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Resolving relationships over a wide taxonomic range in Delphacidae (Homoptera) using the COI gene

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Abstract. Using a combination of different methods to investigate the suitability of a fragment of the cytochrome c oxidase I gene (COI), we succeeded in partially resolving phylogenetic relationships in Delphacidae from the level of species to subfamily. Spectral analysis applied to the relatively noisy COI data proved to be especially useful. It clearly showed when phylogenetic signals were not completely randomized and it was very helpful for identifying problem areas in the dataset. Relationships among the four sampled subfamilies were completely resolved. In contrast to the tree based on morphological characters, we found evidence that *Asiraca* and *Ugyops* are sister groups (supporting monophyly of Asiracinae) and that Stenocraninae are the sister group of Kelisiinae. Contradictory signals were observed within Delphacini, but there are characters that support a close relationship between *Conomelus* and *Megamelus*. Other than this, the COI data gave support for the monophyly of Kelisiinae, Delphacinae, *Chloriona* and *Javesella*. Although third codon positions may appear to be saturated within the 'modern' Delphacidae (Delphacini), they still contain important phylogenetic signals at the deepest taxonomic level. The easiest explanation for this is the difference in amino acid usage between Asiracinae and non-Asiracinae. Overall, this fragment of the COI gene seems to be useful for a rather wide taxonomic range in Delphacidae, except maybe for resolving generic relationships in the large tribe Delphacini.

Introduction

The planthopper family Delphacidae currently comprises over 2000 described species (Asche, 1990). They are relatively host-plant specific and a number are of economic importance, such as *Nilaparvata lugens* (Stål), which feeds on rice. Another interesting feature of Delphacidae is their acoustic communication system, which may play an important role in species recognition and possibly also in speciation, and has therefore been the subject of much research (for review see Claridge & de Vrijer, 1994).

Phylogenetic relationships in Delphacidae have been studied by Asche (1985, 1990). Using morphological characters, including the presence or absence of yeast-like symbionts, the following subfamily relationships were suggested: (((((Delphacinae, Plesiodelphacinae) Stenocraninae) Kelisiinae) Vizcayinae) Asiracinae). All subfamilies (except Asiracinae) are supported by autapomorphies. Asiracinae is divided into two tribes, Asiracini and Ugyopini, and Ugyopini are considered a basal monophyletic group. However, Asiracini may not be monophyletic. A number of genera in this tribe, including *Asiraca* Latreille, possess a genal carina. Non-Asiracinae also have a genal carina, but in these taxa it ends in a different place. For the time being, this character is considered to be a synapomorphy and those Asiracini possessing a genal carina are assumed to be the sister group of the non-Asiracinae (Asche, 1985, 1990). A more detailed morpho-phylogeny of Asiracinae was given by Yemel'yanov (1996), who elevated both the Ugyopini and Asiracini of Asche to subfamily level. Larval characters supported Asche's (1985, 1990) view that Ugyopini are the sister group of all remaining Delphacidae. However, as in Asche (1985, 1990), a number of Asiracini appear to be more closely related to the non-Asiracinae. Throughout this study, we follow the phylogenetic framework of Asche (1985, 1990).

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Most subfamilies contain only a small number of genera (Table 1). Delphacinae is, however, by far the largest group, with about 250 genera. It is subdivided into three tribes with about 1090 species in Delphacini (Wilson *et al*., 1994). In general, relatively little is known about relationships among genera within groups because of lack of suitable morphological characters. Within Delphacini, however, there is a group of genera which cover their eggs with a laclike fluid, which is produced in specialized oviduct glands. Genera possessing this derived trait are known as the 'modern' Delphacidae′ (Asche, 1985, 1990). (In phylogenetic terms this is probably not a very appropriate name, but it is in standard usage and no other collective term exists for this grouping of genera.) The genera without this character or behaviour, e.g. *Conomelus* Fieber and *Megamelus* Fieber, may form a separate lineage within Delphacini, but there are no morphological characters supporting this (Asche, 1985). It is clear from the above discussion that because of the lack of suitable morphological characters, systematic problems not only occur at low taxonomic levels but also at a deep taxonomic level; for example, Asiracinae may not be a monophyletic group. Molecular techniques may be important in providing additional data for further resolving relationships in Delphacidae (Caterino *et al*., 2000).

Molecular data have already been used for investigating delphacid relationships at low taxonomic levels. Jones *et al*. (1993) sequenced the ITS regions to examine species relationships and boundaries in the genus *Nilaparvata* Distant. Roderick & Metz (1997) used a fragment of the cytochrome c oxidase I (COI) gene (which was non-overlapping and differs from the fragment used in our study) to obtain a phylogeny of *Nesosydne* Kirkaldy planthoppers (Delphacinae), which was well enough resolved to detect cospeciation between these planthoppers and their hosts. This already suggested that the COI gene, or parts of it, may be useful for studying delphacid relationships at a low taxonomic level.

In terms of amino acids, the COI gene is the most conserved mitochondrially encoded protein (Lunt *et al*., 1996), but its phylogenetic utility may be at close and intermediate levels of divergence using nucleotide or amino acid characters (Simon *et al*., 1994). Generally, mitochondrially encoded proteins may not be suitable for investigating relationships among families or higher levels because those amino acid positions which are variable may have experienced multiple substitutions (Lui & Beckenbach, 1992; Simon *et al*., 1994), but at which taxonomic level amino acid characters will be saturated in Delphacidae remains to be seen.

The objective of this study was to try to estimate a phylogeny of Delphacidae to resolve questions over their higher level classification and the lower levels in Delphacini (which is by far the largest subfamily). To try to obtain the

Table 1. List of species used in the study. Numbers in parentheses are the number of known genera in the subfamily (following Asche, 1985). WA = Wamel, The Netherlands; IS = Isnas, Finland; MU = Munzeberg, Germany; CP = Canet-Plage, France; AM = Amaliapolis, Greece.

Taxon	Collecting site	Code
Family Delphacidae		
Delphacinae (*c*. 250)		
Delphacini		
('modern' Delphacidae)		
Chloriona dorsata Edwards †	Wamel, The Netherlands	
C. glaucescens Fieber ‡	WA, IS, MU, CP, AM	
C. vasconica Ribaut †	Wamel, The Netherlands	
C. smaragdula (Stål)	'Het Twiske', Landsmeer, The Netherlands	
C. unicolor (Herrich-Schaffer) †	Wilhelminadorp, The Netherlands	CHu
Javesella pellucida (Fabricius) †	Wageningen, The Netherlands	Jpe
J. obscurella (Boheman) †	Wageningen, The Netherlands	
Nilaparvata lugens (Stål)	Mun *et al*. (1999)	Nlu
Sogatella furcifera (Horvath)	Mun *et al*. (1999)	SOf
(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
Kelisia sabulicola Wagner	Tongerse hei, Vierhouten, The Netherlands	Ksa
Asiracinae (23)		
Asiraca clavicornis (Fabricius)	Thues-entre-Valls, France	Acl
Ugyops sp.	Rarotonga, Cook Is, New Zealand	Ugy
Cixiidae		
Tachycixius pilosus (Olivier)	Ginkelse hei, Ede, The Netherlands	Tpi

†Specimen from laboratory rearing.
‡Specimens from laboratory rearing; specimens from Wamel were from F1 generation from field material.

best resolution from the relatively noisy dataset provided by the COI gene, we applied a range of techniques, including parsimony with tree-pruning, spectral analysis and neighbour joining. To provide additional information about the taxonomic range over which this gene might be suitable, or for which groups, we studied divergence levels within and between groups. The existence of rate differences among taxa has also been investigated. Rate differences are interesting in themselves, but also, if species differ in their rate of evolution, parsimony analyses, for example, may be affected and misleading tree topologies may be obtained by long branch attraction (Felsenstein, 1978; but see also Hendy & Penny, 1989).

Materials 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. At least two taxa were sampled across all the major subfamilies, excluding only Vizcayinae and Plesiodelphacinae, which were difficult to obtain and have only one and two genera, respectively. Within Delphacini, two species of non-'modern' delphacids were sampled, and the 'modern' delphacids were sampled widely with the aim of obtaining a range of genera and a range of species within a sample genus (*Chloriona* Fieber). Specimens were also obtained to estimate between- and within-population variation for one sample species (*Chloriona glaucescens* Fieber). In analyses where *C. glaucescens* was represented by a single sequence, it refers to a specimen (no. 70) from the Wamel population. Alcohol-preserved *Ugyops* Guerin-Meneville specimens were obtained from M. Asche. These specimens, along with *Asiraca clavicornis* (Fabricius), were all that was available to us from Asiracinae and each of them comes from a different tribe. The cixiid *Tachycixius pilosus* (Olivier) was used to root the tree. Cixiidae are considered by most authors to be the sister group to Delphacidae (Asche, 1987; Wilson *et al*., 1994; Campbell *et al*., 1995; Bourgoin *et al*., 1997), except Yemel'yanov (1991) who considered them both to be basal within superfamily Fulgoroidea, but not sister groups.

Until now it was believed that, in The Netherlands, *Conomelus* was only represented by *Conomelus anceps* (Germar) (De Vrijer, personal communication), but very recently we have discovered a second species, *Conomelus lorifer* (Ribaut), represented by the subspecies *Conomelus lorifer dehneli* Nast (E. Dijkstra, unpublished data). As it subsequently turned out, our *Conomelus* population sample had been a mixture of these two species. Therefore, the exact identity of the single specimen used for the DNA work is uncertain. However, for the purpose of this study, it is a representative of *Conomelus*.

Sequence data from *Nilaparvata lugens* and *Sogatella furcifera* (Horvath) (Mun *et al*., 1999) were obtained from GenBank (AF222883 and AF2228860). Data generated in this study are accessible by numbers AF304407-AF304428.

DNA extraction

DNA was extracted from individual specimens using the method of Post *et al*. (1993). Mass extractions were done for *Chloriona glaucescens* from Canet-Plage, *Stenocranus major* (Kirschbaum), *Anakelisia fasciata* (Kirschbaum) and *Tachycixius pilosus*. Specimens ($n = 110$–130) from these species were homogenized in liquid nitrogen and transferred to tubes containing 5 ml Bender buffer (Post *et al*., 1993) with 0.25 mg of proteinase K. Following overnight incubation at 48 °C, samples were purified using phenol/chloroform followed by an ethanol precipitation. DNA was resuspended in 0.5 ml sterile water with 5 µg RNAse.

PCR, cloning and sequencing

For each PCR reaction, the mixture consisted of 1 µl (10 pmol/µl) of each primer, 0.25 µl (20 mMol) of dNTP mixture, 0.25 µl (1 U) Taq polymerase, 2.5 µl 10× PCR buffer (Pharmacia Biotech Benelux, Roosendaal, The Netherlands) and 1 µl DNA from a dilution series. The mixture was adjusted with sterile water to a final volume of 25 µl. Primers C1-J-2195 (5′-TTGATTTTTTGGTCATCCAGAAGT) (Simon *et al*., 1994) and MtLys (5′-TTGGTTTAAGAGACCATTAC) (new design, 3′ end base corresponds to position 3788 in the sequence of *Drosophila yakuba* Burla, Clary & Wolstenholme, 1985), were used to amplify a fragment from the middle of the COI gene to the 3′ end of the COII gene. PCR was performed using a Hybaid Omnigene Temperature Cycler HB-TR3-CM (Biozym Nederland bv, Landgraaf, The Netherlands) under the following conditions. An initial denaturation cycle at 94 °C (5 min) was followed by 30–35 cycles of 94 °C (1 min), annealing at 52 °C (30 s), and extension at 72 °C (1 min). The program was completed by a final extension step at 72 °C for 5 min. PCR products were run on an ethidium bromide-stained 1% agarose gel. PCR bands were sliced from the gel and transferred to a 0.5 ml Eppendorf tube, which had a plug of glasswool at the punctured bottom, and kept at −20 °C for at least 1 h. The 0.5 ml tube was placed in a 1.5 ml Eppendorf tube and spun for 5 min. The clean DNA was ethanol precipitated and the pellet resuspended in 10 µl sterile water. Purified DNA was ligated in a T-vector (Promega Corporation BNL, Leiden, The Netherlands). Ligation, transformation, screening and isolation procedures were performed according to the protocol provided by the manufacturer. Plasmid DNA was isolated with the QIAprep Spin Miniprep Kit (QIAgen, Westburg bv, Leusden, The Netherlands), followed by an extra ethanol precipitation. Plasmids were sequenced in one direction on an ABI 373 automated sequencer (Applied Biosystems, Nieuwerkerk a/d IJsel, The Netherlands) using the ABI Dye Terminator Cycle Sequencing Kit.

Data analysis

Sequences from a fragment of the COI gene were aligned with 'pileup' and manually adjusted with 'lineup' (GCG software package version 8.0, University of Wisconsin, Madison, U.S.A). The sequences were translated with 'translate' (GCG 8.0, as above) using the *Drosophila* genetic code (de Bruijn, 1983) and compared to the published *D. yakuba* amino acid sequence (Clary & Wolstenholme, 1985). Unfortunately, the *Sogatella* Fennah GenBank sequence contained a stopcodon (TAA) where all other Delphacini had a tyrosine (TAT or TAC). We believe that this was a minor sequencing error made by Mun *et al.* (1999), and consequently have used this sequence in our analysis but recoded the third codon position of the stop codon as missing (n). The alternative explanation is that the sequence is a pseudogene copy inserted into the nuclear genome (see Zhang & Hewitt, 1996), but we believe that this is not the case because the average ratio of synonymous to non-synonymous substitions is not lower in *Sogatella* than it is in the other 'modern' delphacids (ratios calculated by Comeron's (1995), method were *Sogatella* = 0.49, *Nilaparvata* = 0.55, *Javesella* Fennah = 0.49, *Chloriona* = 0.48). Furthermore, in phylogenetic analyses (see below) *Sogatella* falls within the 'modern' Delphacidae, which would not be expected if this sequence was a non-homologous pseudogene.

We used maximum parsimony for tree construction, but investigated the robustness of the topology by comparison with neighbour-joining. We investigated phylogenetic signal using spectral analysis, which allows the assessment of support for (and alternatives to) particular branches.

For parsimony analyses, we used PAUP (version 3.1.1, Swofford, 1993). For datasets consisting of seventeen taxa, heuristic searches were performed (random addition option, 100 sequence addition replicates, TBR branch swapping). For smaller datasets, branch and bound searches were carried out. Bootstrap values were calculated ignoring uninformative sites (heuristic 1000×, ten sequence addition replicates). Neighbour-joining trees (Treecon, version 1.1, van de Peer & de Wachter, 1994) were obtained using uncorrected distances, and also distances subject to Kimura correction, Tajima–Nei correction and Jin–Nei correction (which allows for among-site variation using Kimura's two-parameter model). The corrected trees had the same topology as the uncorrected tree. Node support was estimated by performing 500 replicates.

Spectral analysis was carried out using 'Spectrum version 2.3' (Hendy & Penny, 1993; Swofford *et al.*, 1996; Charleston & Page, 2000). Spectral analysis specifically aims to investigate phylogenetic signals in datasets. Given a binary matrix, each variable site splits the taxa into two subsets or bipartitions. The more sites showing a certain split, the stronger the signal or support. The total amount of support for splits can be listed above the *x*-axis in a frequency spectrum histogram. There will also be splits that disagree and this can be shown as conflict below the *x*-axis. First, however, the nucleotide characters need to be recoded in binary characters. To capture all the information in the dataset, the DNA characters are recoded in seven ways (A + T/G + C, G/A + C + T, etc.) and the average taken over all mapping ways. Therefore, a single site can contain a number of different splits depending on the number of character states (multiple hits), thus spreading the signal. A tree might then be constructed to visualize the results; the splits (frequencies) are stored as elements of a vector which defines a point in the multidimensional space. This vector contains a number of different trees, depending on the different combination of splits chosen from the main vector. The tree (a subvector) that has the smallest Manhattan (city-block) distance to the main vector is chosen. For each internal branch of the chosen tree, the two local alternative topologies are identified under a nearest-neighbour interchange perturbation (NNI plots). The amount of support for the two local alternative splits around an internal branch (edge) are used for estimating the reliability of that edge (Lento *et al.*, 1995). Thus, the combination of the frequency spectrum, NNI plot and tree facilitates the study of the major signals in the dataset and evaluation of a number of alternative solutions. Unless stated otherwise, we used the average mapping option. For practical reasons we only included a maximum of two species per genus.

Tajima's (1993) rate tests were performed using Molecular Evolutionary Analysis Package (MEA, written by Etsuko Moriyama, Yale University, U.S.A.). This program was also used to calculate uncorrected genetic distances. Relative rate tests were performed using one outgroup only (Table 2). If more than one possible outgroup was available, the one with the shortest terminal branch was used (as obtained from the spectral analysis) to minimize saturation effects.

Results and discussion

Phylogenetic analyses

In total, we obtained a fragment of 504 bp (168 codons), downstream from the middle of the COI gene. The parsimony analysis based on nucleotide characters resulted in six equally parsimonious trees of 693 steps. Phylogenetic relationships are shown in Fig. 1 as a strict consensus tree with *T. pilosus* as the outgroup (the semi-strict consensus tree showed the same topology). Monophyly for both *Chloriona* and *Javesella* is strongly supported and relationships within *Chloriona* are partly resolved with a number of strongly supported nodes. *Chloriona glaucescens*, *C. dorsata* Edwards and *C. vasconica* Ribaut seem to form a group of closely related species, and all eight *C. glaucescens* specimens formed a single group (data not shown). At the deepest evolutionary level within the family, this COI fragment provides strong phylogenetic signals. Both Asiracinae (represented by *Ugyops* and *Asiraca*) and non-Asiracinae are strongly or very strongly supported. This support for a close relationship between *Asiraca* and *Ugyops* is especially pertinent as morpho-phylogenetic studies have indicated Asiracinae to be a paraphyletic group (Asche, 1985,

Table 2. Tajima's relative rate test (1 D method). Upper part of the table shows comparisons between non-asiracines and Asiracinae; the lower part shows comparisons within and between 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 is defined in a similar way. Species 3 is the outgroup taxon.

Species 1	Species 2	Species 3	M1	M2	χ^2
CHu	Ugy	Tpi	51	29	6.05*
Jpe	Ugy	Tpi	55	33	5.50*
Nlu	Ugy	Tpi	58	33	6.87**
SOf	Ugy	Tpi	69	30	15.36***
Can	Ugy	Tpi	42	32	1.35
Mno	Ugy	Tpi	56	29	8.58**
STm	Ugy	Tpi	59	25	13.76***
Ksa	Ugy	Tpi	50	32	3.95*
Afa	Ugy	Tpi	54	35	4.06*
CHu	Acl	Tpi	50	36	2.28
Jpe	Acl	Tpi	51	37	2.23
Nlu	Acl	Tpi	54	37	3.18
SOf	Acl	Tpi	60	29	10.80**
Can	Acl	Tpi	42	40	0.05
Mno	Acl	Tpi	57	38	3.80
STm	Acl	Tpi	59	33	7.35**
Ksa	Acl	Tpi	44	34	1.28
Afa	Acl	Tpi	46	35	1.49
CHu	Can	Afa	37	27	1.56
Jpe	Can	Afa	48	25	7.25**
Nlu	Can	Afa	39	31	0.91
SOf	Can	Afa	50	34	3.05
CHu	Mno	Afa	42	40	0.05
Jpe	Mno	Afa	50	35	2.65
Nlu	Mno	Afa	33	33	0.00
SOf	Mno	Afa	47	39	0.74
Can	Mno	CHu	17	43	11.27***
Jpe	CHu	Can	41	32	1.11
Nlu	CHu	Can	40	32	0.89
SOf	CHu	Can	49	28	5.73*
Nlu	Jpe	Can	41	42	0.01
SOf	Jpe	Can	52	40	1.57
Nlu	SOf	Can	29	42	2.38

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

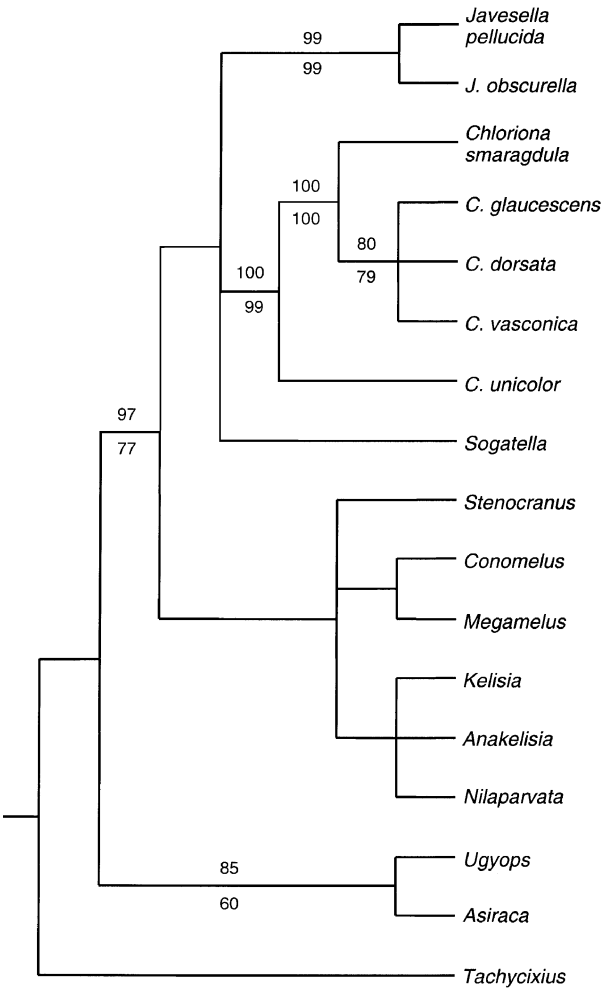

Fig. 1. Strict consensus tree from six equally most parsimonious trees of 693 steps. Values above branches show the bootstrap support based on all codon positions. Below branches are values based on third codon positions only.

1990; Yemel'yanov, 1996). Unfortunately, there is not much resolution within non-Asiracinae, and species of Delphacini appear in various lineages.

The strongly supported groups in Fig. 1 were generally also strongly supported by a neighbour-joining (NJ) analysis (except for the unresolved species complex within *Chloriona*). In addition, NJ indicated a close relationship for *Conomelus* and *Megamelus* with bootstrap support of 70–76%, and monophyly for Kelisiinae was supported by 50–57%.

A parsimony analysis based on amino acid characters resulted in twenty-two equally parsimonious trees of ninety-two steps (only two *Chloriona* species were included, because preliminary analysis showed that the full set of five were monophyletic). The strict consensus tree (Fig. 2, showing the same topology as the semi-strict) shows that there is moderate support for a sister-group relationship between *Asiraca* and *Ugyops* and very strong support for non-Asiracinae, confirming the division at the deepest level. However, two other well supported groups appeared in the tree. A close relationship between *Conomelus* and *Megamelus* is now strongly supported, and the second group strongly suggests that *Stenocranus* Fieber (Stenocraninae) is closely related to Kelisiinae. There was also weak support for a monophyletic Kelisiinae. It has been suggested by Simon *et al.* (1994) that amino acid characters may provide useful information for resolving relationships at intermediate taxonomic levels because at those levels third codon positions may be saturated. To test this, a parsimony analysis was performed on third codon positions only. All groups supported by third codon positions (bootstrap > 49%) were present in Fig. 1. As expected, there is good support for monophyly of both *Chloriona* and *Javesella*. However, it is

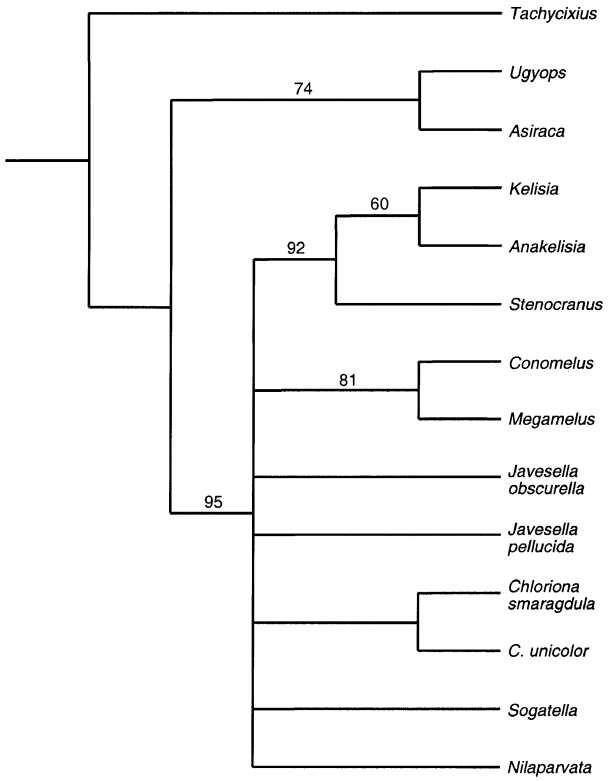

Fig. 2. Strict consensus tree from twenty-two equally most parsimonious trees of 92 steps using amino acid characters. Numbers refer to bootstrap values.

also clear that third codon positions are not completely saturated at the deepest level of the phylogeny because a sister-group relationship between *Asiraca* and *Ugyops*, and non-Asiracinae is still supported (Fig. 1). This is consistent with the signal for first and second codon positions combined (bootstrap values 60% and 77%, respectively). This illustrates that third codon positions may contain an important fraction of the phylogenetic signal and care should be taken in judging the phylogenetic value of these positions (see also, e.g. Yoder *et al.*, 1996; Källersjö *et al.*, 1999). It is not completely clear why amino acid characters provide little information about relationships among 'modern' Delphacidae. It may be that speciation rates have been very fast.

Although it is clear that there are phylogenetic signals at different taxonomic levels, relationships among the non-asiracine subfamilies are still not completely resolved, and there is also no evidence that Delphacini is a monophyletic group. To further investigate the phylogenetic signals in the nucleotide dataset, we performed a spectral analysis. Only two *Chloriona* species were included.

The nearest neighbour interchange (NNI) plot (Fig. 3) shows the amount of support for internal branches. Internal branches occurring in the spectral tree (not shown) are shown in black and are called edges. Each edge is followed by the amount of support for the two local alternative topologies (white bars), and this can be used to estimate how much confidence can be assigned to an edge (Lento *et al.*, 1995). Edges are labelled with letters. Only the five edges with the highest support are discussed here. In all cases, the amount of support was higher than the total amount of conflict, as indicated in the ranked spectrum (not shown).

Edges a and b show the amount of support for *Asiraca* + *Ugyops* and non-Asiracinae, respectively. These groups were also strongly supported using maximum parsimony (Fig. 1) and NJ. The amount of support for a relatively

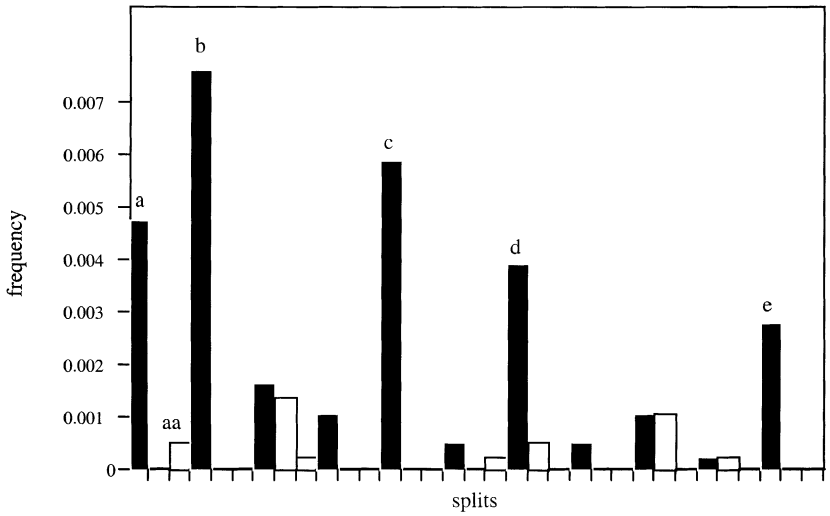

Fig. 3. Nearest neighbour interchange (NNI) plot. Black bars show the amount of support for edges (internal branches) in the spectral tree (not shown). Each edge is followed by two white bars, which show the amount of support for the two local alternatives. Edges: a = *Asiraca* + *Ugyops*; aa = *Asiraca* + non-Aciracinae; b = non-Aciracinae; c = *Conomelus* + *Megamelus*; d = Kelisiinae + *Stenocranus*; e = *J. pellucida* + *Sogatella*.

close relationship between *Asiraca* and the non-Asiracinae, as proposed by Asche (1985, 1990), is much lower (split aa) than for a sister-group relationship between *Asiraca* and *Ugyops*. Notably, as shown by edge c, nucleotide characters give a relative strong signal for a close relationship between *Conomelus* and *Megamelus*, in contrast to Fig. 1. This might be explained in at least two ways. First, the spectral analysis may use more sites than parsimony. For example, a site where *Conomelus* has state A and *Megamelus* has state T, and all the remaining taxa have C. This site is not informative for parsimony but the spectral analysis will use it to partition the taxa. The average mapping option will result in three different bipartions for this site, two of which will only contribute to terminal branch lengths. The third one partitions the taxa in *Conomelus* + *Megamelus*/remaining taxa. Secondly, for example, an informative site where *Conomelus* and *Megamelus* have A and G, and the remaining taxa have T (taxon K + l) or C (taxon M + N). If there are more sites like this but with different character state distributions among the remaining taxa (K + M, L + N), then those sites will proportionally contribute more to a signal for *Megamelus* and *Conomelus*.

In Fig. 3, edge d shows that there is a relatively strong signal in the nucleotide characters for a close relationship between *Stenocranus* and Kelisiinae. Using maximum parsimony, this group was supported in the tree based on amino acid characters but not in the tree based on nucleotide characters. Because edges a to d are not contradicted by local alternatives (Lento *et al*., 1995), as shown by the NNI plot (Fig. 3), these results strongly suggest that the phylogenetic signal in the data is not completely randomized.

Edge e suggests a close relationship between *Sogatella* and *Javesella pellucida* (Fabricius). As can be seen in the NNI plot (Fig. 3), there is no or only very little support for local alternatives. The consequence is, however, that *Javesella* would not be a monophyletic group, which disagrees completely with the results of the parsimony (Fig. 1) and NJ analyses. Possibly, edge e is a result of homoplasies in *Sogatella* and *J. pellucida*.

Neither parsimony nor spectral analyses showed evidence to suggest that Delphacini are a monophyletic group. However, the highest contradictory signal in the ranked spectrum (not shown) proposed a close relationship between *Asiraca* and the *Javesella* species. Because these taxa belong to completely different groups, as we have shown, this was taken as evidence for homoplasious substitutions in rather distantly related taxa. To decrease the level of homoplasy, *Asiraca* and the cixiid outgroup were removed from the dataset. *Ugyops* was used as outgroup in the new analyses.

The pruning of Asiraca and the cixiid outgroup appeared to have a clear effect on the parsimony analysis using amino acid characters. Seven equally parsimonious trees of 74 steps were obtained, and as shown in the strict consensus tree (Fig. 4), Delphacini appear to be a moderately supported monophyletic group, as does Kelisiinae. Spectral analysis and parsimony on nucleotide characters, however, did not give a single tree in which they were monophyletic, but those trees did not confidently support

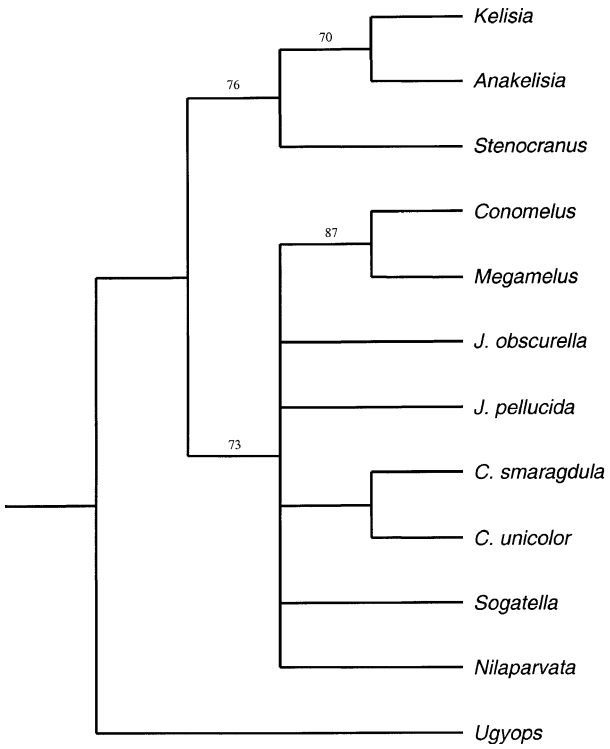

Fig. 4. Strict consensus tree from seven equally most parsimonious trees of 74 steps using amino acid characters. Monophyly for Delphacini supported by 73%.

a different view. Nevertheless, the spectral analysis now showed good support for monophyly for *Javesella*. Thus, the inclusion of *Sogatella* was not the sole problem for establishing monophyly of *Javesella* using the spectral analysis (see above), and therefore we have not excluded this species from further analyses.

Our evidence for monophyly of Delphacini is consistent with results from morphological data (Asche, 1985, 1990). To further resolve relationships within this group, the analyses were continued with the non-asiracine taxa only, using *Stenocranus* and Kelisiinae as outgroup taxa.

Results from the spectral analysis are shown in Figs 5–7. The first thing we can note from the tree (Fig. 6) is that the ingroup and outgroup are separated by a relatively long branch (edge p). Both the ranked spectrum and the NNI plot (Figs 5 and 7, respectively) indicate that there is only a small amount of conflict against edge p. Thus, there is a phylogenetic signal in the nucleotide characters supporting monophyly for Delphacini. In contrast to the above, a parsimony analysis on this (nucleotide) dataset produced four equally parsimonious trees. In two trees, monophyly for Delphacini was rejected (data not shown). Apparently, there is still too much noise in the DNA characters for parsimony to get a clear result.

Another interesting result is that the spectral tree indicates that 'modern' Delphacidae (*Chloriona*, *Javesella*, *Nilaparvata* and *Sogatella*) form the sister group of the strongly supported *Conomelus* + *Megamelus* lineage.

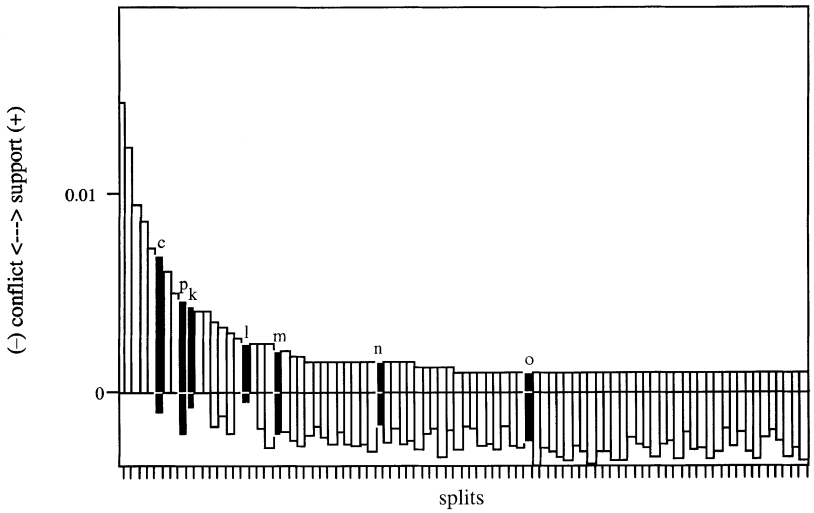

Fig. 5. Ranked sequence (bipartition) spectrum showing the major splits and their frequencies from high to low for the non-asiracine taxa. Amount of support is shown above *x*-axis, amount of (normalized) conflict is shown below *x*-axis. Black edges are the internal branches of Fig. 6. White bars without conflict are terminal branches. Edges: c = *Conomelus* + *Megamelus*; k = *Javesella* species; l = *Chloriona* species; m = *Javesella* and *Chloriona* species; n = *Sogatella* + *Nilaparvata*; o = *Sogatella* + *Nilaparvata* + *Javesella* and *Chloriona* species; p = Delphacini.

However, edge o is not reliable because the amount of conflict is clearly larger than the amount of support (Fig. 5), and there is also some local conflict (Fig. 7). Although Delphacini as a whole are well supported, the ranked spectrum showed considerable signals for a relationship between *J. pellucida* and *Stenocranus*, and also for *Nilaparvata* with *Kelisia* Fieber (frequencies about 0.0035). This may explain, at least in part, the low support (or the high amount of conflict) for 'modern' Delphacidae and Kelisiinae. Nevertheless, monophyly for 'modern' Delphacidae is supported by a morphological character, and a sister-group relationship between this group and the *Conomelus* + *Megamelus* lineage is consistent with Asche's (1985) view.

Monophyly for both *Javesella* and *Chloriona*, edges k and l, is clearly supported as shown in Figs 5 and 7, respectively. For 'modern' Delphacidae, there is an indication that *Chloriona* and *Javesella* (edge m) are relatively closely related. Estimating the reliability of this edge, however, is rather difficult. Although there is very little support for local alternatives, the ranked spectrum shows that the amount of support equals the amount of conflict. Therefore, we consider this edge only weakly supported. A close relationship between *Sogatella* and *Nilaparvata* (edge n) is clearly contradicted by a local alternative. This alternative proposes a relationship between *Chloriona*, *Javesella* and *Sogatella*. Importantly, in addition to signals for members

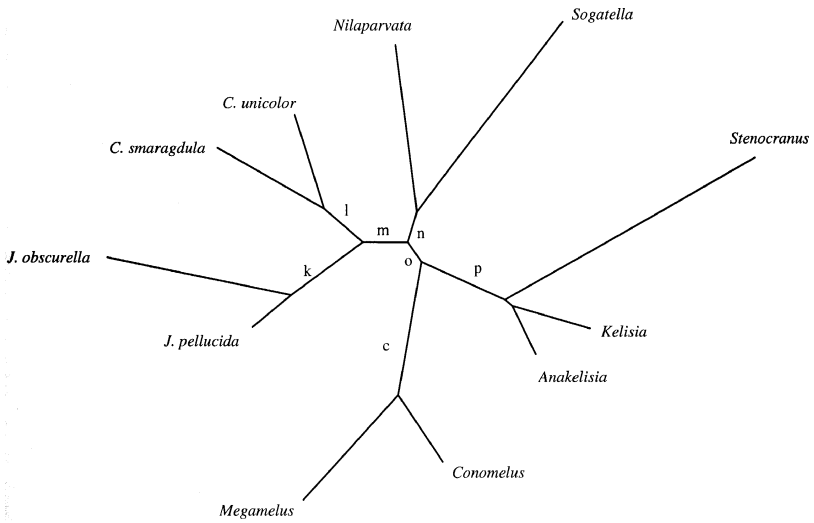

Fig. 6. Optimal tree from the spectrum in Fig. 5 showing relationships within non-Asiracinae. Edges marked as in Fig. 5.

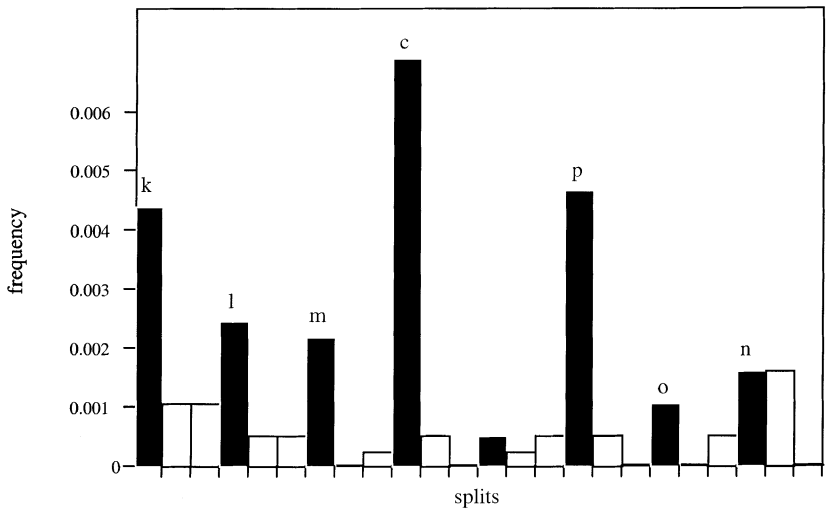

Fig. 7. NNI plot for the tree in Fig. 6 showing the amount of support for the edges (black) and the two local alternatives. Note the comparatively high amount of local conflict for edge n.

from different subfamilies (see above), there is also a fair amount of conflict within 'modern' Delphacidae.

In an attempt to further reduce the amount of noise, signals were studied within the Delphacini only (*Megamelus*, *Conomelus*, *Nilaparvata*, *Sogatella*, *Chloriona unicolor* (Herrich-Schaffer), *C. smaragdula* (Stål), *Javesella obscurella* (Boheman) and *J. pellucida*). Support for a close relationship between *Chloriona* and *Javesella* (Fig. 6, edge m) more than doubled to a frequency of 0.0057, which is relatively high, and there was little conflict. Edge n, however, was strongly contradicted by two local alternatives. Parsimony on nucleotide characters resulted in a single tree. Delphacine relationships were the same as shown in Fig. 6, but relationships among 'modern' delphacids had only low bootstrap support. The parsimony analysis on amino acid characters resulted in eight shortest trees indicating character conflict, although monophyly of 'modern' Delphacidae was never rejected. Thus, resolving relationships within this group may be problematic because of conflict in nucleotide and amino acid characters.

Genetic divergence

Intraspecific variation was estimated for one species only. Five different *C. glaucescens* populations were sampled (Table 1). The Wamel population was represented by four specimens; all other populations were represented by one specimen. Genetic distances are based on uncorrected pairwise comparisons.

Variation within the Wamel population ranged from 0 to 0.6%, and comprised three haplotypes. Between populations the range of variation was 0–1.4%, with the largest difference between the populations from Wamel and Munzeberg. The specimens from Wamel formed a single group within *C. glaucescens* in the phylogenetic tree (not shown). However, the specimens from Finland and Greece shared the same haplotype. Therefore, the COI gene may not be very useful for separating populations of *C. glaucescens*.

Mun *et al.* (1999) sampled specimens of *Nilaparvata lugens* and *Sogatella furcifera* from Malaysia to Korea. Intraspecific variation within *Nilaparvata lugens* and *Sogatella furcifera* ranged from 0 to 0.23% and 0–0.12%, respectively. Even compared to the small number of *C. glaucescens* specimens from Wamel (0–0.6%), intraspecific variation for *Nilaparvata* and *Sogatella* seems to be rather small.

Sequence variation between *Chloriona* species was lowest between *C. glaucescens* and *C. dorsata* (3.6–3.9%). Thus, variation between species appears about 2.5 times larger than within species. The highest interspecific difference within *Chloriona* was observed for the comparison between *C. vasconica* and *C. unicolor* (9.1%). A rather similar percentage (10.3%) was found between the two *Javesella* species.

Divergences between genera within Delphacini ranged from 13.3% (*Conomelus* and *Megamelus*) to 20.1%. (*Sogatella* and *Megamelus*). In contrast, divergences within 'modern' Delphacidae ranged from 16.1% (*C. unicolor* and either *Javesella*) to 19.9% (*C. dorsata* and *Sogatella*). Thus, divergence levels within 'modern' Delphacidae are similar to that within Delphacini as a whole.

The average amount of sequence divergence between Delphacini and *Stenocranus* was 20.2% (range 17.3–22.4%). Comparisons between Delphacini and other taxa did not yield higher divergence levels. Divergences within Kelisiinae and Asiracinae (species from different tribes) were 12.3% and 13.3%, respectively.

Rate differences within and between lineages

Using Tajima's (1993) relative rate test, we first examined whether there is evidence for a relatively faster rate in

non-Asiracinae compared to Asiracinae. *Chloriona* and *Javesella* are represented by *C. unicolor* and *J. pellucida*. Of the two species pairs used in the spectral analysis, terminal branch lengths for these species were shortest. Thus, this is a rather conservative approach.

Non-asiracines have consistently experienced more substitutions than *Ugyops* and *Asiraca* (M1 > M2, Table 2). In the comparisons with *Ugyops,* all differences are significant except the one with *Conomelus*. In the comparisons with *Asiraca*, there are only two cases where rates are significantly different. However, although the comparisons with *C. unicolor* and *J. pellucida* against *Asiraca* showed no significant differences, both *C. smaragdula* and *J. obscurella* did evolve significantly faster than *Asiraca* (data not shown). Thus, these results suggest that there is a general pattern or tendency for a relative faster rate in non-Asiracinae compared to Asiracinae. Multiple hits at third codon positions may have been a problem in these relative rate tests; however, third codon positions still showed phylogenetic signal as indicated in Fig. 1, and were therefore not excluded. It is difficult to hypothesize a likely explanation for these differences, but Asche (1990) has suggested that the radiation of non-asiracine delphacids may have been connected to the evolution of the monocotyledons, and this could have increased the rate of evolution.

With respect to rate differences within Delphacini, we first examined whether there were differences between the two delphacine lineages. All 'modern' Delphacidae have experienced more substitutions than *Conomelus,* although this is only significant in one case. In the comparisons with *Megamelus,* the differences are less extreme and no significant cases were detected. However, a faster rate for *Megamelus* compared to *Conomelus* is highly significant. Therefore, although there may be saturation effects in these comparisons, there is a rather consistent pattern.

Systematic implications

Our results are broadly consistent with the current taxonomy and morpho-phylogeny of Delphacidae, and as far as the taxon sampling allows support the basal position of Asiracinae and the monophyly of non-Asiracinae, Kelisiinae, Delphacini, the non-'modern' delphacids, *Javesella* and *Chloriona*. However, in contrast to evidence based on morphological characters (Asche, 1985, 1990; Yemel'yanov, 1996), our results support a sister-group relationship between *Asiraca* and *Ugyops* (i.e. monophyly of Asiracinae, within the limitations of the taxon sampling) and a sister-group relationship between Stenocraninae and Kelisiinae. Moreover, there is now also good evidence that *Conomelus* and *Megamelus* form a monophyletic group. Until now, there were no data supporting this presumed relationship. A sister-group relationship between this group and 'modern' Delphacidae was proposed by the spectral analysis, but only at a low confidence level. However, this sister-group relationship is consistent with Asche's (1985) view.

Of the two major points where the molecular tree differed from the tree based on morphological characters, the first disagreement involves the asiracines *Asiraca* and *Ugyops*, which according to our data may belong to a monophyletic group. Although there was a signal for a sister-group relationship between *Asiraca* + non-Asiracinae, which would support Asche (1985, 1990), it was much weaker than the signal proposing a sister-group relationship between *Asiraca* and *Ugyops*. Gene trees are not always the same as species trees (e.g. Moore, 1995), but if our results are correct they raise the possibility that the genal carina of *Asiraca* (plus a few other genera of Asiracini) and of non-Asiracinae is not a synapomorphy. Asche (1985) has already discussed the possibility that the genal carina has arisen independently twice because in the *Asiraca* group the genal carina ends distinctly behind of the antennal base, whereas in non-Asiracinae, including Vizcayinae, the genal carina ends beneath or slightly in front of the antennal base (Asche, 1990). If the genal carina has arisen twice, it may serve as a synapomorphy for the taxa in the *Asiraca* group. Alternatively, the carina may have arisen in the common ancestor of Delphacidae and was lost independently twice.

The second point of conflict refers to the position of *Stenocranus*. Molecular data strongly support a sister-group relationship between *Stenocranus* and Kelisiinae. Asche (1985, 1990), however, placed Stenocraninae as sister taxon of Delphacinae (+ Plesiodelphacinae) because the larvae in these groups emerge from the egg through a ring-like hatching-cap at the anterior pole. In the plesiomorphic condition, the larvae hatch through a longitudinal slit at the anterior pole, as do Kelisiinae. Moreover, there were no characters uniting Stenocraninae with Kelisiinae (Asche, 1985). As above, there are again two possible evolutionary scenarios. Hatching of the larvae through a ringlike cap may have arisen independently in Stenocraninae and in the common ancestor of Delphacinae + Plesiodelphacinae, or this character might be ancestral and then have been lost from Kelisiinae. In either case, the instability of the character is illustrated by some Asiracinae larvae, which also hatch through a ringlike cap (Asche, 1985).

Suitability of the COI gene for systematic studies

The fragment of the COI gene used in this study contains variable and conserved regions (Lunt *et al.*, 1996; Tsukihara *et al.*, 1996, for corrections on the transmembrane regions) and appeared to be useful at different taxonomic levels within Delphacidae. By performing parsimony on both nucleotide and amino acid characters and performing spectral analyses, almost all nodes were resolved with at least some level of confidence. Phylogenetic signals could usually be improved by pruning distantly related taxa from the tree, after basal nodes were resolved. Both nucleotide and amino acid characters provided, at least to some extent, information from the subfamily to the genus level. Species relationships within *Chloriona* were partially resolved using nucleotide characters.

Third codon positions supported monophyly of congeneric species which were diverged by as much as 10%. However, monophyly of the kelisiine genera (sequence divergence of 12.3%) and monophyly of the *Conomelus*, *Megamelus* lineage (sequence divergence of 13.3%) was rejected if parsimony was used on third codon positions. This suggests that the information content in these codon positions may be lost at divergence levels between 10% and 13%, and the true signal may become overwhelmed by parallelisms in more distantly related taxa. Still, third codon positions did resolve the most basal node within Delphacidae.

In cytochrome b, Meyer (1994) found that third codon positions seem to be saturated at 15–20% (uncorrected) overall sequence divergence, and suggested that other similarly conserved mitochondrial proteins would show the same pattern. Given a divergence range of 16.1–19.9% for 'modern' Delphacidae it seems likely that, for parsimony, third codon positions were too saturated to give reliable phylogenetic estimates in this group. This in contrast to Delphacidae as a whole, where third codon positions retained information concerning the basal position of Asiracinae.

Generally, amino acid characters were useful from the subfamily to genus level with complete resolution among subfamily relationships, although we had to deal with some noise before monophyly for Delphacinae (or Delphacini) could be shown. Resolving generic relationships within this group was rather problematic, except for the *Conomelus*/*Megamelus* lineage. Both nucleotide and amino acid characters contained too much noise for parsimony to support any group among 'modern' Delphacidae with confidence.

Because third codon positions were probably almost saturated, and considering the existence of rate differences and the large number of genera in this group, this gene fragment might not appear to be the best choice for resolving generic relationships within Delphacini. However, the spectral analysis did support a close relationship for *Chloriona* and *Javesella* (minimum divergence of 16.1%), so there may still be rather strong signals at relative high divergence levels. Moreover, the ranked spectrum and the NNI plot can be used to identify problem areas in the dataset.

Noise is less likely to be a problem for resolving generic relationships within Asiracinae. First, the two genera sampled come from the two different tribes (Asiracini and Ugyopini), and were only diverged by about 13%, suggesting even lower divergence levels within each tribe. Second, there is evidence suggesting that members in this group evolve with a comparatively slow rate.

In any case, it seems worthwhile to use this gene for further investigations on the main branching patterns in Delphacidae, especially as the big advantage of the COI gene is the ease with which all taxa could be amplified, which is in contrast to a number of other genes tested (e.g. elongation factor-1 alpha, histones, opsins and ITS regions). Together with adding more taxa, more characters can be sampled from this gene. If noise is a severe problem, there is an option to remove the most variable amino acid sites from the dataset. These sites may be detected by comparing the dataset with data from other insect orders, e.g. from Lunt *et al*. (1996).

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