Insect Science (2006) 13, 167−177

Resolution of phylogenetic relationships of the major subfamilies of the Delphacidae (Homoptera: Fulgoroidea) using the mitochondrial ribosomal DNA

EDDY DIJKSTRA[1], MICHEL A. SLOTMAN[1] and RORY J. POST[2]

[1]Biosystematics Group, Wageningen University, the Netherlands and [2]The Natural History Museum, London, UK

Abstract Delphacid relationships from the genus level to the subfamily have been completely resolved (among those taxa examined) using sequence data from the 3' end of the 12S gene. Monophyly of the non-asiracine subfamilies was strongly supported and the asiracine *Ugyops* was placed in the most basal position of the tree. Support levels for monophyly of the Delphacini increased after weighting transversions more heavily than transitions and after removing the cixiid outgroup from the dataset. Among the Delphacini, *Conomelus* and *Megamelus* were more closely related to each other than either was to *Chloriona*. These results are in agreement with the tree based on morphological characters. However, in contrast to morphological data our results strongly supported a sister group relationship between the Stenocraninae and the Kelisiinae. Although the 12S gene fragment gave some information about the species relationships within *Chloriona*, neither this fragment nor the 5' end of the 16S gene appear to be very useful for this level. Molecular evolutionary patterns provided evidence that there has been a shift in base composition from T to A during the early evolution of the non-Asiracinae. The non-Asiracinae also had comparatively fast substitution rates and these two observations are possibly correlated. In the 'modern' delphacid *Chloriona*, the AT content was comparatively low in regions free of constraints but this was not the case for 'non-modern' delphacids. The tRNA for valine has been translocated elsewhere, probably before the Delphacidae and Cixiidae diverged from each other.

Key words classification, Delphacidae, evolution, Fulgoroidea, Homoptera, mitochondrial DNA, phylogeny, ribosomal DNA
DOI 10.1111/j.1744-7917.2006.00079.x

Introduction

The planthopper family Delphacidae is the largest of the 20 recognized families of the Fulgoroidea and currently more than 2 000 species have been described (Asche, 1990). They have a number of interesting features which make them a suitable group for ecological and evolutionary studies. For example, the easily recognisable wing dimorphism has been used to study trade-offs between dispersal and reproduction (Denno, 1994), and the acoustic behavior of planthoppers has been used to study species recognition and speciation (Claridge & de Vrijer, 1994). A number of delphacids are of economic importance because they feed on rice, corn or other cereals, and are able to transmit plant pathogens (Wilson & O'Brien, 1987).

Phylogenetic relationships in Delphacidae have been

Correspondence: Rory J. Post, Department of Entomology, The Natural History Museum, Cromwell Road, London SW7 5BD, UK. Tel: +44 20 79425593; fax: +44 20 79425229; e-mail: r.post@nhm.ac.uk

Current address: Eddy Dijkstra, National Plant Protection Service, Department of Entomology, Wageningen, The Netherlands. Michel A. Slotman, Department of Ecology and Evolutionary Biology, Yale University, USA

studied by Asche (1985, 1990) using morphological characters, including the presence/absence of yeast-like symbionts. The following subfamily relationships were suggested: (((((Delphacinae, Plesiodelphacinae) Stenocraninae) Kelisiinae) Vizcayinae) Asiracinae). All subfamilies (except the Asiracinae) were supported by autapomorphies.

There are remarkable differences in the sizes of the subfamilies. The Delphacinae is the largest group with ca. 1 090 described species in the Delphacini. In contrast, the Vizcayinae (Oriental region) and Plesiodelphacinae (South America) are very small subfamilies with both containing less than 10 known species (Asche, 1990; Wilson *et al*., 1994). Phylogenetic relationships at lower taxonomic levels, for example species and genus level, are largely unknown because of a lack of suitable morphological characters (Manfred Asche, 1997, personal communication). Therefore, molecular data may contribute greatly to our understanding of relationships at lower taxonomic levels as well as providing independent datasets to investigate the hypotheses of higher classification based on morphological characters.

The ribosomal internal transcribed spacer (ITS) regions (Jones *et al*., 1993) and the mitochondrial cytochrome c oxidase I (COI) gene (Roderick & Metz, 1997) have been used for inferring phylogenetic relationships at the population and/or species level for two delphacid genera. Unfortunately, despite intensive efforts, the "conserved" primer combination used by Jones *et al*. (1993) failed to work properly for *Chloriona* and *Javesella* (Dijkstra, 1995, unpublished data).

There has been one previous attempt to resolve phylogenetic relationships at the higher level within the Delphacidae using molecular data (Dijkstra *et al*., 2003). Monophyly of both the non-Asiracinae and Asiracinae was supported using nucleotide and amino acid characters from a fragment of the COI gene. In addition, close relationships between *Conomelus* and *Megamelus*, and between Stenocraninae and Kelisiinae were well supported using amino acid characters. Nucleotide characters also supported these two groups but only in a spectral analysis and not in the parsimony analysis of the main dataset. A sister group relationship for *Conomelus* and *Megamelus* was already presumed to exist but there were no morphological characters to support this (Asche, 1985). However, a sister group relationship between the Stenocraninae and Kelisiinae is in conflict with evidence from morphological characters. A number of other groups were also shown to be monophyletic (Dijkstra *et al*., 2003), agreeing with morphological data, but only under certain conditions. For example, monophyly of the Delphacini was only observed in parsimony analyses after pruning distantly related taxa from the dataset. Moreover, species and generic relationships among a sample of Delphacini were not very clear. Therefore, other genes needed to be investigated.

In this study we investigate the utility of parts of the two mitochondrial ribosomal genes for inferring phylogenetic relationships in delphacid planthoppers using parsimony and maximum likelihood. The 5' end of the 16S gene was used to try to resolve relationships within the genus *Chloriona*. The 3' end of the 12S gene was used at all taxonomic levels within the family. Mitochondrial ribosomal genes have been shown to be phylogenetically useful for distantly related taxa, but at the species level these genes may not be variable enough and/or the sites which do vary may have experienced multiple hits (reviewed by Simon *et al*., 1994).

Data from the COI gene (Dijkstra *et al*., 2003) also suggested that the non-Asiracinae evolved faster than *Ugyops*. Since mitochondrial genes are inherited as a single unit we have investigated whether the 12S data also show the same pattern.

Material and methods

Taxon sampling

Species used in this study are listed in Table 1. As far as possible representatives from different lineages (following Asche, 1985) were field-collected or taken from laboratory cultures. Four of the six delphacine subfamilies were sampled for this study. The Asiracinae, Kelisiinae and Stenocraninae are each represented by a single species. The two subfamilies which were not sampled are very small and do not occur in the Palaearctic region (Vizcayinae includes one genus, and Plesiodelphacinae consists of two genera). Within the Delphacinae, there are three tribes, but the Delphacini contains the great majority of genera (90%) and was sampled with the aim of obtaining a range of genera from both the 'modern' and 'non-modern' delphacids (sensu Asche, 1985), and a range of species from a sample genus (*Chloriona*). *Chloriona glaucescens* was represented from France and from The Netherlands. *Conomelus* and *Megamelus* were sampled as representatives of the so-called 'non-modern' delphacids and *Chloriona* and *Javesella* were selected to represent the 'modern' Delphacidae (all Delphacini, see Asche, 1985, 1990). However, the *Javesella* species failed to amplify. Alcohol-preserved *Ugyops* specimens were obtained from Manfred Asche. The cixiid *Tachycixius pilosus* was used to root the tree. A number of studies suggest that the Cixiidae are the sister group of the Delphacidae (Asche, 1988; Campbell *et al*., 1995; Bourgoin *et al*., 1997), although

Yemel'yanov (1991) considers both families to be basal within the Fulgoroidea and not sister groups.

DNA extraction

Single specimens were extracted using the method of Post *et al.* (1993). Mass extractions were done for *Chloriona glaucescens* from France, *Stenocranus major* and *Anakelisia fasciata*. Protocols for mass extractions, cloning and sequencing were as previously described by Dijkstra *et al.* (2003).

PCR

Primers LR-J-13417 (5' ATGTTTTTGTTAAACAGG CG) and SR-N-14588 (5' AAACTAGGATTAGATACC CTATTAT) were used to amplify a section of the 5' end of the 16S, the tRNA valine and the 3' end of the 12S gene (Simon *et al.*, 1994). DNA from *C. glaucescens* TW, *C. vasconica*, *C. smaragdula* and *Javesella dubia* (Kirschbaum), a putative closely related taxon to *Chloriona*, could not be amplified using this primer combination, and DNA from these species (except for *J. dubia*) was amplified using LR-J-12887 (5' CCGGTCTGAACTCA GATCACGT), which is located further downstream in the 16S gene, in combination with an internal primer MtRibIN3 (5' TTGTACCTTTTGTATCAGGGTT) which is located near the 5' end of the 16S gene (Simon *et al.*, 1994). The reverse complement of the internal primer was used in combination with SR-N-14588.

Polymerase chain reaction (PCR) was performed using a Hybaid Omnigene Temperature Cycler HB-TR3-CM with the following conditions. An initial denaturation cycle at 94℃ (4 min) was followed by 30−40 cycles of denaturation at 94℃ (1 min), annealing at 50℃ (30 sec), and extension at 72℃ (1 min). The program was completed by a final extension step of 5 mins. PCR products were ligated in a Promega T-vector and plasmids were sequenced in one direction on an ABI 373 automated sequencer using the ABI Dye Terminator Cycle Sequencing Kit, and the internal primers used to obtain partial overlap. Sequences have been deposited in Genebank under accession numbers AY178032−AY178043.

Data analysis

Sequence fragments were aligned with 'pileup' using parameters set by the program, and manually adjusted with 'lineup' (GCG software package version 8.0, University of Wisconsin, Madison, USA). In order to increase confidence in the homology among the nucleotides positions, ambiguous aligned regions (caused by length differences

Table 1 List of species used in this study.

Taxon	Collecting site	Code
Family Delphacidae		
Delphacinae (ca. 250)		
Delphacini		
(modern Delphacidae)		
Chloriona dorsata Edwards†	Wamel, The Netherlands	
C. glaucescens Fieber†	'het twiske', Landsmeer, The Netherlands	TW
C. glaucescens†	Canet-Plage, France	CP
C. vasconica Ribaut†	Wamel, The Netherlands	
C. smaragdula (Stål)†	'het twiske', Landsmeer, The Netherlands	
C. unicolor (Herrich-Schaffer)†	Wilhelminadorp, The Netherlands	Chu
(non-modern Delphacidae)		
Conomelus sp.	Renkum, The Netherlands	Can
Megamelus notula (Germar)	Veenendaal, The Netherlands	Mno
Stenocraninae (4)		
Stenocranus major (Kirschbaum)	'het twiske', Landsmeer, The Netherlands	STm
Kelisiinae (2)		
Anakelisia fasciata (Kirschbaum)	'het broek', Waardenburg, The Netherlands	Afa
Asiracinae (23)		
Ugyops sp.	Rarotonga, Cook Is, New Zealand	Ugy
Family Cixiidae		
Tachycixius pilosus (Olivier)	Ginkelse hei, Ede, The Netherlands	Tpi

†specimens from laboratory rearing. Numbers in paranthesis are the number of known genera in the subfamily (following Asche, 1985).

for example) were excluded from the data set. The 16S gene, the tRNA valine and the 12S gene were sought by comparison with the nucleotide sequences and secondary structures of *Drosophila yakuba* (Clary & Wolstenholme, 1985) and *Magicicada tredecim* (Simon *et al*., 1994). The tRNA valine gene was not found and separate datasets were compiled for 12S and 16S genes. Sequences from the 5' end of the 16S gene were only obtained for the Delphacini and hence they were only used to try to resolve phylogenetic relationships within *Chloriona*. For 12S two datasets were constructed. The large 12S dataset consisted of all species with 352 unambiguously aligned nucleotides, and the small 12S dataset consisted of only Delphacini because a larger number of sites (391 nucleotides) could then be unambiguously aligned.

Maximum parsimony (MP) and maximum likelyhood (ML) analyses were carried out in PAUP (D. L. Swofford, 2000. PAUP*: Phylogenetic Analysis Using Parsimony [*and other methods] Version 4.0b8 Sinauer, Sunderland, MA.). Parsimony analyses were conducted using the branch and bound option. Remaining gaps were treated as missing. Node support was assessed by performing 1 000 pseudoreplicates with 10 random addition replicates per pseudoreplicate and TBR branch swapping, excluding uninformative sites. Maximum likelihood analyses were performed using the heuristic search option; 100 random addition replicates with NNI branch swapping. Parameters for the GTR + I + G model were estimated during the searches. The estimated parameter values of the best tree were used in the bootstrap analysis: 200 pseudoreplicates with one random addition per pseudoreplicate and TBR branch swapping. In the bootstrap analysis of the small 12S dataset, alpha was estimated during the bootstrap runs because in the best ML tree alpha was estimated to be infinity, which may be an inaccurate estimate due to the small number of taxa (Sullivan *et al*., 1999).

Tajima's (1993) rate tests were performed on the large 12S dataset only, using Molecular Evolutionary Analysis Package (MEA, written by Etsuko Moriyama, Yale University, USA).

Results

Phylogenetic analyses of subfamilies and tribes

The large 12S dataset containing representatives of a number of different subfamilies consisted of 352 unambiguously aligned characters of which 136 were variable. The parsimony analysis resulted in two equally parsimonious trees of 199 steps, one of which is shown in Fig. 1. All nodes above the species level are well supported, indicating strong phylogenetic signals in the dataset. The ML tree had an identical topology and usually similar support values (Fig. 1). Moreover, the relationships shown are consistent with results from the COI gene (Dijkstra *et al*., 2003).

The Delphacini (*Conomelus*, *Megamelus* and *Chloriona*)

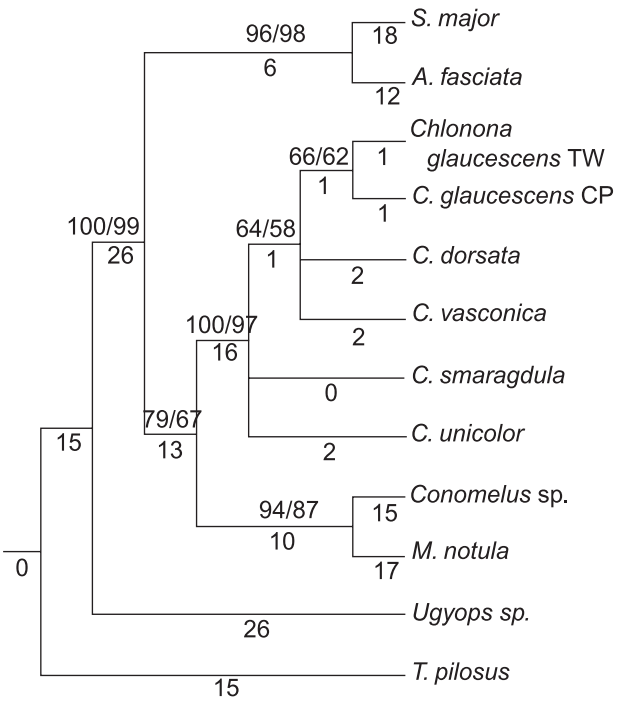

Fig. 1 One of the two equally parsimonious trees. Values above branches show the bootstrap support (maximum parsimony/maximum likelihood). Number of steps are shown below the branches.

form a monophyletic group and are moderately well supported. Within this group there is good evidence that *Conomelus* and *Megamelus* are closely related taxa. Nevertheless, compared to the number of inferred changes (13) on the Delphacini branch, a support of 79% indicates a considerable loss of phylogenetic signal. However, support for the Delphacini increased to 97% (parsimony) after pruning *Tachycixius* from the dataset, indicating the presence of homoplasies in the cixiid outgroup and Delphacini. Weighting transversions two or three times as heavily as transitions in parsimony analyses had a similar but less pronounced effect, with support values increasing to 88% and 91%, respectively.

Phylogenetic analyses within Chloriona

Phylogenetic relationships of the *Chloriona* species were studied using the 16S data (448 characters, 141 variable)

and the small 12S dataset (391 characters, 76 variable). Only Delphacini were included in these datasets. *Megamelus* and *Conomelus* served as outgroups which is consistent with Asche (1985), Dijkstra *et al*. (2003) and Fig. 1. The 12S data resulted in a single MP tree of 84 steps (Fig. 2). The 16S data resulted in a single tree of 180 steps (Fig. 3). Although the two genes are very closely linked, the topologies are different, except for the position of *C. unicolor* and the monophyly of *C. glaucescens*. The 12S tree may be closer to the true tree than the16S tree for two reasons. First, the 12S tree is identical to the tree based on data from the COI gene (Dijkstra *et al*. 2003). Secondly, although not fully resolved, the 12S tree has more nodes supported than the 16S tree. Nevertheless, the resolution and/or bootstrap support is still rather poor for both genes. The maximum likelihood analysis of the 12S gene resulted in the same tree as shown in Fig. 2, differing only in the amount of support at the deepest node within *Chloriona*. This supports our view that the 12S gene may be closer to the true tree than the tree based on 16S data. In the ML tree based on the16S data the same two nodes as in Fig. 3 were supported. However, the topology differed with *C. dorsata* placed in the most basal position and *C. unicolor* the sister species of *C. vasconica*.

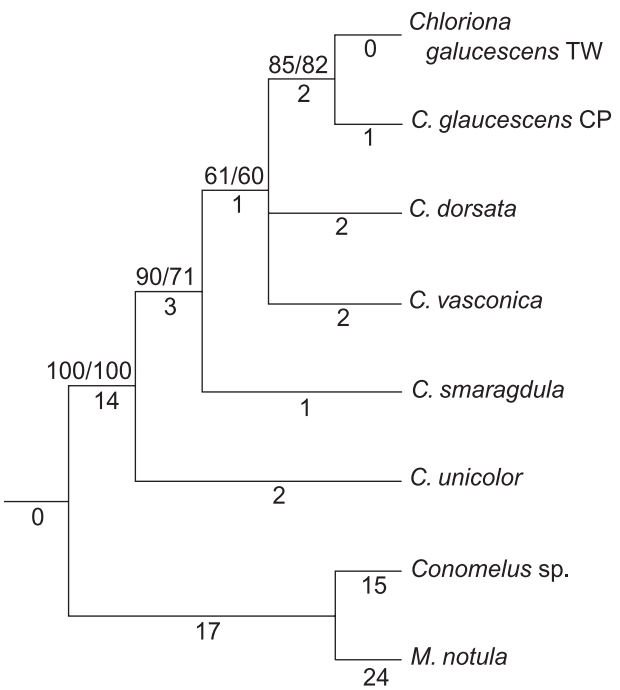

Fig. 2 Single most parsimonious tree showing *Chloriona* relationships using the 12S gene. Values above branches show the bootstrap support (maximum parsimony/ maximum likelihood). Number of steps are shown below the branches.

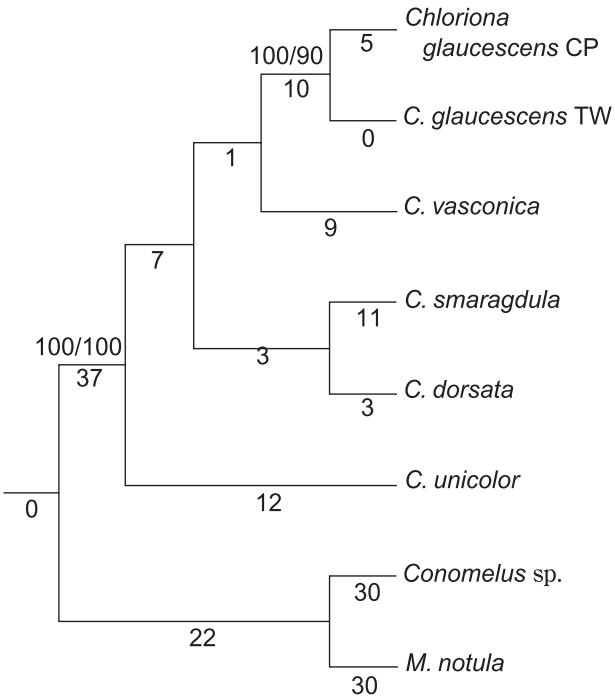

Fig. 3 Single most parsimonious tree showing *Chloriona* relationships using the 16S gene. Values above branches show the bootstrap support (maximum parsimony/ maximum likelihood). Number of steps are shown below the branches.

Nucleotide composition

Distantly related taxa may be joined together in a phylogenetic analysis due to artifactual base compositional effects (Hasegawa & Hashimoto, 1993). To investigate the possibility that the sister group relationship between *Stenocranus* and *Anakelisia,* for example, was a result of this phenomenon we investigated the nucleotide content of the taxa. Base composition data were calculated from the unambiguously aligned datasets.

The average AT content for the large 12S, small 12S and 16S datasets were 73.7% (range 72.3%–76.1%), 75.3% (74.3%–77.6%) and 82.4% (80.4%–87.1%), respectively. Similar values have also been noted for other insects (review Simon *et al*., 1994; Uhlenbush *et al*., 1987). It is also known that the 5' end halves of the ribosomal genes are more variable than the 3' end halves (references in Simon *et al*., 1996) and that the nucleotide bias tends to accumulate in the most variable positions (Simon *et al*., 1994). This could explain why the AT content in the 16S is relatively high and also why the AT content in the small 12S dataset is higher than in the large 12S dataset. The small 12S dataset consists of Delphacini only and there were fewer alignment problems. As in some other hemimetabolous

insects (Uhlenbusch *et al*., 1987; Garcia & Powell, 1998) thymine was always the most common nucleotide and cytosine was the least common in these three datasets.

Nucleotide homogeneity was not rejected in any of the three datasets. However, in the large 12S dataset, the thymine frequency in the non-Asiracinae was lower than *Ugyops* and the cixiid outgroup, with average values of 40.6% (39.1%−41.9%), 45.1% and 47.1%, respectively. Similarly, the adenine content in the non-Asiracinae was comparatively higher with average values of 32.8% (31.7%−35.2%), 28.3% and 29.1% respectively. These differences were even more pronounced if only variable positions were considered (data not shown). Average thymine content for the non-Asiracinae was 40.1% (36.8%−43.7%), but 51.9% and 57.1% for *Ugyops* and *Tachycixius*. Similarly, adenine frequencies were 37.0% (33.6%−42.0%), 24.8% and 27.1% respectively. These results suggest a shift in base composition from thymine to adenine on the coding strand which possibly occurred in the common ancestor of the non-Asiracinae. Separate χ^2 tests between the 10 non-asiracines and *Ugyops* showed significant differences (range $P = 0.041\ 6$−$0.005\ 6$) except for the tests with *C. unicolor*, *Stenocranus* and *Anakelisia*. Comparisons between non-asiracines and *Tachycixius* showed no significant differences.

Since the AT bias tends to accumulate in the most variable positions (Simon *et al*., 1994), datasets consisting of variable sites only may be expected to have a higher AT content than datasets consisting of all positions. Consistent with this, all taxa in the large 12S dataset had higher AT values when constant sites were removed (data not shown). In our 16S dataset with variable positions only, *Megamelus* and *Conomelus* also followed this expected pattern. However, the *Chloriona* species showed a decrease in AT content if constant sites were removed. Moreover, base homogeneity was rejected for this dataset ($P = 0.002\ 55$). The AT content values for all sites considered and for variable sites only were: *Conomelus*, 85.7% and 86.4%; *Megamelus*, 87.1% and 91.5%; *Chloriona*, 81.1% (80.4%−82.0%) and 72.2% (70.0%−75.2%). Whereas the adenine content was fairly constant in the dataset with variable sites only (37.6%−41.4%), the thymine content differed among the taxa. The thymine content in *Conomelus* and *Megamelus* were 52.5% and 45.0%, respectively. In *Chloriona* it was on average 33.0% (31.2%−35.5%). This suggests that the increase of both guanine and cytosine in *Chloriona* is mainly at the cost of thymine.

In the small 12S dataset, the AT content of the *Chloriona* species also decreased when constant sites were removed but, as in the 16S gene, increased in *Megamelus* and *Conomelus*. However, this time the increase in GC content in *Chloriona* appeared to be mainly due to an increase in guanine at the expense of adenine. The guanine content for *Conomelus*, *Megamelus* and *Chloriona* were 9.2%, 7.9% and on average 18.2% (15.8%−19.7%), respectively. For adenine these numbers were 39.5%, 38.2% and on average 29.2% (26.3%−31.6%), respectively. Thus, in both gene fragments the AT content decreases for *Chloriona* but the nucleotides involved seem to differ. Nevertheless, base homogeneity was not rejected ($P = 0.758\ 7$).

In the large 12S dataset, the AT content increased in all taxa, including *Chloriona*, when constant sites were removed (data not shown). The AT content of the *Chloriona* species in the small 12S dataset (74.7%) was also (slightly) higher compared to the value for large 12S dataset (73.2%). Therefore, the short nucleotide strings which could be added to the small 12S dataset must be comparatively AT-rich otherwise the AT content could not have increased in the small 12S dataset. However, a large proportion of these AT-rich sites must be constant or unvaried in the Delphacini otherwise the AT content in the *Chloriona* species cannot decrease if constant sites are removed. Thus, in *Chloriona*, guanine and cytosine increased in those positions which are the most variable (in the aligned datasets), whereas in members of the sister lineage of *Chloriona*, *Megamelus* and *Conomelus*, these positions are proportionally AT-rich.

Relative rates

A number of rate differences were found in the large 12S dataset (Table 2). All taxa tested have experienced more substitutions than *Ugyops*, and for each subfamily there is at least one species evolving significantly faster than *Ugyops* (first five rows). Moreover, although the difference between, for example *C. unicolor* and *Ugyops* is not significant, it is in the same direction, and *C. unicolor* evolves significantly faster than *Anakelisia* (row 6), which in turn evolves significantly faster than *Ugyops* (row 5). Perhaps the difference between *C. unicolor* and *Ugyops* has gone undetected because of saturation effects. Note, for example, that there are more transversions than transitions in these comparisons, even for *Ugyops*.

The three delphacine taxa which were tested have all accumulated more substitutions than both *Anakelisia* and *Stenocranus*. This was significant in only two individual cases, but the overall trend suggests that the *Anakelesia* + *Stenocranus* clade shows a lower rate than the Delphacini. No significant rate differences were detected within the Delphacini.

Intra- and interspecific divergences

Divergence levels (as uncorrected distances) were calculated for comparisons within *Chloriona* from the small 12S

Table 2 Tajima's relative rate test (1 D method).

Species			M1	M2	S1	S2	V1	V2	χ^2		
1	2	3							Total	Ts	Tv
Chu	Ugy	Tpi	38	33	10	15	28	18	0.35	1.00	2.17
Can	Ugy	Tpi	40	29	12	11	28	18	1.75	0.04	2.17
Mno	Ugy	Tpi	47	27	16	12	31	15	5.41**	0.57	5.57**
STm	Ugy	Tpi	41	20	12	10	29	10	7.23***	0.18	9.26****
Afa	Ugy	Tpi	38	20	9	8	29	12	5.59**	0.06	7.05***
Chu	Afa	Ugy	26	13	10	3	16	10	4.33*	3.77	1.39
Can	Afa	Ugy	25	15	7	4	18	11	2.50	0.82	1.69
Mno	Afa	Ugy	33	16	14	3	19	13	5.90**	7.12***	1.13
STm	Afa	Ugy	14	9	7	2	7	7	1.09	2.78	0.00
Chu	STm	Ugy	24	16	7	6	17	10	1.60	0.08	1.82
Can	STm	Ugy	24	19	6	8	18	11	0.58	0.29	1.69
Mno	STm	Ugy	31	19	13	7	18	12	2.88	1.80	1.20
Chu	Mno	Afa	18	25	7	11	11	14	1.14	0.89	0.36
Can	Mno	Afa	11	20	4	9	7	11	2.61	1.92	0.89
Chu	Can	Afa	16	14	7	6	9	8	0.13	0.08	0.06

Rows 1−5 show comparisons between non-asiracines and *Ugyops*. Following rows show comparisons within non-asiracines with the last three rows comparisons within the Delphacini. M1 is defined as the total number of observed differences between species 1, and species 2 and 3, with the latter two species having identical character states. M2 (S1, V1) is defined in a similar way. S1 refers to the number of transitions, V1 the number of transversions. Species 3 is the outgroup species. *$P < 0.05$; **$P < 0.025$; ***$P < 0.01$; ****$P < 0.005$.

dataset because it contained more characters than the large 12S dataset, and with respect to *Chloriona* relationships, we have also assumed that the 12S tree was closer to the true tree than the 16S tree (see above).

For the 12S gene, the two *C. glaucescens* populations differed by 0.3%. The smallest interspecific divergences, 1.0%, were noted for the three comparisons between *C. glaucescens* TW, *C. dorsata* and *C. vasconica*. The same three species also formed the unresolved species group in the phylogeny. A difference of 2.3%, the largest, was observed for *C. glaucescens* CP and *C. unicolor*. Low levels of variation have been reported for the 3' end of the 12S gene (Simon *et al*., 1994) and they are consistent with the lack of resolution in the phylogenetic trees. Still, the range of interspecific variation appears to be rather small. For *Drosophila* and *Magicicada*, interspecific ranges of 1.7%−5.7% and 0.3%−6.3% have been reported for the 3' end of the 12S gene (Simon *et al*., 1996).

The 16S gene showed higher levels of divergence. The two *C. glaucescens* populations differed by 1.1%, while *C. glaucescens* TW and *C. dorsata* differed by 3.6%, *C. glaucescens* CP and *C. unicolor* differed by 7.2%. The latter comparison was also the largest interspecific difference, as in the 12S gene. A comparatively high level of variation was expected for this fragment because the 5' end halves of the ribosomal genes contain fewer conserved regions than the 3' end halves (references in Simon *et al*., 1996).

tRNA valine gene

In order to identify the 12S and 16S junctions with the tRNA valine gene, secondary structures were drawn for sections of the ribosomal genes of *C. unicolor* aided by the known structures of *Drosophila yakuba* (Clary & Wolstenholme, 1985) and *Magicicada tredecim* (Simon *et al*., 1994). The structures in *Chloriona* were very similar to *Drosophila*, helping to identify the ends of the 12S and 16S genes. Sequence similarity near the 12S and 16S junctions among the taxa in our dataset was high enough to be confident about homology of the aligned regions. However, the number of nucleotides between the two junctions (approximately 18 in *C. unicolor*, for example) was far too low to contain the tRNA valine.

Discussion

Delphacid relationships were completely resolved (among the taxa examined) from the subfamily level to the genus

level using the 3' end of the 12S gene. The taxon sampling was rather sparse in this study and hence many relationships remain to be tested. However, it is unlikely that the major phylogenetic relationships are an artefact of this because of the levels of the support and because the results are the same as the relationships inferred by Dijkstra *et al.* (2003) from the COI gene. The precise effects of sparse taxon sampling on phylogenetic accuracy remain controversial in molecular systematics (Rosenberg & Kumar, 2001, 2003; Hillis *et al.*, 2003), but it is clear that adding more DNA sequence data to the delphacid COI gene analysis (Dijkstra *et al.*, 2003) will be expected to contribute much more to the accuracy of the phylogenetic tree than would adding more taxa (Hillis *et al.*, 2003). Hence, the importance of the results from the mt rDNA is that they confirm relationships which were only weakly or ambiguously supported by the COI data (i.e. monophyly of the Delphacini, and a sister group relationship between *Stenocranus* and *Anakelisia*, and between *Conomelus* and *Megamelus*). Both the 12S and COI genes are mitochondrial genes, and hence the phylogeny that has been recovered will represent the phylogeny of the mitochondrial DNA, which can differ from the organismal evolution and the evolution of the nuclear genome, usually as a result of lateral transfer (introgressive hybridisation) or clonal inheritance with lineage sorting. In practice these factors can be important at the species level, but are not significant at higher taxonomic levels.

Monophyly of the Delphacini was indicated by the large 12S dataset, and although data from the COI gene also suggested that the Delphacini were a monophyletic group this was only observed after pruning distant taxa from the dataset. Therefore, the 12S fragment may be more suitable for investigating relationships within the Delphacini than the COI gene, even if only for establishing the main groups within this tribe. The close relationship between *Conomelus* and *Megamelus* observed in this study is especially interesting. Asche (1985) believed that they belonged to a group of genera which are the sister group of genera possessing specialized oviduct glands (which he called the 'modern' delphacids). However, there were no characters supporting this.

In general, the 12S tree is congruent with the tree based on morphological characters (Asche, 1985, 1990), although the 12S data strongly support a sister group relationship between *Stenocranus* (Stenocraninae) and *Anakelisia* (Kelisiinae) whereas morphological data suggest that the Stenocraninae are more closely related to the Delphacinae (although the morphological character which indicates this relationship is also found in some Asiracinae). Data from the COI gene (Dijkstra *et al.*, 2003) also supported a sister group relationship between *Stenocranus* and *Anakelisia* (and between *Conomelus* and *Megamelus*), using amino acid characters, but nucleotide characters only gave support in a spectral analysis and not in the parsimony analysis of the main dataset. 12S support for *Stenocranus* and *Anakelisia* might still be an artifact caused by long branch attraction (Felsenstein, 1978). Since sequence data from related genera are not available in order to break up the branches (Hendy & Penny, 1989) we have tested whether *Stenocranus* could be 'attracted' to any of the Delphacini species seperately. A series of eight parsimony runs were performed each time with *Stenocranus*, *Anakelesia* and a single delphacine species in the dataset. However, in all analyses *Stenocranus* grouped with *Anakelisia* with a support value of at least 96% which suggests that the node is rather stable. The implications of the conflict between the molecular and morphological evidence have been discussed in our previous paper (Dijkstra *et al.*, 2003). In any case, the phylogenetic signals in the 12S fragment appear to be much clearer than in the COI gene (Dijkstra *et al.*, 2003) and thus the 12S gene may be a better choice for inferring relationships at these levels within the Delphacidae.

The large 12S dataset, consisting of the more conserved regions, did not contain enough variable sites to be useful at the species level. Interestingly, the resolution within the genus *Chloriona* increased after adding regions which were more variable (i.e. the small 12S dataset). Such regions are known to harbour homoplasious substitutions (Simon *et al.*, 1994), but since the resolution increased and the result was consistent with data from the large 12S data and the COI gene (Dijkstra *et al.*, 2003) it seems that the regions which could be added did not include misleading homoplasies. Nevertheless, the phylogenetic signals in the small 12S dataset remained weak because there was still a lack of informative sites. Relationships among *C. glaucescens*, *C. dorsata* and *C. vasconica* remained uncertain. This was also the case with data from the COI gene and it is possible that the uncertainty is partly a result of fast speciation rates. The 5' end of the 16S gene was more variable than the 3' end of the 12S gene but the informative sites in this fragment seem to be a result of homoplasious substitutions. Thus, neither of the ribosomal fragments appear to be very useful at the species level, as already suggested by Simon *et al.* (1994). It is to be expected that a much more complete test of phylogenetic relationships would result from improved taxon sampling and to a lesser extent by further integration of morphological and molecular data.

Apart from the very clear phylogenetic signals, there were also some complex patterns at the molecular level, but this data provided no indication that the various sister group relationships (between *Stenocranus* and *Anakelisia*

for example) were caused by base compositional effects.

Insect mitochondrial DNA has a high AT content and the AT bias normally accumulates in the most variable positions (Simon *et al*., 1994, and see Artiss *et al*., 2001 for a recent example). This was also true for the Delphacidae except for *Chloriona* where a decrease of the AT content was observed at the most variable positions. Data from the 16S gene suggested that in this fragment the increase of the GC content was due to substitutions from T to G and C. In the small 12S dataset the decrease of the AT content appeared to be caused by A to G transitions. At least for the small 12S dataset we may assume that these positions are not under very strong functional constraints because they could not be aligned with the sequences of distantly related taxa.

In two forficulid genera (Forficulidae, Dermaptera), third codon positions of the COII gene have become relatively GC-rich whereas the GC content of third codon positions in members from other earwig families was comparable to that of other insects (Wirth *et al*., 1999). Moreover, the change in GC content in the Forficulidae appeared to be accompanied by an increased substitution rate. Whether there is also an increased rate in *Chloriona* is not clear. Relative rate tests (Table 2) do not indicate rate differences between *Chloriona*, *Conomelus* and *Megamelus*. However, those tests are based on the more conserved positions of the large 12S dataset and thus do not include the positions which are of interest here. Of special interest are the comparisons between the *Ugyops* and the non-Asiracinae. In these comparisons there are at least three cases where non-asiracines have accumulated significantly more transversions than *Ugyops*. Although we do not know whether there has been a rate increase in the common ancestor of the non-asiracines (instead of a slowdown in *Ugyops*) this may have been the case and the accumulation of transversions in this group could be a reflection of the shift from T to A. Nevertheless, the shift from T to A and a rate increase may have been correlated phenomena in the common ancestor of the non-asiracines. The shift from T to A would still be consistent with the idea that selection is maintaining an AT bias in insect mitochondrial DNA (Wolstenholme & Clary, 1985), but the decrease of the AT content in *Chloriona* could indicate a relaxation of this process (Wirth *et al*., 1999). The faster rate of evolution of the 12S gene in the non-Asiracinae compared to *Ugyops* is similar to the rate differences in the COI gene observed by Dijkstra *et al*. (2003). This suggests a general pattern for the mitochondrial DNA in the Delphacidae, and this might be related to the radiation of the non-asiracines parallel to the evolution of the monocotyledons, as discussed by Dijkstra *et al*. (2003).

Finally, the absence of the tRNA valine between the 12S and 16S genes raises the question whether this tRNA has been translocated elsewhere or whether we have sequenced pseudogenes. Mitochondrially derived pseudogenes have been found in the nuclear genome of many animals (reviewed by Zhang & Hewitt, 1996). However, there are a number of arguments which suggest that we are dealing with the functional 12S and 16S genes; First, PCR bands on the agarose gels gave no indication for the existence of more than one copy (pseudogenes may still be present but could not be amplified with the primers). Second, using conserved primers, the tRNA gene was absent from all taxa, including the cixiid outgroup. Third, the large 12S dataset contained rather strong phylogenetic signals which were consistent with data from the COI gene (Dijkstra *et al*., 2003) and mostly agreed with the tree based on morphological characters. If these genes really are pseudogenes then this fragment was probably inserted in the nuclear genome before the Cixiidae and Delphacidae diverged. Little is known with certainty about the fossil record of Delphacidae (Asche, 1985), but it is assumed that this family existed around 40 mya (Manfred Asche, 1997, personal communication) although dates as old as 130 mya have been suggested (Wilson *et al*., 1994). It seems rather unlikely that pseudogenes could have retained the phylogenetic signals in the 12S dataset for as long as 40 my. Fourth, the 'deletion' seems to be restricted to the tRNA gene without affecting the 12S and 16S junctions. Fifth, the secondary structures obtained for *C. unicolor* are very similar to the secondary structures of *Drosophila yakuba* and *Magicicada tredecim*. Finally, pairwise comparisons within *Chloriona* indicate a 3-fold substitution rate difference between the 12S and 16S gene, suggesting different selection pressures in this region.

Still, the tRNA itself may be translocated and the remaining 18 nucleotides (seen in *C. unicolor*) may be what is left of the original copy. Mitochondrial gene rearrangements have been observed in a range of different animal phyla, including insects. The most commonly used model to explain mitochondrial gene rearrangements assumes the duplication of a DNA fragment (for example because of slipped strand mispairing, or imprecise termination, during replication) followed by a random loss of one of the copies which may result in a different gene order. This model has been used to explain gene rearrangements and the presence of pseudo-tRNA genes and pseudo-control regions in some snakes and the sea cucumber *Cucumaria*, for example (reviewed by Boore, 1999). To our knowledge, mitochondrial pseudogenes have not yet been reported from arthropods, although the expanded non-coding region of the bark weevil *Pissodes* contains multiple repeat units. Nevertheless, one region where translocations are often observed in arthropods is near the large non-coding region, indicating that the origin of replication may be involved

(Boore, 1999). Supposedly, the fragments sequenced for this study are adjacent to this region.

Acknowledgments

We thank Peter de Vrijer and Theodoor Heijerman for helpful discussions and comments during this research; Peter de Vrijer and Jan Rozeboom for their help collecting and identifying field material. We are also very grateful to Manfred Asche (ZMHB) who kindly provided the *Ugyops* material, and last but not least to Tony van Kampen for giving technical assistance.

References

Artiss, T., Schultz, T.R., Polhemus D.A. and Simon, C. (2001) Molecular phylogenetic analysis of the dragonfly genera *Libellula*, *Ladona*, and *Plathemis* (Odonata: Libellulidae) based on mitochondrial cytochrome oxidase I and 16S rRNA sequence data. *Molecular Phylogenetics and Evolution*, 18, 348–361.

Asche, M. (1985) Zur Phylogenie der Delphacidae Leach, 1815 (Homoptera Cicadina Fulgoromorpha). *Marburger Entomologische Publikationen*, 2, 1–912.

Asche, M. (1988) Preliminary thoughts in the phylogeny of the Fulgoromorpha (Homoptera: Auchenorrhyncha). *Proceedings of the 6th International Auchenorrhyncha Meeting* (eds. C. Vidano & A. Arzone), pp. 47–53. University of Turin, Turin.

Asche, M. (1990) Vizcayinae, a new subfamily of Delphacidae with revision of *Vizcaya* Muir (Homoptera: Fulgoroidea): A significant phylogenetic link. *Bishop Museum Occasional Papers*, 30, 154–187.

Boore, J.L. (1999) Animal mitochondrial genomes. *Nucleic Acids Research*, 27, 1767–1780.

Bourgoin, T., Steffen-Campbell, J.D. and Campbell, B.C. (1997) Molecular phylogeny of Fulgoromorpha (Insecta, Hemiptera, Archaerrhyncha). The enigmatic Tettigometridae: Evolutionary affiliations and historical biogeography. *Cladistics*, 13, 207–224.

Broughton, R.E., Stanley, S.E. and Durrett, R.T. (2000) Quantification of homoplasy for nucleotide transitions and transversions and a reexamination of assumptions in weighted phylogenetic analysis. *Systematic Biology*, 49, 617–627.

Campbell, B.C., Steffen-Campbell, J.D., Sorensen, J.T. and Gill, R.J. (1995) Paraphyly of Homoptera and Auchenorrhyncha inferred from 18S rDNA nucleotide sequences. *Systematic Entomology*, 20, 175–194.

Claridge, M.F. and de Vrijer, P.W.F. (1994) Reproductive behavior: The role of acoustic signals in species recognition and speciation. *Planthoppers: Their Ecology and Management* (eds. R.F. Denno & T.J. Perfect), pp. 216–233. Chapman & Hall, New York.

Clary, D.O. and Wolstenholme, D.R. (1985) The ribosomal RNA genes of *Drosophila* mitochondrial DNA. *Nucleic Acids Research*, 13, 4029–4045.

Denno, R.F. (1994) Life history variation in planthoppers. *Planthoppers: Their Ecology and Management* (eds. R.F. Denno & T.J. Perfect), pp. 163–215. Chapman & Hall, New York.

Dijkstra, E., Rubio, J.M. and Post, R.J. (2003) Resolving relationships over a wide taxonomic range in Delphacidae (Homoptera) using the COI gene. *Systematic Entomology*, 28, 89–100.

Felsenstein, J. (1978) Cases in which parsimony or compatibility methods will be positively misleading. *Systematic Zoology*, 27, 401–410.

Garcia, B.A. and Powell, J.R. (1998) Phylogeny of species of *Triatoma* (Hemiptera: Reduviidae) based on mitochondrial DNA sequences. *Journal of Medical Entomology*, 35, 232–238.

Hasegawa, M. and Hashimoto, T. (1993) Ribosomal RNA trees misleading? *Nature*, 361, 23.

Hendy, M.D. and Penny, D. (1989) A framework for the quantitative study of evolutionary trees. *Systematic Zoology*, 38, 297–309.

Hillis, D.M., Pollock, D.D., McGuire, J.A. and Zwickl, D.J. (2003) Is sparse taxon sampling a problem for phylogenetic inference? *Systematic Biology*, 52, 124–126.

Jones, P., Gacesa, P. and Butlin, R. (1993) A molecular approach to planthopper systematics. *Proceedings of the 8th Auchenorrhyncha Congress* (eds. S. Drosopoulos, P.V. Petrakis, M. F. Claridge & P.W.F. de Vrijer), pp. 7–9. Secretariat of the Eighth Auchenorrhyncha Congress, Delphi, Greece.

Post, R.J., Flook, P.K. and Millest, A.L. (1993) Methods for the preservation of insects for DNA studies. *Biochemical Systematics and Ecology*, 21, 85–92.

Roderick, G.K. and Metz, E.C. (1997) Biodiversity of planthoppers (Hemiptera: Delphacidae) on the Hawaiian silversword alliance: Effects of host plant phylogeny and hybridisation. *Memoirs of the Museum of Victoria*, 56, 393–399.

Rosenberg, M.S. and Kumar, S. (2001) Incomplete taxon sampling is not a problem for phylogenetic inference. *Proceedings of the National Academy of Sciences USA*, 98, 10751–10756.

Rosenberg, M.S. and Kumar, S. (2003) Taxon sampling, bioinformatics and phylogenomics. *Systematic Biology*, 52, 119–124.

Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H. and Flook, P. (1994) Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the Entomological Society of America*, 87, 651–701.

Simon, C., Nigro, L., Sullivan, J., Holsinger, K., Martin, A., Grapputo, A., Franke, A. and McIntosh, C. (1996) Large differences in substitutional patterns and evolutionary rate of

12S ribosomal RNA genes. *Molecular Biology and Evolution*, 13, 923−932.

Sullivan, J., Swofford, D.L. and Naylor, G.J.P. (1999) The effect of taxon samplng on estimating rate heterogeneity parameters of maximum-likelihood models. *Molecular Biology and Evolution*, 16, 1347−1356.

Tajima, F. (1993) Simple methods for testing the molecular evolutionary clock hypothesis. *Genetics*, 135, 599−607.

Uhlenbusch, I., McCracken, A. and Gellissen, G. (1987) The gene for the large (16S) ribosomal RNA from the *Locusta migratoria* mitochondrial genome. *Current Genetics*, 11, 631−638.

Wilson, S.W. and O'Brien, L.B. (1987) A survey of planthopper pests of economically important plants (Homoptera: Fulgoroidea). *Proceedings of the 2nd International Workshop on Leafhoppers and Planthoppers of Economic Importance* (eds. M.R. Wilson & L.R. Nault), pp. 343−360. CAB International Institute of Entomology, London.

Wilson, S.W., Mitter, C., Denno, R.F. and Wilson, M.R. (1994) Evolutionary patterns of host plant use by delphacid planthoppers and their relatives. *Planthoppers: Their Ecology and Management* (eds. R.F. Denno & T.J. Perfect), pp. 7−45. Chapman & Hall, New York.

Wirth, T., Le Guellec, R. and Veuille, M. (1999) Directional substitution and evolution of nucleotide content in the cytochrome oxidase II gene in Earwigs (Dermapteran insects). *Molecular Biology and Evolution*, 16, 1645−1653.

Wolstenholme, D.R. and Clary, D.O. (1985) Sequence evolution of *Drosophila* mitochondrial DNA. *Genetics*, 109, 725−744.

Yemel'yanov, A.F. (1991) An attempt to construct a phylogenetic tree for planthoppers (Homoptera, Cicadina). *Entomological Review*, 70, 24−28. (Author's name transliterated by journal as Emel'yanov).

Zhang, D.X. and Hewitt, G.M. (1996) Nuclear integrations: Challenges for mitochondrial DNA markers. *Trends in Ecology and Evolution*, 11, 247−251.

Accepted February 28, 2006