Zoological Studies **37**(1): 45-55 (1998)

Zoological Studies

Phylogenetic Relationships of the Tropiduchidae-group (Homoptera: Fulgoroidea) of Planthoppers Inferred through Nucleotide Sequences

Wen-Bin Yeh[1], Chung-Tu Yang[1] and Cho-Fat Hui[2,*]

[1]*Department of Entomology, Chung-Hsing University, Taichung, Taiwan 402, R.O.C.*
[2]*Institute of Zoology, Academia Sinica, Taipei, Taiwan 115, R.O.C.*
Fax: 886-2-27858059.

(Accepted November 15, 1997)

Wen-Bin Yeh, Chung-Tu Yang and Cho-Fat Hui (1998) Phylogenetic relationships of Tropiduchidae-group (Homoptera: Fulgoridea) of planthoppers inferred through nucleotide sequences. *Zoological Studies* **37**(1): 45-55. Phylogenetic reconstruction of the relationships within the Tropiduchidae-group of families of planthoppers is conducted based on DNA sequences of 2 mitochondrial genes. The sequence comparisons of 400 bases of the 3' end of the 16S rDNA genes from 9 taxa of 6 families and 120 codons of the 5' end of the cytochrome b genes from 10 taxa of 6 families reveal general congruence. The phylogenetic trees based on these 2 genes show that Flatidae and Tropiduchidae are always grouped together; Meenoplidae and Delphacidae each is a basal lineage among these taxa, and Issidae is a nonhomogeneous group. The ratio of transitions over transversions of the cytochrome b gene in fulgoroids has reached a steady-state when compared with insects of other orders. Both genes can be candidate genes in studying the relationships of Fulgoroidea since the sequence variations of both genes between species of intragenus, close affinity families, and distant affinity families are about 10%, 20%, and 30%, respectively.

Key words: Mitochondrial DNA, 16S rDNA, Cytochrome b, Fulgoroidea.

Although numerous studies of morphological characters indicate that the insect order Homoptera (planthoppers, leafhoppers, treehoppers, cicadas, spittlebugs, aphids, psyllids, scales, whiteflies, etc.) is not a monophyletic group (Goodchild 1966, Schuh 1979, Zrzavy 1992, Campbell et al. 1994 1995), yet there is no question that the Fulgoroidea (planthoppers) is a monophyletic group. Asche (1987) has proposed 5 synapomorphies that include shapes of mesocoxa and metacoxa, position of ocelli, position of antennae, sensory plaque organ of the pedicel, and the special fulgoromorphan face to support Fulgoroidea being a well-defined group. Nymphal characters presented by Chen and Yang (1995) also indicate that Fulgoroidea is a monophyletic group, and they further divide the members of Fulgoroidea into 5 family-groups: (I) the Tettigometridae; (II) the Ricaniidae-group which comprises the families Eurybrachidae, Gengidae, Hypochthonellidae, Lophopidae, and Ricaniidae; (III) the Tropiduchidae-group which comprises the families Flatidae, Issidae, Nogodinidae, and Tropiduchidae; (IV) the Fulgoridae-group which comprises the families Dictyopharidae and Fulgoridae; and (V) the Cixiidae-group which comprises the families Achilidae, Achilixiidae, Cixiidae, Delphacidae, Derbidae, Kinnaridae, and Meenoplidae.

The phylogenetic relationships of fulgoroid families have been studied previously using adult and nymphal characters; however, results are not congruent (Muir 1923 1930, Asche 1987, Emeljanov 1990, Yang and Fang 1993, Chen and Yang 1995). Based on adult morphological characters, fulgoroids have been divided into 2 groups, but the members within each group show great divergence (Muir 1923 1930, Asche 1987). Muir (1923) grouped Cixiidae-Delphacidae-Tropiduchidae-Derbidae-Achilixiidae-Tettigometridae together, with the rest in an individual group; while Asche

*To whom correspondence and reprint requests should be addressed.

(1987) grouped Cixiidae-Delphacidae together, with the rest in an individual group. In contrast, Emeljanov (1990) composed a phylogenetic reconstruction of fulgoroids is in successive dichotomies. Alternatively, based on nymphal characters, Yang and Fang (1993) divided fulgoroids into 2 groups, Delphacidae-Meenoplidae-Cixiidae-Achilidae-Derbidae, with the rest in an individual group, while Chen and Yang (1995) divided them into 5 family groups. Obviously, there is no general congruence concerning fulgoroid phylogeny within the published literature. Thus, it is necessary to use characters other than morphological characters to infer the relationships of fulgoroids.

Mitochondrial genes generally have been used in recent molecular systematic studies of mammals (Honeycutt et al. 1995, Rosel et al. 1995, Wettstein et al. 1995, Arnason and Gullberg 1996), aves (Helm-Bychowski and Cracraft 1993), reptiles (Bowen et al. 1993, Graybeal 1993, Hay et al. 1995, Reeder 1995), and insects (Xiong and Kocher 1991 1993, Martin and Pashley 1992, Fang et al. 1993). Nucleotide sequences of conserved genes can be used to infer ancient phylogenetic affiliations. The mitochondrial 16S rDNA gene has been found to reveal phylogenetic information at the family level in insects and mites (Black IV and Piesman 1994, Dowton and Austin 1994, Yeh et al. 1996 1997, Dietrich et al. 1997). The cytochrome b gene (*COB*) is one of the conserved genes in mitochodrial DNA. The nucleotide sequence of the *COB* gene generally has not been used in the studies of insect systematics, but it should be a candidate gene for studying phylogenetic relationships of higher categories since phylogeny reconstructions using this gene have been informative in vertebrates (Irwin et al. 1991, Bowen et al. 1993, Graybeal 1993, Honeycutt et al. 1995, Arnason and Gullberg 1996).

In this paper, nucleotide sequences of a part of the 16S rDNA gene and a part of the *COB* gene of the Tropiduchidae-group were obtained to study nucleotide information and phylogenetic relationships of members of the Tropiduchidae-group. One motivation for this study is to clarify fulgoroid phylogeny since the results of previous studies have disagreed. Although phylogenetic relationships derived from partial 16S rDNA and *COB* sequences are not always identical, they still could be candidate genes for studying the relationships of fulgoroids as the sequence variations of both genes between species of intragenus, close affinity families, and distant affinity families are about 10%, 20%, and 30%, respectively. A 2nd objective for this study is to evaluate the phylogenetic position of the Issidae family. CT Yang and LB O'Brien (pers. comm.) are planning a nomenclature act to raise the subfamily, Caliscelinae, of Issidae to a new status, Caliscelidae, based on the special characters of tegmina, apical teeth of metatarsus, and the pad of sacs on the sole of metatarsus. Alternatively, SW Wilson (pers. comm.) has suggested that Issidae might not be a monophyletic group from the ecological behavior of its members, and furthermore, Caliscelinae should be a separate family out of Issidae. The problematic composition of Issidae will be discussed in this paper.

MATERIALS AND METHODS

Samples and DNA extractions

There are 10 species included in this study. Seven species are from the Tropiduchidae-group, and three species are from the Cixiidae-group for outgroup comparison. In the Tropiduchidae-group, 4 species are examined from different subfamilies of Issidae that include *Epyhemisphaerius tappanus* (Hemisphaerinae), *Tonga botelensis* (Tonginae), *Eusarima astuta* (Issinae), and *Mushya fasciata* (Caliscelinae); 3 other species are from different families that include *Phylliana serva* (Flatidae), *Mindura subfusciata kotoshoenis* (Nogodinidae), and *Kallitaxila sinica* (Tropiduchidae). The 3 selected species for outgroup comparisons are *Nisia serrata* and *Nisia lansunensis* (Meenoplidae), and *Purohita taiwanensis* (Delphacidae). Live insects were collected, preserved in 95% alcohol, and stored at -70 °C. Crude DNA was extracted from whole insect bodies (about 0.1 g) as described by Yang et al. (1994).

PCR amplification and direct sequencing

A part of the 5' end sequence of the *COB* gene and a part of the 3' end sequence of the 16S rDNA gene were amplified by the polymerase chain reaction (PCR). The 2 primers that amplified the *COB* gene were designed based on the conserved nucleotide sequences of *Drosophila yakuba* (Clary and Wolstenholme 1985), *Drosophila melanogaster* (Garesse 1988), *Anopheles gambiae* (Beard et al. 1993), *Anopheles quadrimaculatus* (Mitchell et al. 1993), *Apis mellifera* (Crozier and Crozier 1993), and *Tetraponera rufoniger* (Jermiin and Crozier 1994). The upstream primer (5'-GATAAACCT AAAGCTCCCTCACA-3') and the downstream

primer (5'-GCGCCTCAAAATGATATTTGTCCCC-3') are located in the *ND4L* (nucleotides 9623-9645) and the *COB* (nucleotides 10945-10921) genes respectively, of the mitochondrial genome of *D. yakuba* (Clary and Wolstenholme 1985). The resulting fragment was about 1.3 kb in length. The 2 primers that amplified the 16S rDNA gene were designed based on the conserved nucleotide sequences of *Lymantria dispar* (Davis et al. 1994), *Locusta migratoria*, *D. yakuba*, *D. melanogaster*, *Aedes albopictus*, *Anopheles gambiae*, and *Apis mellifera*. The locations of the upstream primer (5'-GCCTGTTTATCAAAAACAT-3') and the downstream primer (5'-CCGGTCTGAACTCAGATCA-3') correspond to nucleotides 13416-13396 and 12866-12884, respectively, of the 16S rDNA gene of *D. yakuba* (Clary and Wolstenholme 1985).

Extracted crude DNA was subjected to 39 cycles of amplification in a 100-µl volume reaction that contained 10 mM Tris-Cl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton-X100, 2 units of *SuperTaq* polymerase (HT Biotechnology LTD), 0.2 mM of each dNTP, and 20 pmoles of each primer. These amplifications were carried out with the following temperature profile: denaturation for 50 s at 95 °C, annealing for 70 s at 50 °C, and extension for 2 min at 72 °C. The purification of double-stranded DNA products followed protocols that have been previously described (Jean et al. 1995), and DNA products were sequenced directly using the PCR sequencing kit (Perkin Elmer) for 29 cycles with the following temperature profile: 50 s each for denaturation at 95 °C, annealing at 50 °C, and extension at 72 °C.

DNA analysis

Mitochondrial sequences were aligned using the Pileup program of the GCG software package (Genetic Computer Group, version 7.0) (Devereux et al. 1991), then checked by eye. The *COB* sequences were checked again after the derived amino acid sequences were obtained. Aligned DNA sequences were analyzed using the MEGA program (Kumar et al. 1993) for calculating (1) the proportions of nucleotide compositions of each species; (2) the total substitution, transition, and transversion proportions between all paired sequences; and (3) the ratio of transitions over transversions. The proportions of transition, transversion, and total substitution of the codon positions 1, 2, and 3 of the *COB* sequences were also calculated. Statistical analysis system (SAS) was used to test the nucleotide base composition bias and the plots between total substitutions and Ts/Tv ratio, transition, and transversion for the *COB* gene (SAS Institute Inc. 1996). Phylogenetic analysis was performed by the Neighbor-Joining method (Saitou and Nei 1987) where the pairwise distance estimates were based on models by Jukes and Cantor (1969), Kimura (1980), Tamura (1992), and Tamura-Nei (1993) as implemented by the MEGA program.

RESULTS

DNA sequence compositions

COB

The primers used in the PCR reaction amplified a DNA fragment of approximately 1.3 kb. Within the fragment, the upstream 900 bp segment includes a part of the *ND6*, *Pro-tRNA*, and a part of the *ND4L* genes, and the other downstream 400bp is the 5' end segment of the *COB* gene (Fig. 1). The mean compositions of guanine, adenine, thymine, and cytosine of the *COB* gene are 11.1%, 37.9%, 36.3%, and 14.7%, respectively. The nucleotide divergences between taxa increase with taxonomic distance (Table 1). The average nucleotide divergences are 10% within the genus *Nisia* of the Meenoplidae, 21% within the Tropiduchidae-group, and 32% between the Cixiidae-group and the Tropiduchidae-group. The results show that due consideration has to be given if the *COB* gene is to be used in studies of fulgoroid relationships since the nucleotide differences have nearly reached a saturation level. Thus, selecting a codon position with suitable nucleotide differences, transversion, or transition of protein coding gene shall be a necessary criterion in analysis of phylogenetic relationships of the fulgoroids. The ratios of transitions over transversions of all paired sequences of the *COB* gene (Table 2) decrease with increasing taxonomic distance, in which the average ratio between Meenoplidae and the other fulgoroids is 1:2.12; between Delphacidae and the other fulgoroids (excluding Meenoplidae) is 1:1.91; among Issidae, Nogodinidae, and Flatidae is 1:1.50; and 1:1.39 if Tropiduchidae is included, as the value for Tropiduchidae is highly biased; and among subfamilies of Issidae is 1:1.47.

16S rDNA

When gaps were added for alignment, a total of 400 sites were used in analysis, of which 207

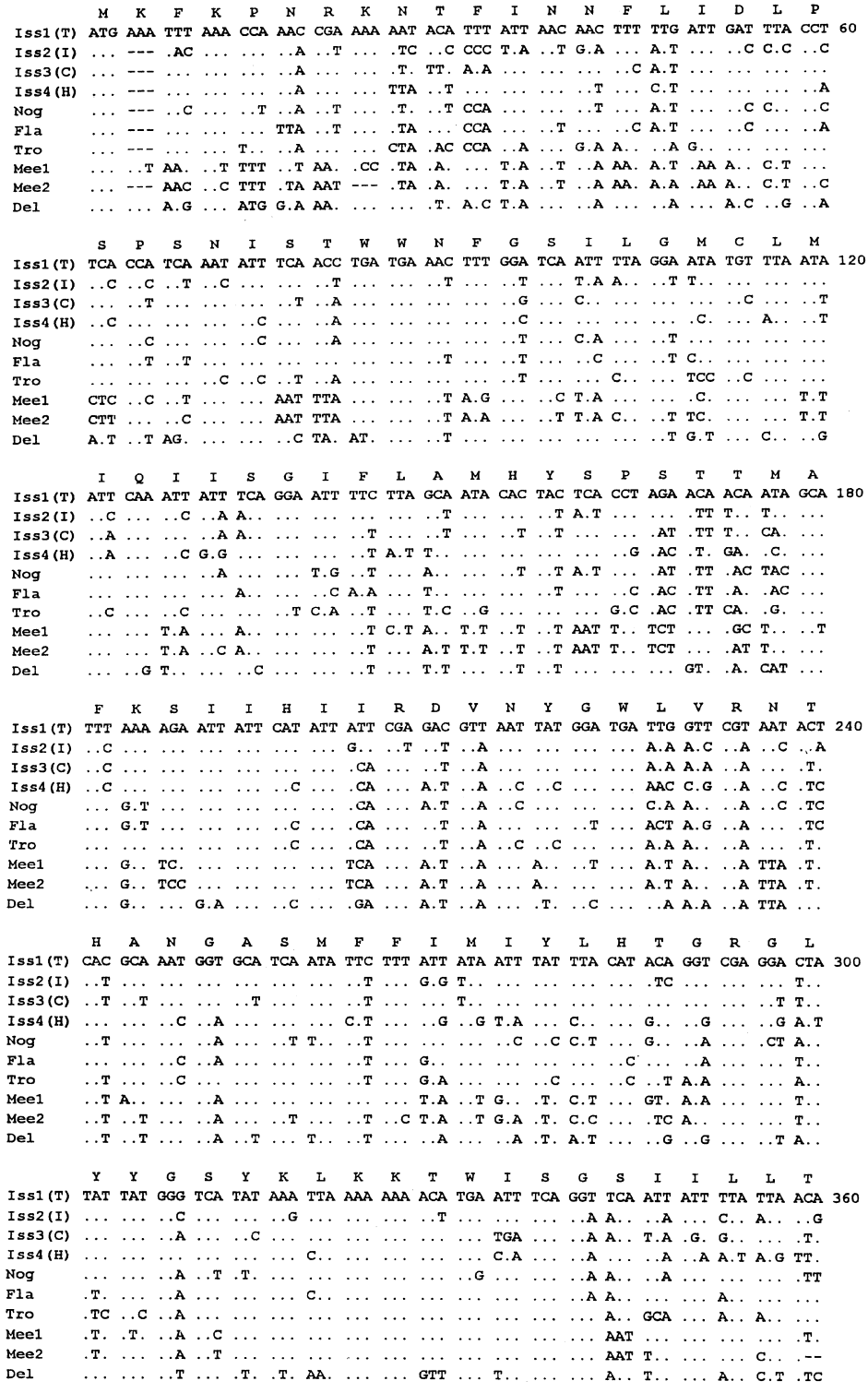

Fig. 1. Alignment of the fulgoroid cytochrome b sequences with derived amino acid sequence of the 1st taxon presented in single letter forms. Taxa are abbreviated as the first 3 letters of family names while numbers represent the different subfamilies of Issidae, and the different species of Meenoplidae which are listed as follows: Iss1 = *Tonginae, Tonga botelensis*; Iss2 = Issinae, *Eusarima astuta*; Iss3 = Caliscelinae, *Mushya fasciata*; Iss4 = Hemisphaerinae, *Epyhemisphaerius tappanus*; Nog = Nogodinidae, *Mindura subfusciata kotoshoenis*; Fla = Flatidae, *Phylliana serva*; Tro = Tropiduchidae, *Kallitaxila sincia*; Mee1 and Mee2 = Meenoplidae, *Nisia serrata* and *N. lansunensis*, respectively; Del = Delphacidae, *Purohita taiwanensis*. A deletion is denoted by a hyphen (-), and a dot (.) is identical to the 1st nucleotide sequence.

Table 1. Proportion of nucleotide divergence (above diagonal) and the ratio of transition over transversion (below diagonal) of the *COB* gene

	Iss1(T)	Iss2(I)	Iss3(C)	Iss4(H)	Nog	Fla	Tro	Mee1	Mee2	Del
Iss1 (T)		0.207	0.168	0.210	0.182	0.182	0.215	0.311	0.332	0.275
Iss2 (I)	0.762		0.204	0.277	0.212	0.207	0.238	0.324	0.301	0.322
Iss3 (C)	0.538	0.698		0.218	0.190	0.184	0.215	0.324	0.326	0.271
Iss4 (H)	0.705	0.707	0.660		0.229	0.218	0.226	0.355	0.377	0.330
Nog	0.773	0.727	0.581	0.519		0.182	0.240	0.305	0.304	0.294
Fla	0.711	0.609	0.610	0.773	0.711		0.198	0.322	0.329	0.294
Tro	0.974	0.889	0.750	0.884	0.911	0.821		0.347	0.338	0.333
Mee1	0.436	0.547	0.381	0.460	0.416	0.494	0.550		0.108	0.352
Mee2	0.463	0.514	0.386	0.446	0.390	0.487	0.545	1.000		0.323
Del	0.523	0.494	0.426	0.532	0.479	0.522	0.700	0.366	0.295	

Table 2. Average ratio of transition over transversion for the different level comparisons

Level	Ts : Tv
Order-Order[a]	1 : 2.26
Order-Suborder[b]	1 : 2.23
Mee-fulgoroids	1 : 2.12
Del-fulgoroids (excluding Mee)	1 : 1.91
Interfamilies[c]	1 : 1.50
Intersubfamilies of Issidae	1 : 1.47
Intragenus (*Nisia, Anopheles*, and *Drosophila*)	1 : 0.81

[a] The average ratio of the 3 insect orders Diptera, Hymenoptera, and Homoptera.
[b] The ratio involves the values of suborder to suborder of Diptera.
[c] The ratio comes from Tropiduchidae-group, excluding Mee, Del, and Tro.

sites were constant. The partial sequence data of the 16S rDNA gene for all 9 species are presented in Fig. 2. The nucleotide compositions of guanine, adenine, thymine, and cytosine are 15.2%, 32.0%, 45.5%, and 7.3%, respectively and the composition is similar to that of other insects (Fang et al. 1993, Xiong and Kocher 1993) and mites (Black IV and Piesman 1994). The average substitution proportion (Table 3) between cicadellids and fulgoroids is 0.30, which is more significant than the comparisons within fulgoroids (0.20). The observed nucleotide divergences increase with increasing taxonomic distances, in which the average nucleotide differences between Delphacidae and the other fulgoroids is 0.25; and between Meenoplidae and the other fulgoroids (excluding Delphacidae) is 0.21. The average difference among the subfamily

Table 3. Proportion of nucleotide divergence (above diagonal) and the ratio of transition over transversion of the 16S rDNA gene (below diagonal)

	Iss1(T)	Iss2(I)	Iss3(C)	Iss4(H)	Nog	Fla	Tro	Mee2	Del	Cic
Iss1(T)		0.183	0.180	0.160	0.147	0.167	0.179	0.221	0.245	0.313
Iss2(I)	0.224			0.173	0.148	0.191	0.175	0.219	0.248	0.275
Iss3(C)	0.321	0.278		0.209	0.176	0.196	0.221	0.238	0.255	0.283
Iss4(H)	0.378	0.288	0.373		0.145	0.180	0.178	0.225	0.248	0.309
Nog	0.213	0.357	0.360	0.267		0.158	0.189	0.217	0.257	0.292
Fla	0.300	0.321	0.267	0.429	0.356		0.164	0.224	0.266	0.321
Tro	0.169	0.155	0.133	0.211	0.197	0.370		0.212	0.239	0.309
Mee2	0.217	0.277	0.233	0.284	0.169	0.349	0.212		0.243	0.318
Del	0.329	0.306	0.329	0.418	0.324	0.295	0.197	0.282		0.322
Cic	0.229	0.284	0.230	0.272	0.220	0.271	0.182	0.311	0.319	

```
                                                                                                                  80
Iss1(T) GCTAAGGTAG CATAATAATT AGTCTTTTAA TTGAGGTCTG GAATGAATGG ATTCACGAGG AATATACTTT ATTAATTTAA
Iss2(I) .......... .......... .......... .......... .G........ T.AG.....A ....A..... ...T.A..T.
Iss3(C) .......... .......... .......... ..A......T .......... T.GG.T..AA ..A.A..... ........TT
Iss4(H) .......... .......... .......... .........A .......... T.GA.....A ...T...... ...GT...T.
Nog     ..A....... .......... .......... .......... .......... T.GG.....A ...T.T.... ...TT...T.
Fla     ..G....... .......... .......... .......... .T........ T.GG..A... ..AT...... ...TT.....
Tro     ..A....... .......... .......... .......... .......... T.GG.....A ..A....... ...TT..A..
Mee2    ..A....... .......... .......... .........T .......... G..A..A.AA T.ATAG.... .....A....
Del     A......... .......... .......... .......... .......C.. T..A..A... TGA.A..... C...T..A..
Cic     ..A....... ..A....... ...T.C.... ..AGAAG..A .......... ..AT.T.T.A T.....T... ....GG..T.

                                                                                                                 160
Iss1(T) TTTTTTTTGA ATTTTATATT TTTGTTAAAA AGCTTAAGTT AAAAAGAGTG ACGATAAGAC CCTATAGATC TTTAAAATTT
Iss2(I) .......... ....GT.T.. ..A....... TT..A..A.G TTTT....G. ....C..... .......... .AA.G..GA.
Iss3(C) ...G.A.... .......T.. .AAT...... ..A....A.. TTTG.AG.G. .......... .......... ..A.....A.
Iss4(H) .......... .......T.A ..G....... ...CATTA.G TTT.....G. .......... ...G...... ....T.....
Nog     ....A..... .....TAT.. ..G....... TT.CA..A.. TTT.....G. .......... .......... .........-.
Fla     .....G.... ......G... ..G....... TT.CA..A.. TTT..A..G. .......... .......... ....T....-
Tro     A...A...-. ....A..T.. A......... C..AAT.A.. .TTTGA.... .......... .......... ...---....
Mee2    A....-.... .A..A..T.. AAA....... TT...T.A.. .TT...G.G. .......... .......... ..A....A.A
Del     ..AC.-.... ....A..T.. ..A....... ....A..A.. TTT..AT.G. -..-...... .......... ..A..TT...
Cic     AAA..-AA-. ......AT.. .GG....... T...C..T.. CTTTTT..G. .......... ........A. ....------

                                                                                                                 240
Iss1(T) ---TTTTTTT TTAATTTTTT TTGTTG--TT TATTGGTTAT TTAAATTT-A TTTTTTTTGT TGGGGAGATA GATAAAATTT
Iss2(I) ..T..A.... A.-T..... .......... .T..AT...A .AT..-A..T .......... .....T..C. AT.......
Iss3(C) ....A.A... A..TA.A... ......TT.A ....TA.A.A AATT..A.T. ...-...... .....T..C. A......A..
Iss4(H) AAA.AA...- ...T...... G....TGT.. .T..T..... .--.T..G.. ...A...... .....T..C. .T.......
Nog     AAT..A...A ...T...... ......TA.. ....TT.... .--T..A..T .......... .....T.... .......AC.
Fla     ..C..A.... ..TG...... .......... .TAA.A.... ...TT.A.G. ...A...A.. .....T...T A......A..
Tro     TAT....... ..TT..C... ...A..GT.. .TA.TTAA.. ....T.A.A. --AA...... .....T..C. TT.......
Mee2    AAT....A.A ..T.G..... ..-----.TA. .T..TAAA.. ..------.. .......AA. .........T T......AA.
Del     TTT.A.C.-- -A.T..C..G G.TAATAAAA .G..AAAAT. .....AAA.. --A....... .....T..C. TTA...T...
Cic     ..C.AAA.A. AATT.AG..G G.T..TTT.. ...ATAAAT. .ATT.AA.TT .A.....A.. .....T..C. .T....T...

                                                                                                                 320
Iss1(T) TAA-ACTTTA ATTTTTTTTT TACTTTTTTT ATGAATT-T- -TTGATCCTT AATTTTGGAT TATAAGAATA AGATACCTTA
Iss2(I) .......... .........C ACA....... ...-T..A.. .......... CT..G.T... ..A....TA. ..........
Iss3(C) .......... T......... ACA...G... .....A.T.. .......... --........ A.A.....A. ..........
Iss4(H) .......... G......... ACA.A.A... ...GT..G.. ..A....T.. ......T... ATC....TC. .........C.
Nog     .........T T...A..A.. ACA...A... ...GT..T.. .......... ......T... .TC....T.. ..........
Fla     .T..T..... T..A...... ACA....... T..G-..A.. .......T.A .......... .........A. ..........
Tro     ....T..... T..A...... ACA....... T...G..... .....A..A. ......T.T. ...T....A. ..........
Mee2    .......... TA......... ACA..AA..G T..TT..A.. A.......A. TT..AGT... .TA....TA. ..........
Del     ...AT....T .A.CA..... ACA.AAA.AA ...-TA.A.. .......T.A .CA...A... ..A..A.T.. ..........
Cic     A.-.-..... .....GA.AA .CA..A.... ..--G.AG.T T.......A. .TA.-AT... A......TA. ..T.......

                                                                                                                 400
Iss1(T) GGGATAACAG CGTTATAAAT CTGGAAAGTT CTAATTGATA GATTTGTTTG CGACCTCGAT GTTGGATTAA TTTGTTACTG
Iss2(I) .......... ...A...T.A T....T.... .A........ AT.A...... .......... .......... AC.T.ATT..
Iss3(C) .......... .......TT. .....G.... .....CA... ..A....... .......... .......... C..AAATT..
Iss4(H) .......... .......G.. T....G.... ..T....... A......... .......... .......... AG.T......
Nog     .......... .......T.. .....G.... .......... ...A...... .......... .......... ...AAAG..A
Fla     .......... .A.A....T. .....G.... .....C.... ..C....... T......... .......... AA.T.ATT..
Tro     .......... ...A...T.. T....T.... ..T....... A..A.T.... .......... .......... AA.T..TT..
Mee2    .......... ...A...... T...GG.... .AT...T... A......... .....A.... ....A..... AA.T...GGA
Del     .......... ...A...... T..A.T.... .AT....... A........A .......... ....A..... .A.AAA.---
Cic     .......... ...A..TTTA G...GG.... .AC..CT... CTAAAT.... .......... ....A..... GA.AAA.AAA
```

Fig. 2. Alignment of the fulgoroid 16S rDNA sequences. Designations used here are the same as in Fig. 1. The sequence of Cic = Cicadellidae, *Macrosteles fascifrons*, is from Fang et al. (1993).

members of Issidae is 0.18, which is indistinguishable from the average difference among Issidae, Flatidae, Nogodinidae, and Tropiduchidae (~ 0.18). The same extent of nucleotide differences are shown between Issidae and the other Tropiduchidae-group members in the 16S rDNA gene as that in the *COB* gene. These results, obtained from both genes, suggest that the divergent time of the subfamily members of Issidae might be similar to that of the other family members (excluding Meenoplidae and Delphacidae) of the Tropiduchidae-group. If such an inference was correct, then the phylogenetic relationships of the Issidae might not be easily resolved using molecular data (Yeh et al. 1997). The ratio of transitions over transversions does not exist in a normalized way in this gene. The ratio values between Flatidae and the others are the largest, and the ratio values between Tropiduchidae and the others are smaller than in the comparisons of the other taxa.

Phylogenetic analysis

For the *COB* gene sequences only substitutions of the 1st and 2nd codon positions were included in the calculations of the nucleotide distances, since the transitions of the 3rd codon position might have evolved to saturation (Irwin et al. 1991, Bowen et al. 1993). The phylogenetic trees reconstructed using the proportion distances and the pairwise distance models of Jukes-Cantor, Kimura 2-parameter, Tamura, and Tamura-Nei are all identical (Fig. 3A). Obviously, these molecular trees suggest that Meenoplidae is the most basal lineage with the next one being Delphacidae, and these are consistent with the information from proportion distances of the gene. Flatidae is grouped with Tropiduchidae, then clusters to Iss4(H). The 4 subfamilies of Issidae are not grouped together, which might be similar to the inferences of Yang and O'Brien, and Wilson which suggest that Issidae is not a monophyletic group. In the analysis of the 16S rDNA sequences, only transversions are included, because transition substitutions in other insects, such as black flies (Xiong and Kocher 1991 1993) and leafhoppers (Cicadellidae) (Fang et al. 1993) have evolved rapidly to saturation. The phylogenetic tree (Fig. 3B), reconstructed with the 16S rDNA nucleotide sequences, is identical regardless of distance models used, and reveals general congruence with that of the *COB* gene. Delphacidae is the most basal lineage, while the next one is Meenoplidae. Flatidae is grouped with Tropiduchidae, then grouped to 2 Issidae subfamilies, Iss1(T)-Iss4(H). The 4 subfamilies of Issidae are not grouped together. The 2 phylogenetic tree reconstructions always group Flatidae with Tropiduchidae, with Meenoplidae and Delphacidae each being a distinct basal lineage. The relationships of Issidae and Nogodinidae are ambiguous, and the 4 subfamilies of Issidae are not always grouped together.

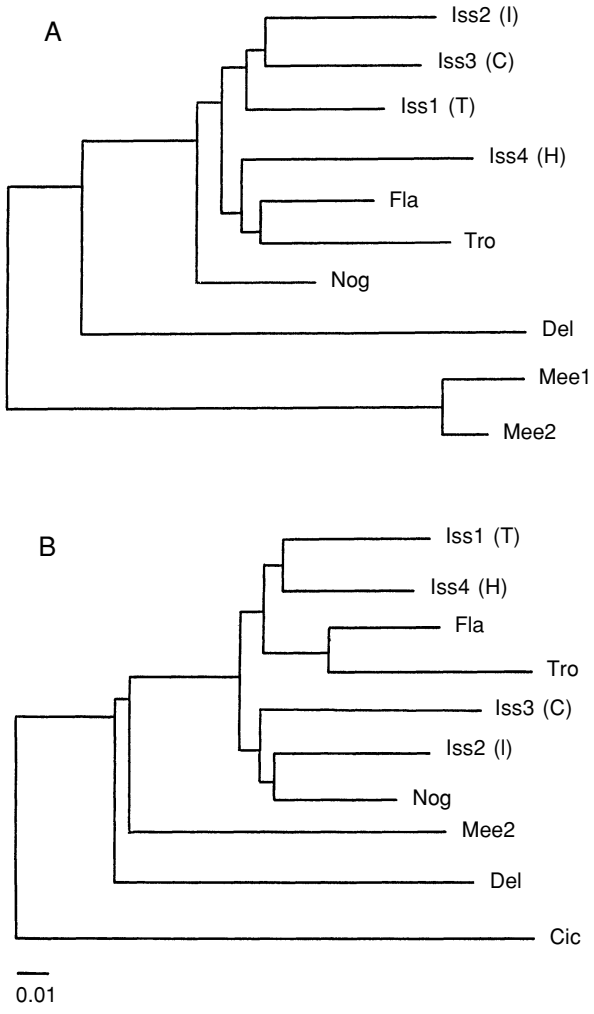

Fig. 3 (A). Phylogenetic trees inferred from the partial *COB* sequence data by the Neighbor-Joining method of the following pairwise distance models including proportion distance, Jukes and Cantor, Kimura 2-parameter, Tamura, and Tamura-Nei. Designations used here are the same as in Fig. 1. (B). Phylogenetic tree inferred from the partial 16S rDNA gene sequences by Neighbor-Joining method of the following pairwise distance models including proportion distance, Kimura 2-parameter, Tamura-Nei, Gamma (Kimura 2-parameter). Designations used here are the same as in Fig. 1

DISCUSSION

It is well known that nucleotide sequence compositions of insect mtDNA are rich in adenine and thymine. When *COB* gene nucleotide sequence compositions of the 3 insect orders Diptera, Hymenoptera, and Homoptera are compared (Table 4), all of them are AT rich in this gene. However, among these 3 insect orders there are some significant differences in nucleotide compositions. In the total nucleotide sequence compositions, guanine and cytosine are lower in Hymenoptera than in the other 2 insect orders, Diptera and Homoptera. The thymine proportion is similar to adenine in Homoptera, but is 10% higher than adenine in Diptera and Hymenoptera. There are many variations among these taxa; Diptera and Hymenoptera show great divergence from each other, although both are holometabolous insects. Thus, we might be able to obtain a base composition cline among members within the same order. Highly biased base compositions at different codon positions are seen in these 3 insect orders. Bias at the 2nd codon position is against adenine, and thymine is distributed equally in each codon position. Bias at the 3rd codon position against guanine and cytosine could be observed in these 3 insect orders, and the same observation has been made in the cytochrome oxidase I gene of beetles (Howland and Hewitt 1995). It is very interesting that the guanine proportion is very low in this codon position, and even reaches 0 in Hymenoptera. Although, it is well known that most mutations at codon position 3 are synonymous substitutions, yet our results show that substitutions at this codon position might not be random.

Table 2 indicates that the ratios of transitions over transversions of the *COB* nucleotide sequences increase linearly with taxonomic distance below the level of suborder; therefore substitutions in the *COB* nucleotide sequences are adequate to provide phylogenetic information within fulgoroids. The average ratio between Meenoplidae and the other fulgoroids is 1:2.12, which is almost identical to the values among the 3 orders (1:2.26). Thus, the transition over transversion ratio of the *COB* gene in fulgoroids has reached a steady-state level, whereas the increase in pairwise nucleotide differences between distant affinity families is due to transversion substitutions. Furthermore, scatter plots between total substitutions and transversions are linear, whereas transversions increase with total substitutions (data not shown), therefore, transversions can be used as a suitable criterion in analyzing the distant affinity family relationships of fulgoroids.

Molecular data analyses of both the *COB* and 16S rDNA genes agree with Emeljanov's conclusions that Delphacidae and Meenoplidae are more basal in Fulgoroidea (Emeljanov 1990) but are not consistent with other previous results (Muir 1923, Asche 1987). But, in the 16S rDNA gene analysis, Delphacidae is the most basal, while in the *COB* gene analysis it is Meenoplidae. These results may be due to different properties of the genes. In addition, the relationship of Meenoplidae and Delphacidae is distant, which is in disagreement with the morphological data of nymphs in which Meenoplidae clusters with Delphacidae and is situated on the advanced evolutionary lineage (Chen and Yang 1995). Different morphological characters appear to reveal different relative phylogenetic positions of Meenoplidae and Delphacidae. These contrasting phylogenetic positions may be due to the phenomenon of convergent evolution. The molecular results from either distance matrix (Tables 1, 3), or the phylogenetic analyses (Fig. 3) of both genes, strongly support Meenoplidae and

Table 4. Total base composition and at 1st, 2nd, and 3rd codon positions for 3 insect orders Diptera, Hymenoptera, and Homoptera

	Total				First				Second				Third			
	G	A	T	C	G	A	T	C	G	A	T	C	G	A	T	C
Diptera[a]	14.4	30.6	40.8	14.2	23.1	27.1	35.5	14.3	19.2	23.1	37.9	19.8	1.0	41.6	48.8	8.6
Hymenoptera[b]	8.6	36.7	45.3	9.4	11.7	42.4	35.8	10.1	14.2	24.2	47.5	14.2	0.0	43.3	52.5	4.2
Homoptera[c]	11.1	37.9	36.3	14.7	14.1	41.3	30.7	13.9	16.1	24.8	39.7	19.4	3.2	47.5	38.5	10.9

[a] Data from *D. yakuba, D. melanogaster, A. gambiae*, and *A. quadrimaculatus*.

[b] Data from *A. mellifera*.

[c] Data from fulgoroids in this paper.

Delphacidae being located on the basal lineages among fulgoroids. This concurs with results obtained from nuclear 18S rDNA nucleotide sequences (Campbell et al. 1995). Flatidae and Tropiduchidae are grouped together when both genes are used, and that is consistent with the morphological data (Yang and Fang 1993). Nogodinidae is closer to Issidae than to Flatidae, but their phylogenetic position is unclear; thus it may be necessary to analyze the remaining families of Fulgoroidea in order to clarify the relative position of Nogodinidae. The 4 Issidae subfamilies are not grouped together using either gene. Using the *COB* gene, Hemisphaerinae is grouped with Flatidae-Tropiduchidae, and the other Issidae subfamilies are grouped together. But when the 16S rDNA gene is used, Hemisphaerinae-Tonginae are grouped with Flatidae-Tropiduchidae and Issinae, and Nogodinidae is grouped with Caliscelinae. It is reasonable to conclude that members of Issidae are not clustered together if Issidae is not a monophyletic family. Furthermore, what evolutionary process did Issidae go through if it is homogeneous? The proposal that the speciation process can take place in a short period of time has increasingly turned out to be a rule rather than an exception (Helm-Bychowski and Cracraft 1993). Thus the phylogenetic position of Issidae could be easily misjudged if members of Issidae evolved according to this proposal. In addition, it is generally accepted that hot spot mutation of a gene would increase over time with the mutation proportion eventually reaching saturation. Thus, in a higher category, even if the ancestral lineage of each clade is still extant, it might still be difficult to deduce the correct phylogenetic relationships by DNA sequence due to a saturation effect (Yeh et al. 1997).

Using the nuclear 18S rDNA nucleotide sequence to evalvate the relationships of holometabolous insects, the sequence divergences within the orders of Lepidoptera, Coleoptera, and Diptera have been found to be 5%-8%, 8%, and 11%-14%, respectively (Carmean et al. 1992). The nucleotide proportion distance of the 18S rDNA gene between hymenopterous families is 1.8% (Pashley et al. 1993). The same gene sequences have been used to infer that homopterous insects are paraphyletic, in which 5 families of fulgoroids are involved (Cixiidae, Delphacidae, Dictyopharidae, Flatidae, and Issidae), and the proportion distances of the conserved gene among these 5 families are 1.5%-6.3% (Campbell et al. 1995). These results show that nucleotide information of the nuclear 18S rDNA gene might not be suitable for analysis of phylogenetic relationships of close affinity families since its nucleotide variations are relatively low. The nucleotide sequence divergences of the partial 16S rDNA and *COB* genes between species within a genus, close families, and distant families are about 10%, 20%, and 30%, respectively. MtDNA sequence divergences begin to plateau at 30% variation; presumably the genome becomes saturated with substitutions at the variable sites (Moritz et al. 1987). The proportions of nucleotide distances of the 16S rDNA gene (Table 3) show that the proportion distances between leafhoppers (Cicadellidae) and planthoppers (Fulgoroidea) is about 30%, which means that the nucleotide sequence divergences of the 16S rDNA gene at the superfamily level may have reached saturation. The proportion distances (Table 1) and transitions over transversions ratio (Table 2) of the *COB* nucleotide sequence comparisons show that the nucleotide differences have nearly reached a saturation level. Usually, in protein coding genes the mutation rate of transition substitution is faster than that of transversion substitution, and different codon positions have different adaptive evolutionary properties. Selecting codon positions with suitable nucleotide divergences should be a necessary criterion to analyze phylogeny. Nucleotide information of both 16S rDNA and *COB* genes reveals that the 2 genes can be candidate genes for studying the relationships of Fulgoroidea.

The basic phylogenetic proximities of Fulgoroidea lineages have been debated since 1923. A variety of phylogenetic trees based on different adult morphological characters have been obtained. Fulgoroid phylogeny based on nymphal characters appears different from that based on adult characters (Yang and Fang 1993, Chen and Yang 1995). Molecular data from fulgoroids according to nuclear 18S rDNA sequences (Campbell et al. 1995) suggest that this gene is too conserved to be usful in judging affinities of distal families within the same lineages. In our studies, based on the nucleotide sequences of the partial 16S rDNA and *COB* genes, some resolution of these difficulties has been obtained. We conclude that (1) Meenoplidae and Delphacidae each is a basal lineage in fulgoroids; (2) Flatidae are closer to Tropiduchidae than to the other fulgoroids; (3) Issidae is a nonhomogeneous group except that the members of Issidae have special adaptive evolutionary processes; and (4) both genes can be candidate genes for studying the relationships of fulgoroids.

Acknowledgments: This work was supported by Academia Sinica and the National Science Council of the Republic of China (Grant No. NSC-84-0409-B003-330).

REFERENCES

Arnason U, A Gullberg. 1996. Cytochrome b nucleotide sequences and the identification of five primary lineages of extant cetaceans. Mol. Biol. Evol. **13:** 407-417.

Asche M. 1987. Preliminary thoughts on the phylogeny of Fulgoromorpha (Homoptera: Auchenorrhyncha). In Proc. 6th Int. Auchen. Meeting; 7-11 Sept; Turin, Italy: Univ. of Turin Press, pp. 47-53.

Beard CB, DM Hamm, FH Collins. 1993. The mitochondrial genome of the mosquito *Anopheles gambiae*: DNA sequence, genome, organization, and comparisons with mitochondrial sequence of other insects. Insect Mol. Biol. **2:** 103-124.

Black IV W, J Piesman. 1994. Phylogeny of hard- and soft-tick taxa (Acari: Ixodida) based on mitochondrial 16S rDNA sequences. Proc. Natl. Acad. Sci. USA **91:** 10034-10038.

Bowen BW, WS Nelson, JC Avise. 1993. A molecular phylogeny for marine turtles: trait mapping, rate assessment, and conservation relevance. Proc. Natl. Acad. Sci. USA **90:** 5574-5577.

Campbell BC, JD Steffen-Campbell, RJ Gill. 1994. Evolutionary origin of whiteflies (Hemiptera: Sternorrhyncha: Aleyrodidae) inferred from 18S rDNA sequences. Insect Mol. Biol. **3:** 73-89.

Campbell BC, JD Steffen-Campbell, JT Sorensen, RJ Gill. 1995. Paraphyly of Homoptera and Auchenorrhyncha inferred from 18S rDNA nucleotide sequences. Syst. Entomol. **20:** 175-194.

Carmean D, LS Kimsey, ML Berbee. 1992. 18S rDNA sequences and the holometabolous insects. Mol. Phyl. Evol. **1:** 270-278.

Chen S, CT Yang. 1995. The metatarsi of the Fulgoroidea (Homoptera: Auchenorrhyncha). Chinese J. Entomol. **15:** 257-269.

Clary DO, DR Wolstenholme. 1985. The mitochondrial DNA molecule of *Drosophila yakuba*: nucleotide sequence, gene organization, and genetic code. J. Mol. Evol. **22:** 252-271.

Crozier RH, YC Crozier. 1993. The mitochondrial genome of the honeybee *Apis mellifera*: complete sequence and genome organization. Genetics **133:** 97-117.

Davis RE, TJ Kelly, EP Master, BS Thyagaraja, CA Rote, RB Imberski. 1994. Complete base sequence for the mitochondrial large subunit ribosomal RNA of the gypsy moth *Lymantria dispar* (L.). Insect Mol. Biol. **3:** 219-228.

Devereux J, P Haeberli, P Marquess. 1991. Genetic computer group manual, version 7.0. Madison, WI: Univ. Wisconsin.

Dietrich CH, J Jokela, WC Black IV. 1997. Utility of DNA sequence data for leafhopper and treehopper (Homoptera: Membracoidea) phylogenies. In Program & Abstract book of 9th Int. Auchen. Congress; 17-21 Feb; Sydney, Australia: Australian Academy Sci. and Australian Entomol. Soc. Press, pp. 49-50.

Dowton M, AD Austin. 1994. Molecular phylogeny of the insect order Hymenoptera: apocritan relationships. Proc. Natl. Acad. Sci. USA **91:** 9911-9915.

Emeljanov AF. 1990. An attempt of construction of phylogenetic tree of the planthoppers (Homoptera, Cicadina). Rev. D'Ent. **1:** 31-40.

Fang Q, WC Black IV, HD Blocker, RF Whitcomb. 1993. A phylogeny of new world *Deltocephalus*-like leafhopper genera based on mitochondrial 16S ribosomal DNA sequences. Mol. Phyl. Evol. **2:** 119-131.

Garesse R. 1988. *Drosophila melanogaster* mitochondrial DNA: gene organization and evolutionary considerations. Genetics **118:** 649-663.

Goodchild AJP. 1966. Evolution of the alimentary canal in the Hemiptera. Biol. Rev. Cambridge Philos. Soc. **41:** 97-140.

Graybeal A. 1993. The phylogenetic utility of cytochrome b: lessons from bufonid frogs. Mol. Phyl. Evol. **2:** 256-269.

Hay JM, I Ruvinsky, SB Hedges, R Maxson. 1995. Phylogenetic relationships of amphibian families inferred from DNA sequences of mitochondrial 12S and 16S ribosomal RNA genes. Mol. Biol. Evol. **12:** 928-937.

Helm-Bychowski K, J Cracraft. 1993. Recovering phylogenetic signal from DNA sequences: relationships within the corvine assemblage (class Aves) as inferred from complete sequences of the mitochondrial DNA cytochrome b gene. Mol. Biol. Evol. **10:** 1196-1214.

Honeycutt RL, MA Nedbal, RM Adkins, LL Janecek. 1995. Mammalian mitochondrial DNA evolution: a comparison of the cytochrome b and cytochrome c oxidase II genes. J. Mol. Evol. **40:** 260-272.

Howland DE, GM Hewitt. 1995. Phylogeny of the Coleoptera based on mitochondrial cytochrome oxidase I sequence data. Insect Mol. Biol. **4:** 203-215.

Irwin DM, TD Kocher, AC Wilson. 1991. Evolution of the cytochrome b gene of mammals. J. Mol. Evol. **32:** 128-144.

Jean CT, CF Hui, SC Lee, CT Chen. 1995. Variation in mitochondrial DNA and phylogenetic relationships of fishes of the subfamily Sparinae (Perciformes: Sparidae) in the coastal waters of Taiwan. Zool. Stud. **34:** 270-280.

Jermiin LS, RH Crozier. 1994. The cytochrome b region in the mitochondrial DNA of the ant *Tetraponera rufoniger* : sequence divergence in Hymenoptera may be associated with nucleotide content. J. Mol. Evol. **38:** 282-294.

Jukes JH, CR Cantor. 1969. Evolution of protein molecules. *In* HN Munro, ed. Mammalian protein metabolism. New York: Academic Press, pp. 21-132.

Kimura M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. **16:** 111-120.

Kumar S, K Tamura, M Nei. 1993. MEGA: molecular evolutionary genetics analysis, version 1.01. University Park, PA: Pennsylvania State Univ., 130 pp.

Martin JA, DP Pashley. 1992. Molecular systematic analysis of butterfly family and some subfamily relationships (Lepidoptera: Papilionidae). Ann. Entomol. Soc. Am. **85:** 127-135.

Mitchell SE, AF Cockbum, JA Seawright. 1993. The mitochondrial genome of *Anopheles quadrimaculatus* species: a complete nucleotide sequence and gene organization. Genome **36:** 1058-1073.

Moritz C, TE Dowling, WM Brown. 1987. Evolution of animal mitochondrial DNA: relevance for population biology and systematics. Ann. Rev. Ecol. Syst. **18:** 269-292.

Muir F. 1923. On the classification of the Fulgoroidea (Hemiptera). Proc. Hawaiian Entomol. Soc. **5:** 205-247.

Muir F. 1930. On the classification of the Fulgoroidea. Ann. Mag. Nat. Hist. **10:** 461-478.

Pashley DP, BA McPheron, EA Zimmer. 1993. Systematics of helometabolous insect orders based on 18S ribosomal RNA. Mol. Phyl. Evol. **2:** 132-142.
Reeder TW. 1995. Phylogenetic relationships among phrynosomatid lizards as inferred from mitochondrial ribosomal DNA sequences: substitutional bias, and information content of transitions relative to transversions. Mol. Phyl. Evol. **4:** 203-222.
Rosel PE, MG Haygood, WF Perrin. 1995. Phylogenetic relationships among the true porpoises (Cetacea: Phocoenidae). Mol. Phyl. Evol. **4:** 463-474.
Saitou N, M Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:** 406-425.
SAS Institute Inc. 1996. Logistic regression, examples using the SAS system. Cary, NC, USA: SAS Institute Inc.
Schuh RT. 1979. Review of Evolutionary trends in Heteroptera. Part II. Mouthpart-structures and feeding strategies, by R. H. Cobben. Syst. Zool. **28:** 653-656.
Tamura K. 1992. Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G+C-content biases. Mol. Biol. Evol. **9:** 678-687.
Tamura K, M Nei. 1993. Estimation of the number of nucleotide substitution in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. **10:** 512-526.
Wettstein PJ, M Strausbauch, T Lamb, J States, R Chakraborty, L Jin, R Riblet. 1995. Phylogeny of six *Sciurus aberti* subspecies based on nucleotide sequences of cytochrome b. Mol. Phyl. Evol. **4:** 150-162.
Xiong B, TD Kocher. 1991. Comparison of mitochondrial DNA sequence of seven morphospecies of black flies (Diptera: Simuliidae). Genome **34:** 306-311.
Xiong B, TD Kocher. 1993. Phylogeny of sibling species of *Simulium venustrum* and *S. verecundum* (Diptera: Simuliidae) based on sequences of the mitochondrial 16S rRNA gene. Mol. Phyl. Evol. **2:** 293-303.
Yang CT, SJ Fang. 1993. Phylogeny of Fulgoromorpha nymphs, first results. *In* S Drosopolus, PV Petrakis, MF Claridge, PWF de Virijer, eds. Proc. 8th Auchen. Congress; 9-13 Aug.; Delphi, Greece, pp. 25-26.
Yang YJ, YS Lin, JL Wu, CF Hui. 1994. Variation in mitochondrial DNA and population structure of the Taipei treefrog *Rhacophorus taipeianus* in Taiwan. Mol. Ecol. **3:** 219-228.
Yeh WB, CF Hui, CT Yang. 1997. Some considerations of evolutionary process in Fulgoromorpha. *In* Program & Abstract book of 9th Int. Auchen. Congress; 17-21 Feb; Sydney, Australia: Australian Academy Sci. and Australian Entomol. Soc. Press.
Yeh WB, CT Yang, CF Hui. 1996. The phylogenetic relationships of Fulgoromorpha by DNA sequence analyses. *In* P Congressi, C Affari, F Basso, eds. Proc. 20th Int. Congress of Entomol.; 25-31 Aug; Firenze, Italy.
Zrzavy J. 1992. Evolution of antennae and historical ecology of the hemipteran insects (Paraneoptera). Acta Entomol. Bohemoslov. **89:** 77-86.

軍配飛蝨科群(Homoptera: Fulgoroidea)科間類緣關係之研究

葉文斌[1] 楊仲圖[1] 許祖法[2]

本研究運用粒線體兩個基因的去氧核醣核酸序列重構軍配飛蝨科群(Tropiduchidae-group)科間之類緣關係，分別是 16S 核醣體去氧核醣核酸(16S rDNA) 3'端的 400 個鹼基及細胞色素 b 基因(cytochrome b)之 5'端的 120 個密碼位。整體而言，此二基因分析六個科十個分類單元(於 16S 核醣體去氧核醣核酸為九個分類單元)的結果彼此相符。類緣關係的分析顯示，軍配飛蝨科(Tropiduchidae)及蛾蠟蟬科(Flatidae)聚類在一起；縞飛蝨科(Meenoplidae)及飛蝨科(Delphacidae)分別位於基部；而圓飛蝨科(Issidae)不是一個自然的分類單元。當其它各目昆蟲細胞色素 b 基因的 DNA 序列加入比對時顯示，轉換取代(transition)及顛換取代(transversion)的比值在蠟蟬總科(Fulgoroidea)內已趨近於穩定。另外，此二基因均可應用於蠟蟬總科科間之類緣關係的研究，因為核酸序列的比對於屬內、近緣科間及疏遠科間的差異各約 10%、20%及 30%。

關鍵詞：粒線體 DNA，16S 核醣體去氧核醣核酸，細胞色素 b，蠟蟬總科。

[1]國立中興大學昆蟲學系
[2]中央研究院動物研究所