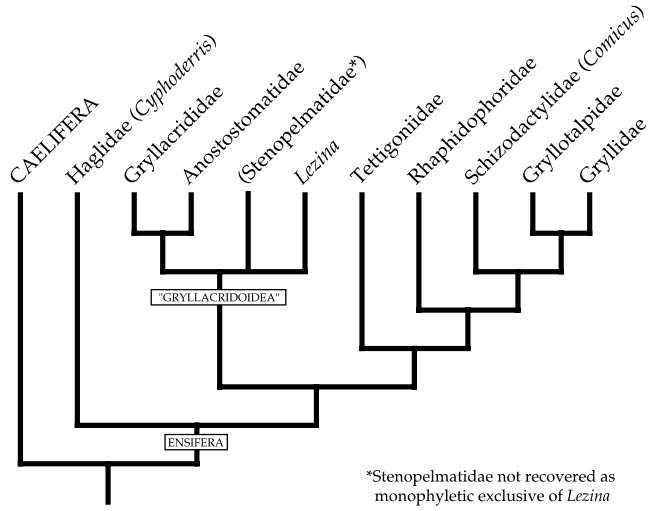


Fig. 14. Simplified version of phylogenetic relationships of Ensifera, as found by our analyses of three molecular loci.

criterion, two-tailed Kishino–Hasegawa tests also found our Bayesian estimate to be significantly better than three alternative trees where the deep branches were rearranged to match the phylogenetic hypotheses of Gwynne (1995), Gorochov (1995a), and DeSutter-Grandcolas (2003) (p values always <0.05). The results and p values for all tree comparison tests are given in Table 6.

3.5. Tree comparisons: morphological and behavioral data

When all characters from Gwynne (1995) were unordered and multistate taxa were interpreted as polymorphism (as Gwynne intended), the most parsimonious tree (Gwynne's) had a length of 121 steps (CI = 0.826; RI = 0.772). Our simplified Bayesian estimate had a length of 149 steps (CI = 0.671, RI = 0.467), regardless of whether Haglidae or our "Gryllacridoidea" clade was placed basal. A fully resolved version of Gorochov's (1995a) estimate that places the Gryllacridoidea (including Schizodactylidae) as the sister to Tettigoniidae + Haglidae had a length of 138 (CI = 0.725, RI = 0.587); both of the other two resolved topologies of Gorochov's (1995a) hypothesis had lengths of 144 (CI = 0.694, RI = 0.522). DeSutter-Grandcolas's (2003) hypothesis had a length of 124 (CI = 0.806, RI = 0.739).

Templeton, winning-sites, and Kishino–Hasegawa tests all found Gwynne's phylogeny to be significantly better than our phylogeny and Gorochov's (1995a) phylogeny ($p < 0.05$), but not significantly better at explaining the data than DeSutter-Grandcolas's (2003) hypothesis (also based on morphology). Results and p values for all tree comparison tests are given in Table 6.

4. Discussion

4.1. Usefulness of ribosomal RNA for phylogenetic inference in Ensifera

Nuclear ribosomal RNA genes are often useful for reconstructing some of the earliest divergences in animals. Data from 18S rRNA have been used to study phylogenetic splits as old as the Cambrian (reviewed in McHugh, 1998 and Winnepenninckx et al., 1998 for Metazoa) and sequences from 28S rRNA have been used to reconstruct

Table 6
p values for tests comparing our parsimony and Bayesian estimates with prior phylogenetic hypotheses for Ensifera

Concatenated molecular data Topology	Kishino–Hasegawa	Templeton	Winning sites	
Tree comparisons: parsimony				
Our most parsimonious trees	Best	Best	Best	
18 MPTs under Gwynne's (1995) constraints	<0.0001*	<0.0001*	<0.0001*	
3 MPTs under Gwynne's (1995) constraints	<0.0001*	<0.0001*	<0.0001*	
6 MPTs under DeSutter-Grandcolas (2003) constraints				
3@	0.0058*	0.0064*	0.0053*	
3@	0.0061*	0.0062*	0.0104*	
Topology	$-\ln L$	$\Delta -\ln L$	p value	
Kishino–Hasegawa tests: likelihood				
Our Bayesian estimate	23647.683	Best	Best	
Our rearranged to match Gwynne (1995)	23679.409	31.726	0.030*	
Our rearranged to match Gorochov (1995a)	23793.010	145.327	<0.001*	
Our rearranged to match DeSutter-Grandcolas (2003)	23691.921	44.238	0.022*	
Topology	Length	Kishino–Hasegawa	Templeton	Winning sites
Morphology and behavior matrix (Gwynne, 1995) (Table 3)				
Ours (simplified to families) Haglidae or Gryllacridoidea basal	149	<0.0001*	<0.0001*	<0.0001*
Gwynne (1995) (origin of the data)	121	Best	Best	Best
Gorochov (1995a), all three resolved alternatives	138	0.0012*	0.0016*	0.0026*
	144	<0.0001*	0.0001*	0.0001*
	144	<0.0001*	0.0001*	0.0001*
DeSutter-Grandcolas (2003)	124	0.3697	0.3657	0.5488

* $p < 0.05$.

divergences from the Paleozoic through the Mesozoic, particularly within arthropods (e.g., Whiting et al., 1997 for holometabola, Hedin and Maddison, 2001 and Hausdorf, 1999 for arachnids, Giribet et al., 1999 for chilopods, Jarman et al., 2000 and Braga et al., 1999 for crustaceans). In our own analyses, the results for the nuclear ribosomal data provide better support for the deepest splits in Ensifera than the 16S data. Pairwise distances and tree branch lengths for our taxa reflected the general tendency for 18S rRNA sequences to be more conserved than 28S rRNA sequences, and for these nuclear genes to be more conserved than the mitochondrial 16S rRNA (see Hillis and Dixon, 1991 for a review of the evolutionary rates and phylogenetic utility of different ribosomal loci). Compared to the Gryllidae, the Tettigoniidae showed relatively poorer clade resolution in the consensus of most parsimonious trees, as well as low bootstrap and decay support indices, for all three single-gene data sets (with some exceptions; see Section 3).

4.2. Phylogenetic relationships and synapomorphies within Ensifera

The Ensifera have usually been viewed as monophyletic, or at least taxonomically distinct from the Caelifera; however, the fossil studies of Sharov (1968) and Gorochov (1995a) both argue that all Orthoptera are descended from the Upper Carboniferous family Oedischiidae, which resembled modern Ensifera more than Caelifera. While this could imply a paraphyletic Ensifera that contains an internally derived Caelifera, our results and those of Flook and Rowell (1997a,b), Rowell and Flook (1998), and Flook et al. (1999) support the status of these two suborders as sister taxa. A monophyletic Ensifera implies that the characters shared by Ensifera should be considered true synapomorphies of this suborder: long, filiform antennae usually longer than the body and composed of more than 30 segments; the elongate sabre- or needle-shaped morphology of ovipositors in females; and auditory tympana, when present, located on the front tibiae. The diversification of Ensifera into its modern families probably occurred prior to the onset of the Cretaceous, since most Triassic and Jurassic ensiferan fossils can be assigned to families with modern living representatives (Carpenter, 1992; Sharov, 1968; Zeuner, 1939). Extant caeliferan lineages are believed to be of similar age, with most extant lineages dating back to the Triassic and Cretaceous (Carpenter, 1992; Sharov, 1968).

We have shown that there is a high degree of conflict between the morphological and molecular data that are available for Ensifera. The morphological and behavioral characters compiled by Gwynne (1995) significantly favor his family-level hypothesis over ours, and cannot reject DeSutter-Grandcolas's, 2003 topology (also based on morphology). However, under parsimony-based tree comparison tests, the combined molecular data from three different genes reject the prior phylogenetic hypotheses of Gwynne (1995), Gorochov (1995a), and DeSutter-Grandcolas (2003). This may indicate a high degree of morphological homoplasy in Ensifera, particularly in regard to acoustic characters (such as tegminal structures) which are likely to evolve under strong selection, and which have been lost several times independently in multiple lineages. Of the 16 characters from Gwynne's (1995) matrix that require additional steps on our topology (characters 7, 8, 9, 11, 16, 18, 22, 26, 27, 28, 29, 33, 43, 47, 52, and 59), seven are characters pertaining either to stridulatory tegmina or auditory organs (characters 9, 11, 18, 22, 26, 27, and 28). Thus, approximately half of the observed conflict is due to acoustic characters. Six of the characters that require additional steps on our tree (characters 8, 16, 43, 47, 52, and 59) are due to character states (absence) shared by Gryllacrididae and our clade of Rhaphidophoridae, Schizodactylidae, Gryllidae, and Gryllotalpidae. In Gwynne's (1995) hypothesis, the presence of these six characters would have been derived within his clade of Stenopelmatidae, Tettigoniidae, and Haglidae.

The phylogeny implied by our parsimony and Bayesian analyses represents a novel hypothesis for ensiferan relationships, one that differs significantly from all prior hypotheses, but contains elements of each. Our phylogeny is simlar to those of Zeuner (1939) and Ragge (1955) (Figs. 2 and 4) in grouping the largest Ensiferan families, Gryllidae and Tettigoniidae, within a clade exclusive of the gryllacridoid taxa. Our finding of Schizodactylidae as sister to the Grylloidea is consistent with Ragge (1955) and Gwynne (1995). Only Judd (1948) left open the possibility of a close Grylloidea/Rhaphidophoridae relationship, placing these groups in a polytomy with Stenopelmatidae + Tettigonioidea (Fig. 3). Our finding of Haglidae (*Cyphoderris monstrosus*) being basal to all other tegminally stridulating Ensifera, may be supported by the fact that haglid-like insects are among the oldest ensiferan fossils (Sharov, 1968) and show an ambidextrous stridulatory configuration, with a file present on both tegmina (gryllids have the file on the right tegmen only, while tettigoniids have it on the left tegmen only). Our finding of a monophyletic Gryllacridoidea is consistent with the classification of Karny (1932) and similar to the phylogenetic hypothesis of Sharov (1968), with the exception that both authors also included the Rhaphidophoridae within this group. Two characters in Gwynne's (1995) morphological matrix which could be synapomorphies of a Gryllacrididae + Stenopelmatidae + Anostostomatidae clade are: numerous tegminal anal veins (25) and tegmina (when present) that wrap around the body (26). These families have several other morphological character states in common, but they are also shared with either Rhaphidophoridae, Tettigoniidae, or both, and most would not require additional steps by the assumption of a Gryllacrididae + Stenopelmatidae + Anostostomatidae clade. Our phylogenetic analyses also proposes a monophyletic Gryllacrididae (albeit based on only three taxa) as a distinct lineage in our putative clade of Gryllacridoidea.

Johns (1997) argued that certain stenopelmatid-like species are morphologically distinct and should be reclassified within a new family, the Anostostomatidae. Our results support this classification, with regard to the genera *Hemideina*, *Hemiandrus*, *Australostoma*, and *Hypocophus*. The position of *Lezina* is particularly interesting since the true phylogenetic affinity of this genus has always been a subject of debate: Kirby (1906) placed *Lezina* (as well as anostostomatids) within the Stenopelmatidae; Karny (1932) considered it to be closely related to the Stenopelmatidae; Johns (1997) recognized the Lezinidae as a separate gryllacridoid family; and Otte (2000) classified it as a genus within the Gryllacrididae. Our results are most consistent with Karny (1932) and Johns (1997), in that *Lezina* is closely related to the Stenopelmatidae; however, since the Stenopelmatidae are not resolved by our phylogeny, it is unknown whether *Lezina* is well nested within this group or belongs to a separate lineage.

The position of Gryllotalpidae as the sister family to the Gryllidae is consistent with virtually every other prior study of ensiferan phylogeny, based on a number of characters relating to wing venation, tibial spines, abdominal morphology, and male sexual anatomy. The grouping of *Gryllotalpa* with *Neocurtilla* (both members of the tribe Gryllotalpini) is consistent with current morphology-based classification which defines the Gryllotalpini as having four tibial dactyls (*Scapteriscus* has 2). Within the Gryllidae, several relationships we found are consistent with prior and/or current morphology-based classification (e.g., Chopard, 1967; Chopard, 1968; Otte and Alexander, 1983; Otte, 1997a) and the morphological phylogenetic work of DeSutter (1987) which used genitalic characters. All expected groupings of exemplars within putative subfamilies were supported, with high bootstrap values and decay indices. A close relationship between gryllines and brachytrupines is consistent with the classification schemes of Chopard (1967) and Otte and Alexander (1983), who did not recognize the Brachytrupinae as a distinct subfamily, but rather placed them within the Gryllinae. A Gryllinae–Brachytrupinae–Podoscirtinae clade, with the Homeogryllinae as an outgroup, is consistent with the phylogenetic hypothesis of DeSutter (1987), although the positions of Nemobiinae and Oecanthinae in our analyses do conflict with DeSutter's topology. For gryllids, our parsimony analysis for the 28S rRNA data is more consistent overall with DeSutter's phylogeny, and this result may require further invesigation; however, in the absence of any strong criteria to guide the proper placement of these subfamilies, we give here their positions as suggested by our combined analyses for all three genes, with the caveat that our 28S rRNA analysis did lend some support to DeSutter's placements of the Oecanthinae and Nemobiinae. Finally, our results for a clade of Mogoplistinae and Malgasiinae are in concordance with DeSutter (1987), who argued that these were sister families, and placed them into a new superfamily, her Mogoplistoidea.

We found a monophyletic Tettigoniidae in our analysis of 18S, and in both parsimony and Bayesian analyses for all three loci. Parsimony bootstrap support for this family was low (<70%) but the posterior support for Tettigoniidae in the Bayesian tree was 100%. Overall, there is good reason to be confident about the monophyly of this group, which is also supported by morphological work by Naskrecki (Ph.D. Thesis, 2000). However, we are less confident about our results for relationships between tettigoniid subfamilies, since these results were not robust in the parsimony analyses. Nevertheless, two of our findings from some analyses are consistent with Naskrecki's phylogeny, namely: a paraphyletic Listroscelidinae (*R. verticalis* and *Yutjuwalia* sp. never clustered in any analysis); and a close relationship between Zaprochilinae and Meconematinae, which were closely related in our Bayesian estimate. However, we believe that branching relationships between tettigoniid subfamilies would be better resolved in the future with additional data, and a larger taxon sample than the one we present.

4.3. The evolution of acoustic structures and communication

Male tegminal stridulation is not the only type of stridulation utilized by Ensifera. In the Stenopelmatidae, Anostostomatidae, Rhaphidophoridae, and Gryllacrididae, femur-abdomen stridulation may be used for both courtship and defense, although the Gryllacrididae appear to use femoral-abdominal stridulation for defensive purposes only (reviewed in Rentz, 1996). Within these four groups, the only taxa that possess tibial tympana are some femur-abdominal stridulating stenopelmatids. One hypothesis proposed by DeSutter-Grandcolas (2003) was that femur-abdomen stridulatory mechanisms in these groups may have evolved after tegminal stridulation was lost. In addition, novel stridulatory structures and mechanisms have apparently evolved multiple times in female tettigoniids, involving modified areas of the wing or other body parts not homologous with the stridulatory morphology of males.

Most studies have viewed tegminal stridulatory morphology and tibial tympana of Ensifera as being too similar in structure and position to have evolved more than once in this suborder (Alexander, 1962; Otte, 1992; Ragge, 1955; Sharov, 1968; Zeuner, 1939). In all tegminally stridulating Ensifera, the presence of a toothed stridulatory file on a vein in the rear region of the front wing, as well as the presence of ear-like auditory tympana on both front tibiae, fulfill at least the structural and positional criteria for homology of these characters. Permian representatives of Ensifera (the extinct family Oedischiidae) appear to have an S-shaped stridulatory vein in the anal/cubital area of the wing (Sharov, 1968), which is where it is found in extant tegminally stridulating Ensifera, offering further support that this character may be plesiomorphic.

Ensiferan acoustic characters satisfy the homology criteria of structural similarity, positional similarity, developmental identity (if the stridulum exists on the same vein;

but see below), and non-redundancy in a single organism. However, some researchers have pointed out differences between grylloids and tettigoniids which may indicate multiple origins of tegminal stridulation (reviewed in Gwynne, 1995). For example, tettigoniids bear the stridulum on the left tegmen while gryllids have it on the right. Thus, tettigoniids always stridulate with the left tegmen over the right, while most grylloids stridulate right-over-left. The haglid stridulatory configuration (as in *Cyphoderris*) is ambidextrous, in that males can stridulate with either tegmen. Based on our phylogenetic results which give Bayesian support for a Haglidae basal to all other tegminally stridulating Ensifera, the ambidextrous configuration in Haglidae may represent an ancestral condition that led to both the left-over-right arrangement seen in tettigoniids, and the right-over-left arrangement found in gryllids. Triassic fossils of haglid-like insects (Prophalangopsidae, sometimes used synonymously with Haglidae) appear to be among the earliest known fossils of Ensifera (Carpenter, 1992; Zeuner, 1935, 1939), and both Gorochov (1995b) and Morris and Gwynne (1978) have viewed Haglidae as being the "ancestral" Ensiferan stock, describing haglid morphology as "primitive."

It has also been argued that both the stridulatory file and tibial auditory organs show considerable anatomical differences between grylloids and tettigoniids, despite their positional and mechanical similarities. Although there has been a long-standing debate as to whether the stridulatory file of tettigoniids exists on the same wing vein as that of grylloids, each of these groups has been described at one time or another as having the stridulatory file on the 2nd cubital vein, or else on the 1st anal vein. Zeuner (1939) identified the stridulatory vein in grylloids and tettigonioids to be homologous as the 1st anal vein (1A); Ragge (1955) also argued for homology of the vein, but identified it as the 2nd cubital vein (Cu2), noting the fusion of 1A with Cu2 near the stridulum (as also described by Ander, 1939). Sharov's opinion (1968) was that the stridulatory vein was 1A in Tettigoniidae, but Cu2 in grylloids and Haglidae. These conflicting views do not reflect diversity in the families, but rather, disagreements between researchers on which vein is involved, following the insect wing nomenclature of Comstock (1918). Therefore, in order to question whether the vein bearing the stridulatory file is the same in these two groups, one must contend with inconsistent opinions of vein identity—a subjective problem that undermines the exercise. Even if some consensus is reached on vein identity in tegminally stridulating Ensifera—perhaps through developmental work—we do not think that the identity alone (1A or 2Cu) of the stridulatory vein in tettigonioids compared to grylloids provides a strong criterion for homology of this structure, since these veins are immediately adjacent to each other on the wing and are even fused together in *Cyphoderris* and some tettigoniids; Ragge, 1955.

Prior studies of the Ensifera have attempted to integrate phylogeny and comparative morphology with a parsimony based model of evolution, often to determine the origin (and thus, homology) of tegminal stridulation and tibial tympana, even though the acoustic structures in question were often used (problematically) to reconstruct the phylogeny in the first place (e.g., Ragge, 1955; Sharov, 1968; Zeuner, 1939). In his study of fossil Ensifera using mainly fossil wing characters, Zeuner (1939) placed the Grylloidea as the sister group to a haglid–tettigoniid clade, and argued for a single origin of tegminal stridulation in the Ensifera, at the base of that clade. The similar phylogeny of Ragge (1955) would suggest that tegminal stridulation was plesiomorphic (and homologous) for all tegminally stridulating Ensifera, and was lost only once within the Schizodactylidae. Sharov's (1968) hypothesis, which doesn't propose a grylloid–tettigoniid–haglid clade, nevertheless implies a single ancestral origin of tegminal stridulation (in the fossil family Oedischiidae, which, he argued, had a stridulatory vein) followed by a single loss at the root of a derived Gryllacridoidea. However, other phylogenies proposed by Ander (1939), Judd (1948), and Gwynne (1995) put greater distance between the Grylloidea and the Tettigonioidea, requiring one to choose between alternative scenarios of multiple gains or multiple losses of tegminal stridulation, and thus, drawing into question the homology of the stridulatory apparatus and auditory tympana.

In phylogenetic studies, both Gwynne (1995) and DeSutter-Grandcolas (2003) invoked parsimony to conclude that tegminal stridulation and tibial tympana both had multiple origins in Ensifera, since such scenarios require fewer evolutionary changes than if Ensifera had a plesiomorphic acoustic condition followed by multiple subsequent losses at deep family levels. However, it has been demonstrated that reconstructing ancestral states using parsimony and an assumption of equally likely gains and losses becomes less and less reliable as one or both of the following variables are increased: (1) the total amount of evolution on the tree (or the probability of change on any given branch—a measure of homoplasy) (Schluter et al., 1997) and/or (2) the actual loss/gain ratio at which the character evolves (Cunningham et al., 1998; also Ree and Donoghue, 1998, 1999). We believe that both of these variables are elevated in the Ensifera, with regard to the evolution of acoustic characters. With respect to both variables, even Gwynne's less-parsimonious alternative of five secondary losses of acoustic characters (for example) is a severe underestimate of the frequency at which acoustic characters have been lost in the Ensifera—primarily within the Grylloidea, in which tegminal stridulation, stridulatory files, tegmina, and/or tibial auditory organs are missing from at least 75 phylogenetically diverse genera (DeSutter-Grandcolas, 1997; Huber et al., 1989; Otte, 1992, 1994; Walker and Masaki, 1989). Overall, there is strong reason to believe that acoustic characters are under strong selection and undergo frequent evolution in Ensifera, and that an elevated amount of homoplasy is to be expected—particularly among taxa that have lost acoustic characters in parallel. Both of these factors would greatly

decrease the accuracy of parsimony-based ancestral state reconstructions.

We also believe that the assumption of equally probable gains and losses of acoustic characters is not well founded: partially based on the fact that secondary losses in Ensifera seem to be commonplace, but also based on the inherent nature of complex traits (see Cunningham et al., 1998). The acoustic complex in Ensifera is not a simple trait that could have evolved in a single "gain," because it involves at least six functionally integrated components, both morphological (stridulatory file, tibial tympana, resonating or amplifying tegminal areas), and behavioral (tegminal stridulation, female phonotaxis, temporal control of song structure). The evolution of the acoustic complex in Ensifera must therefore have occurred in stages, as selection favored the specialization of multiple components that had a cumulative effect on fitness. In contrast to gains, the loss of the acoustic mating system only requires the loss or degeneration of a single component, after which the entire complex ceases operating and contributing to fitness (at least, as a mating system). Once the complex is no longer functional, its components could be lost readily and rapidly as selection favored conservation of energy.

Our finding of a basal position for Haglidae, which are ambidextrous stridulators with tibial tympana, is consistent with the fossil record and suggests an ancestral acoustic condition of Ensifera that was subsequently lost multiple times, particularly within the Gryllidae. Alternative stridulatory mechanisms (such as femur-abdomen stridulation) would then have evolved secondarily in some lineages where stridulatory tegmina were lost. However, despite a long history of phylogenetic studies on ensiferan relationships, and several attempts to examine the evolution of acoustic communication in this group within a phylogenetic context, the lack of a consensus among morphological workers on the homology of acoustic structures makes it doubtful that phylogeny alone—even a very robust one with a large taxon sample—is sufficient for reconstructing the evolution of acoustic communication in Ensifera. More specifically, we believe that phylogeny and parsimonious character optimizations cannot be used as the primary tests of character homology, especially in situations where other criteria of homology are satisfied, as they are in ensiferan acoustic characters. Although modern definitions of homology usually contain some criterion of common ancestry, we do not believe that ancestral inference alone provides a sound test of homology, especially when the method used to reconstruct the ancestral state is prone to error or when systematists disagree about the identity and degree of similarity of complex structures. We feel that molecular data has brought a much-needed perspective to the study of ensiferan lineages that are largely defined by their acoustic morphology; however, for the future, we believe that studies of Ensifera (and other organisms) could best address the problem of character homology through a multidisciplinary approach that more closely integrates phylogenetic methods with both morphological and developmental studies.

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