

Variation and the Phylogenetic Utility of the Large Ribosomal Subunit of Mitochondrial DNA from the Insect Order Hymenoptera

JAMES N. DERR,* SCOTT K. DAVIS,* JAMES B. WOOLLEY,† AND ROBERT A. WHARTON†

*Department of Animal Sciences and †Department of Entomology, Texas A&M University, College Station, Texas 77843

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Nucleotide sequence variation from a 573-bp region of the mitochondrial 16S rRNA gene was determined for representative hymenopteran taxa. An overall bias in the distribution of A and T bases was observed from all taxa; however, the terebrants (parasitoids) displayed significantly lower AT ratios as well as a higher degree of strand asymmetry. Moreover, a strong positive correlation was observed between relative AT richness and sequence divergence, suggesting selection at the nucleotide level for A and T bases as well as functionality. Overall sequence difference ranged from 2.3 to 53.4%, with the maximum divergence between members of the two Hymenopteran suborders. These data were used in a phylogenetic analysis to illustrate the utility and degree of resolution provided by this information at various hierarchical levels within this taxonomically diverse order. Parsimony analysis revealed strong evidence for monophyly of the aculeates and the terebrants. Most noteworthy was a strongly supported clade containing the two terebrant superfamilies Icheumonoidea and Chalcidoidea. Conversely, high sequence divergence values resulted in instability at the base of the tree and limited resolution at the higher taxonomic levels. Nevertheless, these results do identify those taxonomic levels for which 16S rRNA sequences are phylogenetically informative. © 1992 Academic Press, Inc.

INTRODUCTION

The Hymenoptera is one of the more diverse and thoroughly studied insect orders. Traditionally, this order is divided into two suborders, Symphyta and Apocrita, that together contain some 110,000 described species. The Symphyta is composed of the sawflies, horn-tails, and parasitic wood wasps, whereas the Apocrita contains the majority of the parasitic Hymenoptera, the ants, solitary and social wasps, and bees. The evolution of parasitism and social behavior in this order has generated considerable interest, and recently Carpenter (1989) proposed that phylogenetic methods may

provide pivotal information for investigating these questions. Nevertheless, clearly supported and robust models of the phylogenetic relationships for major components of the Hymenoptera are lacking.

The most recent and comprehensive investigations regarding hymenopteran phylogeny are those of Königsmann (1976, 1977, 1978a,b) and Rasnitsyn (1980, 1988). A number of other recent investigations have examined either specific superfamilies within the Hymenoptera (Brothers, 1975; Carpenter, 1986; Gibson, 1986) or various character systems across taxa (e.g., Saini and Dhillon, 1980; Gibson, 1985; Darling, 1988; Johnson, 1988; Whitfield *et al.*, 1989). While these studies have generally increased our understanding of the morphological basis for interpreting relationships, there is nonetheless little congruence among researchers regarding higher classification.

Composition of the Symphyta varies depending on whether the Cephidae (Königsmann, 1977) or the Orusidae (Rasnitsyn, 1980) are removed and placed as the sister group to the Apocrita. Even when both are retained in the Symphyta (the traditional arrangement), there are problems in establishing a symphytan lineage based on shared, derived features relative to the Apocrita (Gibson, 1986; Gauld and Bolton, 1988; Johnson, 1988) and the group is now widely regarded as paraphyletic (Gauld and Bolton, 1988). More detailed resolution of the Symphyta is crucial for understanding the origins of the Apocrita and in particular for establishing character polarities within Apocrita using outgroup arguments.

The Apocrita are generally interpreted as monophyletic largely on the basis of the fusion of the first abdominal segment to the thorax. It is usually subdivided into the Aculeata and the Terebrantes (Parasitica). The aculeate Hymenoptera (ants, solitary and social wasps, and bees) almost certainly form a monophyletic group (Brothers, 1975; Carpenter, 1986); however, serious questions remain regarding the monophyly of the terebrants ("parasitoids") due to the lack of demonstrated synapomorphies.

The parasitoids belong to at least nine currently recognized superfamilies whose relationships and compo-

sition are the subject of some debate (Königsmann, 1978a; Rasnitsyn, 1980, 1988; Naumann and Masner, 1985; Gibson, 1986; Gauld and Bolton, 1988). Of particular interest here is the recent suggestion that the Aculeata and the terebrant superfamily Ichneumonoidea (Ichneumonidae + Braconidae) are sister groups (Rasnitsyn, 1988; Mason, unpublished observation).

Two attributes of the order Hymenoptera make it particularly appealing for investigations using molecular techniques. First, although a number of morphological-based studies are available for various groups within this order, few provide well-supported phylogenetic hypotheses. For example, many of these studies are not based on sound phylogenetic methods and even when they are, reductional features (such as loss of wing veins), which are commonplace throughout the order, often confuse attempts to assess character homology. In addition, lack of agreement on even fundamental relationships hinders the interpretation of character polarity. Second, the very small size of many of the parasitic wasps (many <1 mm) prevents a molecular genetic evaluation due to the lack of sufficient material. However, techniques such as polymerase chain reaction (PCR) offer new and powerful tools that promise to provide new information that will greatly facilitate future systematic research on this group of insects.

In this study we use PCR to illuminate DNA sequence variation from the large ribosomal subunit (16S rRNA) of the mitochondrial genome from members of six superfamilies representing both suborders (Symphyta and Apocrita) of Hymenoptera. These data are used in a phylogenetic analysis to illustrate the utility and degree of resolution provided by this information at various hierarchical levels within this taxonomically diverse order. For comprehensive reviews of the utility of mitochondrial DNA (mtDNA) for evolutionary studies see the work of Moritz *et al.* (1987) and Harrison (1989).

METHODS AND MATERIALS

DNA Extraction and Purification

DNA was extracted from frozen (-80°C) or ethanol-preserved specimens using the proteinase K digestion protocol of Maniatis *et al.* (1982). Final DNA pellets were washed in ice-cold 70% ethanol, redissolved in sterile water, and stored at 4°C . From the small-bodied parasitic species, DNA was isolated following whole-body digestion of 5–15 individuals from a single isolate. Head, thorax, or larva provided the tissue source from larger specimens. High-molecular-weight DNA of sufficient quality for PCR was recovered from both frozen and alcohol-preserved samples. Refer to the Appendix for details regarding the specimens examined.

DNA Amplification

At the time we initiated this study, few oligonucleotide primers were available for amplified specific DNA sequences from insect taxa. We used an edited version of the primers first designed by T. Kocher (personal communication) to amplify a region of the 16S rRNA gene. These primers were chosen because they produced consistent PCR products from a number of different hymenopteran taxa. Primer sequences are represented by the light (L) or heavy (H) strand of mtDNA and their position relative to the complete *Drosophila* mtDNA sequence (Clary and Wolstenholme, 1985). Both primers were constructed with 5' terminal *EcoRI* restriction enzyme sites plus one additional base to facilitate plasmid cloning of the amplified products. The sequences of these two primers are as follows: primer 16saEcoRI (L-12,883), 5'-GGAATTCCTCCGGTTGAACTCAGATC-3'; and primer 16sbEcoRI (H-13,398), 5'-GGAATTCCTCCGCTGTTTATCAAAAACAT-3'. Subsequently, "nested" primers were developed based on our initial sequence in comparison with the published 16S rRNA sequences from *Drosophila* (Clary and Wolstenholme, 1985), *Aedes* (Hsu-Chen *et al.*, 1984), and *Apis* (Vlasak *et al.*, 1987). The sequences of these primers are 16saW (L-12,943), 5'-TAATAATCAAACATAGAGTTCG-3'; and 16sbW (H-13,332), 5'-GACTGTTCAAAGGTAGCATAAT-3'.

Template DNAs were amplified in 50 or 100- μl total reaction volumes with 30–35 PCR cycles (Saiki *et al.*, 1988). Most primer/template combinations were amplified using the thermal stable DNA polymerase *Taq* (International Biotechnologies, Inc.) although Vent DNA polymerase (New England BioLabs) was also used. All amplifications were performed with a Perkin Elmer-Cetus thermal cycler with the following protocol: 35 cycles of DNA denaturing at 93°C for 30 s, primer annealing at 42 – 50°C for 60 s, and primer extension at 72°C for 2 min. With some template/primer combinations the insertion of a 4-min step cycle between annealing and extension increased the yield of the final amplification product. One explanation may be the rapid stabilization of partially mismatched primer/template complexes by the DNA polymerase as the reaction slowly warms from the annealing temperature to (42 – 50°C) to the extension temperature (72°C).

Following PCR, the amplified products were separated by electrophoresis in 1.5% agarose minigels (Maniatis *et al.*, 1982). Gels were stained with ethidium bromide and the DNA was visualized by fluorescence under uv light. Amplified products were concentrated using Centricon 30 microconcentrators (Amicon Div., W.R. Grace) by washing with 2 ml of ddH₂O through three rounds of centrifugation (5000 rpm, 3000 rcf) with a Sorvall (Du Pont) RC-5 high-speed centrifuge using a SA-600 rotor. Typically, 50 μl of PCR product

was recovered after inversion of the Centricon 30 tube and centrifugation at 750 rpm (80 rcf) for 3 min.

Amplified products were cloned into plasmid vectors using two different protocols. First, following concentration, PCR products and supercoiled pUC plasmids were digested with *EcoRI* and then ligated to form circular hybrid DNA molecules, transformed into competent bacterial cells (GIBCO BRL), and screened for recombinant clones. Other PCR products were ligated, transformed, and grown using the TA Cloning kit (Invitrogen) following the manufacturer's directions. Although recent studies document a low error rate frequency for *Taq* polymerase in PCR (Eckert and Kunkel, 1990; Kwiatowski *et al.*, 1991), we sequenced two to six clones from each individual to ensure polymerase fidelity. No PCR-based errors were observed. As an additional test of polymerase accuracy, we used purified double-stranded PCR products in combination with unbalanced priming to generate single-stranded product for direct sequencing from selected individuals following the methods of Allard *et al.*, (1991). All sequencing reactions were accomplished using Sequenase 2 (U.S. Biochemical) following the Sanger method (Sanger *et al.*, 1977). Priming for template DNAs was accomplished using the forward or reverse M-13 sequencing primers or one of the 16S rRNA PCR primers. Both strands were sequenced for all template DNAs in order to obtain sequence information close to the primer and to verify internal sequence.

Sequence Alignment and Analysis

Sequence alignment was accomplished using the CLUSTAL package for multiple sequence alignments of Higgins and Sharp (1988, 1989) and by hand. A number of sequence alignments using the subroutines CLUSTAL1, CLUSTAL2, and CLUSTAL4 with different penalties for inferred gap events were considered. Aligned sequences of the study group and outgroup taxa were analyzed cladistically by the maximum parsimony procedures available in PAUP 3.0 using an exhaustive search or the branch-and-bound algorithm (Swofford and Olsen, 1990; Swofford, 1985). The most reasonable sequence alignment, judged by simply evaluating the alignment by eye and comparing the length and consistency of the final trees from the parsimony analyses, was that found using the following CLUSTAL parameters: a gap penalty equal to 15 and a penalty of 5 for each additional position inserted within a gap. Because of the difficulties in coding and in determining homology among inferred gap events, we conservatively coded all inferred gaps as missing data. Although some useful information may be disregarded,

removing inferred gaps from the analysis prevents the inclusion of spurious information resulting from the loss of one to a few nucleotide bases in these apparently hypervariable regions. Initial analyses included alignment with the published 16S rRNA sequences from *Drosophila* (Clary and Wolstenholme, 1985) and *Aedes* (Hsu-Chen *et al.*, 1984) for outgroup comparison. Bootstrap resampling procedures were performed with 100 replicates to investigate the "robustness" of the data set, and tree length distributions were examined to test for "skewness" using the g_1 statistic of Sokal and Rohlf (1981). This statistic is a common measure of skewness and is negative for distributions with left-skew, 0 for symmetric distributions, and positive for distributions with right-skew (Hillis and Huelsenbeck, 1992).

RESULTS AND DISCUSSION

Sequence Variation

Multiple sequence alignment of the amplified region from selected Hymenoptera taxa to published homologous sequences from *Aedes* and *Drosophila* resulted in a matrix consisting of 573 positions (Fig. 1) due to numerous inferred deletion/insertion events (Table 1). Fragment sizes from individual taxa ranged from 510 bp (*Apis*) to 552 bp (*Allorhogas*). Multiple individuals, each with identical nucleotide sequence, were analyzed from the genera *Allorhogas* (2 animals), *Tremex* (2 animals), *Xanthopimpla* (2 animals), and *Aphytis lingnanensis* (10 animals from a single isoline). The 2 *Digonogastra* individuals examined differed by one nucleotide substitution at position 184 (T-C). Two species, *Polistes versicolor* and the isoline of *Aphytis yanonensis*, were analyzed by cloning the double-stranded PCR fragments and by direct sequencing of single-stranded PCR products. No sequence differences were observed between the cloned and the single-stranded PCR products.

Nucleotide sequence information to date suggests that insect mtDNA is very A and T rich (Clary and Wolstenholme, 1985; Hsu-Chen *et al.*, 1984; Vlasak *et al.*, 1987; Crozier *et al.*, 1989; Simon *et al.*, 1990). We found an overall bias in the number of A and T bases expressed from all taxa ranging from a high of 0.794 in *Tremex* to a low of 0.533 in *Digonogastra* (Table 1). However, representatives of the superfamilies Ichneumonoidea and Chalcidoidea (i.e., *Xanthopimpla*, *Digonogastra*, *Allorhogas*, and *Aphytis*) have significantly less AT-rich 16S rRNA sequences than the remaining taxa based on a least significant difference test using a confidence interval of 95% (Table 1). In addition, the

FIG. 1. Aligned nucleotide sequence from a 573-bp region of the 16S rRNA gene of selected hymenopteran taxa and three previously published sequences: *Drosophila* (Clary and Wolstenholme, 1985), *Aedes* (Hsu-Chen *et al.*, 1984), and *Apis* (Vlasak *et al.*, 1987). Sequences are shown in reference to the *Drosophila* sequence with nucleotide substitutions expressed as the new bases, inferred gap events as hyphens, and positions identical to the *Drosophila* sequence as periods.

60
Drosophila --GTAAGAAT-TTAAAAGTCGAACAGACTTAAA--ATTTGA--ACGGCTACACCCAAAAT
Aedes AT.....C...G.....C.....C...A....TT.....T...A
Tenthredinidae AT...-.....G.....C.....T...A...G.TT..T...T...T.
Tremex AT...A.T.....G.....C....AA..AAAT..--ATT.T...TTT.T.
Apis AT.....T.....AAC...A...-TA..G.G..T..TT.
Polistes AT.....T.....AAC...A...TA..G.G..T...T.
Xanthopimpla AC...G..C.....TC..T....A...--G.ACC...A.TAG....G....-TCGG
Allorhogas AC...G..C.....TC..T....A...--G.ACC...A.TAG....G....-TCGG
Digonogastra AC...G..C.....TC..T....A...--G.ACC...A.TAG....G....-TCGG
A. lingnanensis AC...G..C.....TC..T....A...--G.ACC...A.TAG....G....-TTTG
A. yanonensis AC...G..C.G...TC..T....A...--G.ACC...A.TAG....G....-TTTG

120
Drosophila TATATCTTAATCCAACATCGAG-GTCGCAATCTTTTTATCGATATGAACTCTCCAAAAA
Aedes ..AT.....G.....A.....AA.....
Tenthredinidae .-...T.....TA...A...T.A-.....
Tremex .T..AT....T.....A.....TA...A...T..A....
Apis .-C.....T...G.....A.A.C.....T....GT..A..A..G.
Polistes .-C.....T...G.....A.A.C.....T....GT..A..A..G.
Xanthopimpla G..G..CGT.G.....A...T..A.CC.A..G.T.....G....AG..T.G
Allorhogas G..G..C.G.....T..A.CC.A..G.T.....G....AG..T.G
Digonogastra G..G..C.G.....T..A.CC.A..G.T.....G....AG..T.G
A. lingnanensis GG.G..C.G.....T..A.C..C..G.....G.G..G..TG..G..
A. yanonensis GG.G..C.G.....T..A.C..A..T.T.....GG...TG..G..

180
Drosophila AATTACGCTGTTATCCCTAAAGTAACTTAATTTTTAATCATTATTAATGGATCAATT--
AedesG.....A..AA.T.....A.--
TenthredinidaeG...T...T.C.AA...TT...AG..-A....TA.TT
TremexT.....GG...T...-AC.C-...T-...ATT-A....TA.TT
Apis T.....G...T...T.C.....T.CA...T..AAT...AAAT
Polistes T.....G-...T...T.C..A...T..A.AA...TATC.TTAAAG
Xanthopimpla G...G.....GG.....GT.CCG..GG...AGT-..T.....GA
Allorhogas G...G.....GG.....GT.CCG..GG...AGT-..T.....GA
Digonogastra G...G.....GG.....GT.CCG..GG...AGT-..T.....GA
A. lingnanensis G...G.....GGG.....GG..CGA.....G.TA..C...G.....TT
A. yanonensis G..GG.....GG.....GG..CAA.....GCTG..C...G....ATT

240
Drosophila ATTCATAAATT-AATGTTTTTAA-AATTAAAA----GTTTTT-----TAAA
Aedes ..A...T.....AAACA..T.A.....AA.....
TenthredinidaeAT.....T...A.AAT...A.....AA.....AAA.....
Tremex .A-.AG.TA...T..CA..A.T.ATCAA.T.TTATT...CA.ATA.....
Apis .-.T.T.TA.C..AA..AAA.CT.T--.T.AA.....A.....
Polistes T...-T-TA.CT.....A.....-TT.AA.....CAA.....
Xanthopimpla G.AT.GT.G..CGC.T.GAC.GGTG..G.CTT.GCAT..AC.GCTCGGAGG...T.GGG
Allorhogas G.AT.GT.G..CGC.T.GAC.GGTG..G.CTT.GCAT..AC.GCTCGGAGG...T.GGG
Digonogastra G.AT.GT.G..CGC.T.GAC.GGTG..G.CTT.GCAT..AC.GCTCGGAGG...T.GGG
A. lingnanensis .GATT.G.C..GT...C..GGT.AG...TT-.....C..ATGCTCGGAAG..T.T.G
A. yanonensis .---.GGC..GTG..GCC.GGGTAT..-G.GGGAA...C.CATGGTATTGGAAG.TC.

300
Drosophila TTTTAA-T---AT-CACCCCAA-TAAAATTTTTAATTTATTA-AA--ATTAA--ATTAA
AedesC.....G.....T.....TAAA.C..T.....T.....T
TenthredinidaeTTC.....G.....A..TTAA..A-..T.....T.....C.T.
TremexTT.TAA..ATT.....T.A.ATT.ACC.....TAT..T...A..C
Apis ...-CCA.....CT.....C...T.AA.CT.A-A..T.TTT.....--
Polistes ...-T...C..CT.....-AAA..T.A-C..T..TA.....--
Xanthopimpla ..C.GC.CCGAGGT.G.....CCG...T...-A.TGCAGG.TTGGATG..T.GG.CCTG
Allorhogas ..C.GC.CCGAGGT.G.....CCG...T...A.TGCAGG.TTGGTAG..T.GG.CCTG
Digonogastra ..C.GC.CCGAGGT.G.....CCT...T...-A.TGCAGG.TTGGTAG..T.GG.CCTG
A. lingnanensis ...-TT.CCTA.GT.G.....C.T...AT.....GCCA---TAGCT.----AG.C.TG
A. yanonensis ...GTTCCAAGT.G.....CCG...G---.GC.A---TT.GT.----GGG----

Drosophila TCTTTATAA-T--TAAAATAAATAA-AA--T-ATAAAGATTTATAGGGTCTTCTCGTCTT
AedesT...C.....T...T...-.....A.....C.....
Tenthredinidae .T.A.T...T.AA.....T.....TG..T.ATTC.....C.
Tremex .T.A.....-TTT.....TATAA..T..ATTC-.....A
Apis -T.A-TA..TAAA..TT.A.....-..T..ATTC.....A....CC
Polistes ATCACTA..TAAA.TTT.....CA..TT.T..ATTC-.....A.....A
Xanthopimpla .GGG.T.GTTAGG..CTG.TTGC.TT..TAAAT.....C.CC.....
Allorhogas .GGG.T.GTTAGG..CTG.TTGC.TT..TAAAT.....C.CC.....
Digonogastra .GGG.T.GTTAGG..CTG.TTGC.TT..TAAAT.....C.CC.....
A. lingnanensis .A.-.T..GT.GT.--TG.T-----TAAAT.....C.CC.C.....
A. vanonensis ----.T...TAGG.--T-----..AGT.T...C..C.C.....

Drosophila TTAAATTAATTTAG---CTTTTGTACTAAAAATAAAATCTATTTTAAATTTAAATG
AedesT.AT.....A.....A.T.....AA-.....AA
TenthredinidaeAA..A...A...G.....A...T.....T.....A.....T..A...AA
Tremex .A.TT.A.....T.TTCA.....T..A...TT.T.....A.AAA.GTT.AA..G..
Apis A...T.A.-...TA...GAA...A.....T.T.T...A--.T...AATTTA.
Polistes A.....T.-...AAA...G.A...A...TT...T.T.....A.-A.....AA.TTA.
Xanthopimpla GCTGT---G.CAT.CCCG.C.C.C..GGGC.CC.C.T...AC.GG.T..-AAGT.A.
Allorhogas GCTGT---G...T.CCCG.C.C.C..GGGC.GG.C.T...AC.GG.T..-AAGT.A.
Digonogastra GCTGT---G...T.CCCG.C.C.GGC..GGGC.GG.C.T...AC.GG.T..-AAGT.A.
A. lingnanensis A.GGG---G...T.CCAG...GT...GGC.G..C.GT...AC.GA.TTG-.....T
A. vanonensis A.ATT---G.CATTCTAG...GT...GG..G..C.GT...AAT.GA.GGG-...C.A.

Drosophila AAACAGTTAATATT-TCGTCCAACCATTTCATCCAGCCTTCAAT-TAAAAGACTAATGAT
Aedes .G....AT....C...A.T.....A.A.....-.....C....
Tenthredinidae .G....AATT..C...A..A..T.....T.....T...-.....T.T..
Tremex .G....ATT.....AA.....A..TT..T...-.....A..A.T..G..
Apis .G.....T.....A.TA.TT.T...A.A.TT.....-.....A.T.T..
Polistes --..A.T.....TA..A.TT.T...A..TT.....C.....A..A..
Xanthopimpla .G....-TGA.CCC...GG.G.....A.AG.T.CCT-.T...GGA..A.GA..
Allorhogas .G....-CTGA.CCC...GG.G.....A.AG.T.CCT-.T...GGA..A.G...
Digonogastra .G....-CTGA.CCC...GG.G.....-AG.T.CCT-.T...GGA.TA.G...
A. lingnanensis .G....A.-.C..CC..A.TT.G.....A.TG.T.ACT-.T...GG...A.G...
A. vanonensis .G.....TGG.C.C..A.GTGG.-.....A.TG.T..A.-.T...T...A.....

Drosophila TATGCTACCTTTGCACAGTCAAAATACTGCGGCCATTTAAATTT---TCAGTGGGCAGG
AedesC.....C.....T..C.A..CCA.....
Tenthredinidae .T.....A.....T.....AAT...TA.A...A
TremexA.....C.....A.T.C..C..---AAT.....A...
ApisT.....C.....A..T.....--AAT...T..A...A
Polistes .T.....T.....T.....A..T.....T.-AAT...A.....A
XanthopimplaT.GGG...C.....G..A..C..G.G.....C.....
AllorhogasG..T.GGG...C.....G..A..C..G.G.....C.....
DigonogastraG..T.GGG...C.....G..A..C..G.G.....C.....
A. lingnanensis ..C.....G..T.GG...C.....G..A..G-.G.T...C.....
A. vanonensis ..C.....G..T.GGG...C.....G..A..G..A.T.C...C.....

Drosophila TTAGACTTTATATA---TAATT-CAAAAAGAC
AedesA..T...A..A.....
Tenthredinidae ..G.....A..T...A.T.T.....
Tremex CC.T.TA.ATA..T...A..A..T...TA.A
Apis .CGC...AA.AT.....-T...C.G...
Polistes A..A...GTC..T.....C-T...C...
Xanthopimpla CGGTG.C.CTA...CTGG.G..G..T.G.G.TG
Allorhogas CGGTG.C.CTA...CTGG.G..G..T.G.G..T
Digonogastra CGGTG.CACTA...CTGG.G..G..T.G.G..G
A. lingnanensis CG.C..C..TA...CTTG..T.G..T...G.CT
A. vanonensis CATTG.C..TA...CTGG.TTGGG.T...G.TT

FIG. 1—Continued

TABLE 1

Sizes of DNA Fragments Amplified and Sequenced,
A + T Ratio, and Strand Asymmetry

	Base pairs	A + T ratio ^a	Strand asymmetry ^b
<i>Drosophila yakuba</i>	512	0.766	0.066
<i>Aedes albopictus</i>	515	0.755	0.146*
<i>Apis mellifera</i>	510	0.776	0.078
<i>Polistes versicolor</i>	516	0.777	0.093
Tenthredinidae	525	0.787	0.061
<i>Tremex columba</i>	528	0.794	0.053
<i>Xanthopimpla stemmator</i>	551	0.534	0.111
<i>Allorhogas pyralophagus</i>	552	0.536	0.123
<i>Digonogastra kimballi</i>	546	0.533	0.132
<i>Aphytis yanonensis</i>	527	0.594	0.127
<i>Aphytis lingnanensis</i>	518	0.573	0.154

^a Significant difference (indicated with vertical bars) among A + T ratios and among strand asymmetry values is calculated with a 95% confidence interval.

^b Strand asymmetry calculated by the following formula: $(\text{abs}(\text{No. A} - \text{No. T}) + \text{abs}(\text{No. G} - \text{No. C})) / (\text{No. A} + \text{No. T} + \text{No. G} + \text{No. C})$. Values range from 0.0 (No. purines = No. pyrimidines) to 1.0 (one strand all purines the other all pyrimidines) (Smith *et al.*, 1983).

* This value is not significantly different from that of the lower group.

ratio of A and T bases is not consistent throughout this region of the 16S rRNA gene. For example, as shown by the four representatives depicted in Fig. 2, there are areas of the sequence that display a trend toward higher A and T ratios (roughly corresponding to positions 175, 280, 350, and 500) and other areas relatively rich in G and C bases around positions 80 and 460. These trends are apparent across all taxa examined.

In order to examine the relationship of nucleotide composition to sequence divergence, we calculated an adjusted divergence (Adj. Div.) value for all pairwise combinations of sequences using the equation

$$\text{Adj. Div.} = \frac{[1 - 4(0.25)^2]}{1 - (\text{PA}^2 + \text{PT}^2 + \text{PG}^2 + \text{PC}^2)} D.$$

The numerator represents the probability of a replacement by a different base if the nucleotide ratio is balanced. The denominator is the probability of a replacement by a different base given the observed nucleotide ratio within a "window" of 25 to 50 bp. The values PA, PT, PG, and PC are the percentage of each nucleotide expressed from both sequences in the window. The value *D* is the total number of nucleotide replacements divided by the total number of positions in the window. The advantage of using Adj. Div. values of this fashion is explained by the following example. In comparisons between two sequences that are highly A and T rich, the probability that a single base substitution will not result in a different nucleotide base at that position

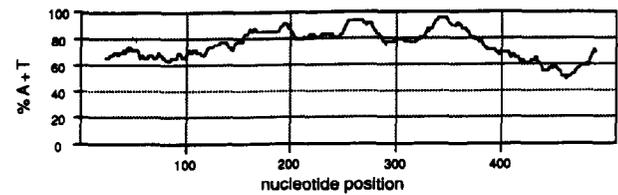
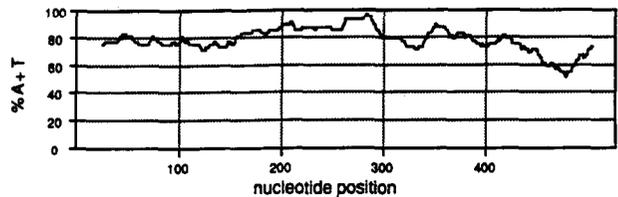
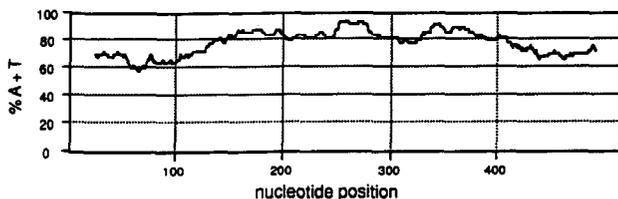
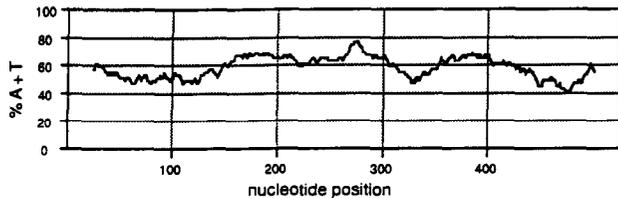
Aedes albopictus*Tremex columba**Polistes versicolor**Aphytis lingnanensis*

FIG. 2. Distribution of A and T bases throughout the 16S rRNA region sequenced from four representative taxa. The sequence of the mosquito (*Aedes albopictus*) was previously reported by Clary and Wolstenholme (1985) and is the only dipteran shown. Of the hymenopteran taxa, *Tremex columba* is a representative of the suborder Symphyta, whereas both the aculeate (*Polistes versicolor*) and the terebrant (*Aphytis lingnanensis*) are in the suborder Apocrita. Values were calculated using a 50-bp sliding window with the sequence analysis package available in MacVector (International Biotechnologies, Inc.).

approaches 50%. This is compared with the probability of replacement in sequences with balanced nucleotide ratios, i.e., approximately 25%. Therefore, comparisons of sequences with a nucleotide bias will give lower percentage divergence estimates. Although a number of published statistics are available for estimating nucleotide substitution, for our purposes it was necessary to limit sequence comparisons to user-defined and relatively small "windows" that were sensitive to rate changes over short regions. We found that 25- to 50-bp windows were long enough to show trends in the data but not so short as to be overly influenced by unequal inferred gap events. In addition, this equation does not weight transversions over transitions because regions

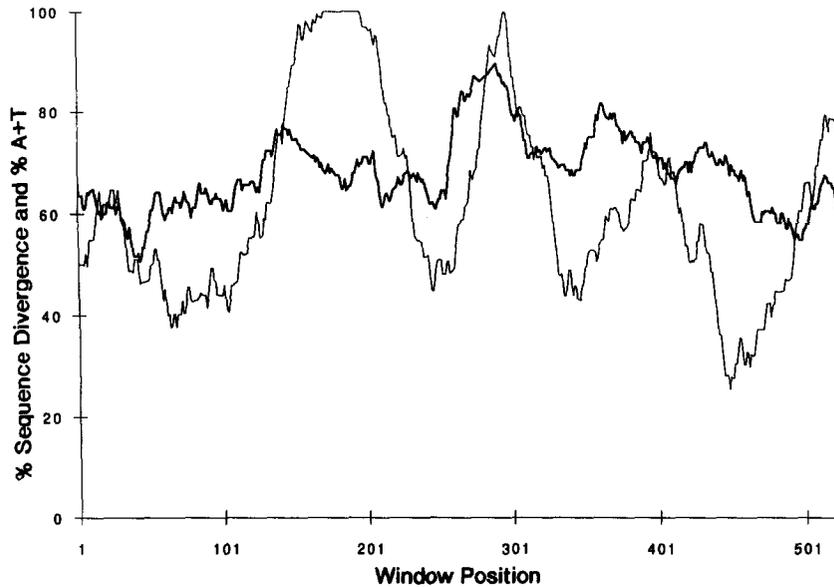


FIG. 3. The combined A and T ratios (upper line) from a region of the 16S rRNA gene compared with the adjusted divergence (Adj. Div.) (lower line) values from *Apis* and *Aphytis lingnanensis*. See text for details regarding the calculation of plotted values.

rich in A and T bases show an unusually high degree of transversion mutations; i.e., any substitution (A-T or T-A) is a transversion mutation.

The graph in Fig. 3 was constructed by limiting our analysis to homologous sequences 50 bp long (window size) and calculating the Adj. Div. between two sequences based on the observed ratio of nucleotides. This 50-bp window was then moved down the sequence 1 base and the divergence estimate was recalculated. Repetitive cycles of this procedure were completed for the entire sequence for all pairs of taxa. Figure 3 shows the Adj. Div. values and combined A + T ratios of two representative sequences (*Apis* and *Aphytis lingnanensis*) plotted by window position. This analysis highlighted multiple areas rich in A and T bases and corre-

sponding areas of high sequence divergence. A test of this observation was completed by comparing nonoverlapping 25-bp windows between pairs of sequences using a correlation coefficient (r) (Sokal and Rohlf, 1981). Specifically, we tested to see if areas high in A and T bases were statistically correlated with higher degrees of sequence divergence. Resulting r values for all pairwise comparisons (Table 2) were >0 (indicating a positive correlation between divergence and A-T richness) for 52 of 55 tests. These results imply selection for nucleotide sequence at two distinct levels. The first involves selection for functionality of the ribosomal RNA molecule. However, selection must also proceed at a second level; in areas where the sequence appears less constrained by functional requirements, nucleotide re-

TABLE 2

Matrix of Correlation Coefficients (r) from Comparisons of Combined A and T Ratios to Nucleotide Divergence Estimates from a Region of the 16S rRNA Gene from Insect Species

	1	2	3	4	5	6	7	8	9	10	11
1. <i>Drosophila yakuba</i>	—	0.498	0.538	0.393	0.612	0.490	0.490	0.358	0.436	0.761	0.752
2. <i>Aedes albopictus</i>		—	0.466	0.476	0.558	0.452	0.466	0.371	0.436	0.692	0.781
3. Tenthredinidae			—	0.372	0.750	0.588	0.390	0.283	0.350	0.665	0.768
4. <i>Tremex columba</i>				—	0.607	0.428	0.424	0.381	0.415	0.563	0.683
5. <i>Apis mellifera</i>					—	0.490	0.313	0.289	0.276	0.505	0.768
6. <i>Polistes versicolor</i>						—	0.375	0.306	0.341	0.710	0.846
7. <i>Xanthopimpla stemmator</i>							—	-0.424	-0.356	0.185	0.289
8. <i>Digonogastra kimballi</i>								—	-0.200	0.206	0.276
9. <i>Allorhogas pyralophagus</i>									—	0.220	0.289
10. <i>Aphytis yanonensis</i>										—	0.441
11. <i>Aphytis lingnanensis</i>											—

Note. Critical t values: $t_{0.1} = 1.72$ $r > 0.360$, $t_{0.05} = 2.08$ $r > 0.420$, $t_{0.01} = 2.84$ $r > 0.537$.

placement seems to involve a propensity for the incorporation of A and T bases. Accordingly, these areas would display higher rates of sequence divergence, due to the lack of functional constraints, as well as a relatively higher number of A and T bases. What remains unexplained is the strong bias for A and T bases among nonvertebrate mtDNA sequences.

Nucleotide composition among different sequences may also be compared on the basis of the degree of strand asymmetry as expressed by the number of purines and pyrimidines on each DNA strand. As depicted in Table 1, taxa with significantly lower ratios of A and T bases (i.e., the superfamilies Ichneumoidea and Chalcidoidea) also display significantly higher degrees of strand asymmetry. Although the correlation between function and strand asymmetry is at present unclear (Smith *et al.*, 1983), these findings may well represent differences that are responding to factors above the nucleotide position level.

A nucleotide substitution matrix was constructed from all pairwise combinations of sequences using Kimura's (1980) two-parameter model with the REAP computer package of McElroy *et al.*, (1991) (Table 3, below diagonal). This algorithm computes distance values based on the K estimates of Kimura (1980, Eq. (10); Nei, 1987, Eq. (5.5)) that represent an estimate of the expected number of nucleotide substitutions per site between any pair of sequences. For comparison, the percentage sequence difference based on each pairwise grouping of sequences from Fig. 1 is also provided (Table 3, above diagonal). For Hymenoptera taxa, percentage sequence difference ranged from less than 2.5% ($d = 0.023$, *Digonogastra* vs *Allorhogas*) to more than 50% ($d = 0.534$, *Tremex* vs *Aphytis lingnanensis*).

Phylogenetic Analysis and Implications

mtDNA Sequences. Two hundred (35%) of the 573 nucleotide positions were identical in all taxa examined and 86 (15%) variable positions differed by a single substitution in one taxon. This left 287 (50%)

nucleotide positions with the potential to contribute phylogenetic information.

This 16S rRNA nucleotide sequence was used to estimate hymenopteran phylogeny by unweighted parsimony. Initial analyses using sequence from the two dipteran taxa as outgroups (*Drosophila* and *Aedes*) resulted in the production of a single most parsimonious tree consisting of 752 steps and a consistency index (CI, excluding uninformative characters) of 0.711. However, this tree could not be rooted with the Hymenoptera as monophyletic in comparison with two dipteran outgroups. Given that Hymenoptera are certainly monophyletic (e.g., see Gauld and Bolton, 1988), this may indicate that Diptera are too remote an outgroup to be useful in polarizing these data. However, it is noteworthy that overall sequence divergence values (Table 3) between Diptera and some ingroup taxa are less than the divergence between some ingroup (Hymenoptera) taxa. In addition, exhaustive examination of all topologies resulting from the use of two dipteran outgroups revealed two trees of 753 steps, one of which has Hymenoptera as monophyletic. A second analysis limiting outgroup comparisons to sequence variation from *Aedes* produced a single tree with 701 steps (CI = 0.753) (Fig. 4a). A tree-length distribution containing trees from 701–1200 steps was significantly more skewed than expected from random ($g_1 = -0.76$; $P < 0.01$). Asymmetrical tree distributions of this nature have been proposed to be more informative due to the possibility of increased resolution among the near-optimal solutions (Hillis and Huelsenbeck, 1992). A bootstrap majority-rule consensus tree (Fig. 4b) has a topology identical to that of the most parsimonious tree. Two major groups of hymenopteran taxa emerged: the first includes both members of the suborder Symphyta (*Tenthredinidae* and *Tremex*) and the two aculeates; the second is composed entirely of the terebrants. In addition, all branches are well supported in the bootstrap consensus tree except for the two internodes below the Symphyta and Aculeates and below

TABLE 3

Percentage Sequence Difference (Above) and Kimura's Evolutionary Distance (1980, Eq. (10)) Below

	1	2	3	4	5	6	7	8	9	10	11
1. <i>Drosophila yakuba</i>	—	0.143	0.209	0.329	0.302	0.298	0.462	0.452	0.467	0.420	0.470
2. <i>Aedes albopictus</i>	0.164	—	0.206	0.322	0.307	0.309	0.452	0.441	0.459	0.422	0.482
3. <i>Tenthredinidae</i>	0.224	0.225	—	0.293	0.268	0.273	0.483	0.473	0.489	0.453	0.495
4. <i>Tremex columba</i>	0.363	0.346	0.307	—	0.352	0.356	0.511	0.497	0.513	0.499	0.534
5. <i>Apis mellifera</i>	0.325	0.342	0.295	0.390	—	0.185	0.497	0.488	0.500	0.503	0.497
6. <i>Polistes versicolor</i>	0.311	0.337	0.301	0.400	0.185	—	0.507	0.499	0.513	0.511	0.520
7. <i>Xanthopimpla stemmator</i>	0.576	0.566	0.649	0.747	0.648	0.693	—	0.024	0.036	0.284	0.331
8. <i>Digonogastra kimballi</i>	0.582	0.577	0.662	0.754	0.648	0.712	0.044	—	0.023	0.265	0.323
9. <i>Allorhogas pyralophagus</i>	0.560	0.552	0.637	0.723	0.629	0.689	0.037	0.028	—	0.281	0.335
10. <i>Aphytis yanonensis</i>	0.449	0.464	0.529	0.640	0.611	0.642	0.295	0.291	0.278	—	0.216
11. <i>Aphytis lingnanensis</i>	0.498	0.545	0.596	0.689	0.604	0.653	0.336	0.336	0.323	0.201	—

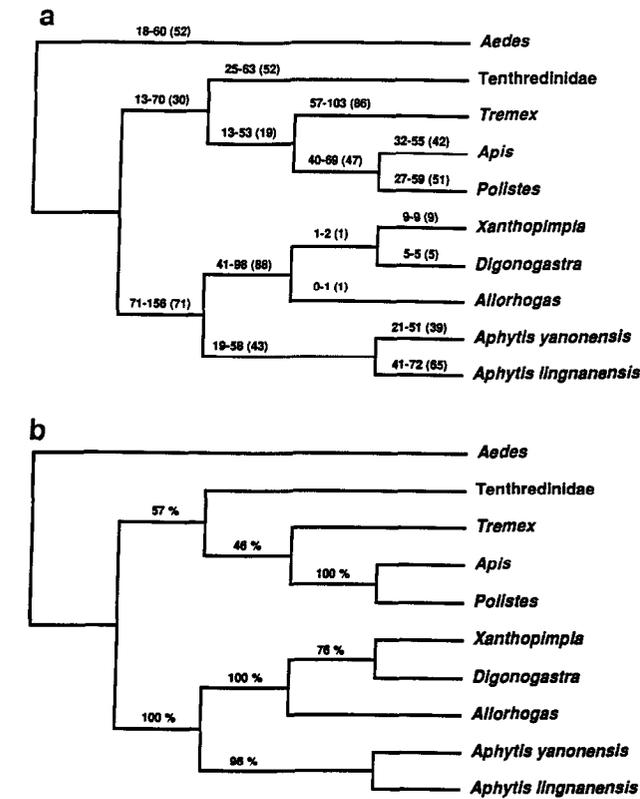


FIG. 4. (a) Parsimony tree of Hymenoptera based on 573 bp of nucleotide sequence from the 16S rRNA gene using a dipteran (*Aedes*) as an outgroup. The minimum, maximum, and assigned numbers of character state transformations are provided along each branch. This tree is 701 steps in length and has a CI of 0.753. (b) Bootstrap majority-rule consensus tree based on the same input matrix as the parsimony tree above. Numbers at each node represent the percentage of bootstrap trees supporting each branch.

Tremex and the Aculeates, where only 57 and 46% of the resampling procedures provided topologies supporting these relationships. Although this analysis shows clear relationships among the terebrants and among the aculeates, the basal branches of the tree, corresponding to the relationships of the hymenopteran suborders, are not strongly supported.

Current views of Hymenoptera classification support a paraphyletic Symphyta basal to a monophyletic Apocrita. Support (Fig. 4a) for a monophyletic clade that includes the Symphyta and the aculeates may be due to the AT richness of these taxa relative to that of the remainder of the ingroup. Examination of less parsimonious topologies revealed two trees of 702 steps that differ from Fig. 4a only in partial resolutions of the ichneuemonoid clade. A single tree of 703 steps is found with the following relationships: (Tenthredinidae, (*Tremex*, (Aculeata, Terebrantes))); in other words, it is congruent with current views. A strict consensus of these four most parsimonious trees is substantially less resolved than the tree presented in Fig. 4a. Tenth-

redinidae, *Tremex*, the Aculeata, and the Terebrantes form a basal multifurcation in the consensus tree, but the sister group relationship between Chalcidoidea and Ichneuemonoidea is retained.

Restricting the parsimony analysis to 16S rRNA sequence variation from Hymenoptera and using either of the symphytan sequences as an outgroup resulted in a single tree with 639 steps and a CI of 0.79 (Fig. 5a, shown with Tenthredinidae as the outgroup). As before, the aculeates and terebrants form distinct monophyletic groups that are reconstructed in 100% of the bootstrap replications (Fig. 5b) and the tree-length distribution is significantly skewed to the left ($g_1 = -0.76$; $P < 0.01$). Among the terebrants, the three representatives of the superfamily Ichneuemonoidea also consistently cluster together. Sequences from this region of the 16S rRNA gene differ at 19 nucleotide positions among members of this superfamily; however, most of these substitutions represent autapomorphies and do not provide strong support for any of the three possible relationships within this clade. Therefore, this

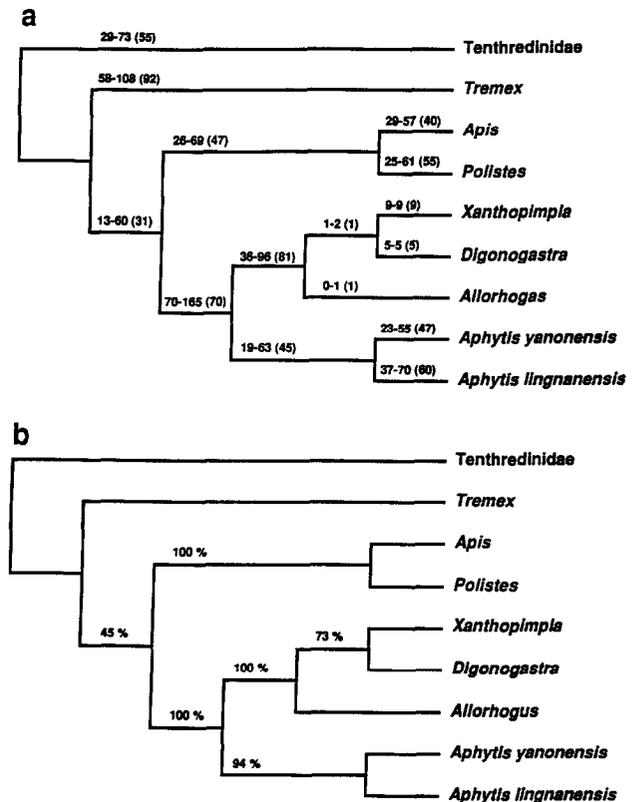


FIG. 5. (a) Parsimony tree of Hymenoptera based on 573 bp of nucleotide sequence from the 16S rRNA gene using a member of the suborder Symphyta (Tenthredinidae) as an outgroup. The minimum, maximum, and assigned numbers of character state transformations are provided along each branch. This tree is 639 steps in length and has a CI of 0.79. (b) Bootstrap majority-rule consensus tree based on the same input matrix as the parsimony tree above. Numbers at each node represent the percentage of bootstrap trees supporting each branch.

sequence seems inadequate for resolving relationships among the ichneumonid *Xanthopimpla* and the two braconids. Conversely, the two chalcidoids, both in the genus *Aphytis*, are clearly divergent and distinguished on the tree by 47 and 60 character state transformations. Although their placement suggests that they are each other's closest relative in our analysis, the amount and type of nucleotide variation expressed from this genus were unexpected. This observation is consistent with either very long divergence times, which is not supported by their morphological similarity, or phylogenetic sorting of ancestral mtDNA lineages among recently speciated taxa (Neigel and Avise, 1986). Nevertheless, it seems a more detailed analysis of this gene region is warranted to better understand the relationships among the chalcidoids.

Utility of 16S rRNA Sequence for a Phylogenetic Analysis of Hymenoptera. We can now address the utility of this region for reconstructing hymenopteran evolutionary history. First, 16S rRNA nucleotide sequence variation may provide detailed information regarding the relationships of the aculeate Hymenoptera. Although our sample was restricted to two members of this group, *Apis* (honeybee) and *Polistes* (paper wasps), their sequence difference ($d = 0.185$) and the strong evidence for monophyly (Figs. 4 and 5) suggest that a more detailed examination at this taxonomic level based on these nucleotide sequences might be fruitful. This reasoning can also be applied to the Chalcidoidea. Both representatives that we examined from this superfamily are members of the genus *Aphytis* and the unexpected high sequence difference ($d = 0.216$) suggests further research with additional representatives may provide a more detailed understanding of the evolutionary history of this group. In comparison, ichneumonoid sequences were remarkably conserved, showing little variation among the two families and three different subfamilies examined. Based on these results, there seems little likelihood of resolving relationships at the subfamily level or above using these sequences. It will be necessary to sequence other members of the ephialtine Ichneumonidae, doryctine Braconidae, and braconine Braconidae to determine if such sequences can be used to elucidate relationships among these genera. Conversely, high sequence divergence between members of the two hymenopteran suborders results in instability at the base of the tree. Accordingly, nucleotide sequence information from this 16S rRNA region is not informative in reconstructing the relationships among the suborders of Hymenoptera.

Although nucleotide sequence variation holds great promise for enhancing our understanding of many taxonomic groups that have proved intractable using traditional characters, the choice of an appropriate region for a given taxonomic level is paramount. As demon-

strated here, ribosomal sequence variation can provide new insight into hymenopteran relationships; however, this sequence is clearly not a panacea. For example, problems associated with sequence alignment, primarily in regions with multiple gap events, complicates the phylogenetic analysis. In addition, strong biases in A and T bases render analyses restricted to these characters less informative due to increased levels of nucleotide divergence between distantly related taxa. Finally, any examination across broad taxonomic groups such as the Hymenoptera might well prove difficult based on sequence divergence limited to one specific region. Nevertheless, studies such as the one presented here provide baseline information on the amount and type of nucleotide variation from specific regions and allow subsequent investigations on selected taxonomic groups to focus on areas of the genome most likely to produce phylogenetically useful information.

Sequence Availability

These sequences have been deposited in GenBank.

APPENDIX I

Specimens Examined, Collection Localities, Method of Tissue Preservation, and Clone Numbers

Voucher specimens of all species have been deposited as number 555 in the Insect Collection, Department of Entomology, Texas A&M University.

Order Hymenoptera

Suborder Symphyta

Superfamily Siricoidea

Siricidae

Tremex columba (Linnaeus)—U.S.A.: Texas, Walker Co., 10 mi. W. New Waverly. Frozen specimen. TAMU Clones S-3-29 and S-15.

Superfamily Tenthredinoidea

Tenthredinidae—U.S.A.: Texas, Brazos Co., Lick Creek Park. EtOH preserved specimen. Clone T4-36.

Suborder Apocrita

Superfamily Ichneumonoidea

Ichneumonidae

Xanthopimpla stemmator (Thunberg)—U.S.A.: Texas, Brazos Co., College Station, lab culture. Frozen specimen. Clones C-7, C7-1, C7-12, and C7-18.

Braconidae

Digonogastra kimballi Kirkland—U.S.A.: Texas, Brazos Co., College Station, lab culture. Frozen specimen. Clones C5-21 and C5-22.

Allorhogas pyralophagus Marsh—U.S.A.: Texas, Brazos Co., College Station, lab cul-

ture. Frozen specimen. Clones C1-5, C1-13, C1-28, and C-41.

Superfamily Chalcidoidea

Aphelinidae

Aphytis yanonensis DeBach and Rosen—U.S.A.: Texas, Brazos Co., College Station, lab culture, originally from Shimizu-shi, Japan, T89-145, frozen specimens from isolines. Clone C2-5.

Aphytis lingnanensis Compere—U.S.A.: Texas, Brazos Co., College Station, lab culture, originally from Homestead, Florida, T89-076. Clones C10-23 and C10-24.

Superfamily Vespoidea

Vespidae

Polistes versicolor—Venezuela: Maracay nests larva. EtOH preserved specimen. Clones C6-3 and C6-4.

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