Abstract

**Background:** With some exceptions, mitochondria within the class Insecta have the same gene content, and generally, a similar gene order allowing the proposal of an ancestral gene order. The principal exceptions are several orders within the Hemipteroid assemblage including the order Thysanoptera, a sister group of the order Hemiptera. Within the Hemiptera, there are available a number of completely sequenced mitochondrial genomes that have a gene order similar to that of the proposed ancestor. None, however, are available from the suborder Sternorrhyncha that includes whiteflies, psyllids and aphids.

**Results:** We have determined the complete nucleotide sequence of the mitochondrial genomes of six species of whiteflies, one psyllid and one aphid. Two species of whiteflies, one psyllid and one aphid have mitochondrial genomes with a gene order very similar to that of the proposed insect ancestor. The remaining four species of whiteflies had variations in the gene order. In all cases, there was the excision of a DNA fragment encoding for cytochrome oxidase subunit III\((COIII)\)-tRNA\(\delta\)-NADH dehydrogenase subunit 3\((ND3)\)-tRNA\(\delta\)-tRNA\(\alpha\)-tRNA\(\alpha\) from the ancestral position between genes for ATP synthase subunit 6 and NADH dehydrogenase subunit 5. Based on the position in which all or part of this fragment was inserted, the mitochondria could be subdivided into four different gene arrangement types. PCR amplification spanning from \(COIII\) to genes outside the inserted region and sequence determination of the resulting fragments, indicated that different whitefly species could be placed into one of these arrangement types. A phylogenetic analysis of 19 whitefly species based on genes for mitochondrial cytochrome b, NADH dehydrogenase subunit I, and 16S ribosomal DNA as well as cospeciating endosymbiont 16S and 23S ribosomal DNA indicated a clustering of species that corresponded to the gene arrangement types.

**Conclusions:** In whiteflies, the region of the mitochondrial genome consisting of genes encoding for \(COIII\)-tRNA\(\delta\)-NADH dehydrogenase subunit 3 can be transposed from its ancestral position to four different locations on the mitochondrial genome. Related species within clusters established by phylogenetic analysis of host and endosymbiont genes have the same mitochondrial gene arrangement indicating a transposition in the ancestor of these clusters.
Background
Whiteflies, psyllids, and aphids correspond to superfamilies within the suborder Sternorrhyncha (Hemiptera) [1]. These insects share a number of common properties that are a consequence of their utilization of plant phloem as their diet. This mode of feeding is accomplished by means of needle-like stylets that probe plant tissues between plant cells until they enter the phloem-sieve elements. Due to this mode of feeding, some species are of major agricultural importance in that they vector plant pathogens and in high numbers may cause plant debilitation due to excessive nutrient consumption [1]. Whiteflies, psyllids, and aphids have an obligate association with prokaryotic endosymbionts localized in specialized cells called bacteriocytes that constitute a larger structure called the bacteriome [2-4]. In the past, numerous studies have been performed on the phylogeny of some of these endosymbionts and their hosts [2-5]. The results have indicated congruence between the endosymbiont and the host derived phylogeny. This observation has been interpreted as being the consequence of an infection of an insect ancestor by a prokaryote and the vertical transmission of the endosymbiont resulting in cospeciation or cocladogenesis. In a recent study of whiteflies, we compared the phylogeny based on endosymbiont 16S-23S rDNA to the phylogeny of the host based on several mitochondrial genes [6]. During this study, we found that in the whitefly, Bemisia tabaci, the order of some of the mitochondrial genes was quite different from the frequently found order of genes in the mitochondria of the class Insecta. This observation led us to obtain the full sequence of the mitochondrial genome of representatives of the suborder Sternorrhyncha. Due to the observed differences in the order of genes in the mitochondrial genome of whiteflies, we obtained additional mitochondrial sequences from species representative of the major phylogenetic clusters previously established on the basis of whitefly mitochondrial and endosymbiont genes [6]. Previous studies of the phylogenetic relationships of member of the Sternorrhyncha, using host 18S rDNA, indicated that it is a monophyletic group [7-9]. These studies also showed that aphids and whiteflies were more closely related to each other than to psyllids.

In animals, the mitochondrial genome is generally circular (14–17 kb), is maternally transmitted and has a relatively simple genetic structure, and a rapid rate of sequence change [10-12]. Of the thirty seven genes found in animal mitochondria, thirteen encode for proteins, consisting of three subunits of cytochrome oxidase (COI, COII, COIII), two subunits of ATP synthase (atp6, atp8), seven subunits of NADH dehydrogenase (ND1, ND2, ND3, ND4, ND4L, ND5, ND6), and cytochrome b (cytB). Two genes encode for the large subunit of ribosomal RNA (16S) and the small subunit of ribosomal RNA (12S). In addition, there are 22 tRNAs, two for leucine and two for serine, and one tRNA each for the remaining eighteen amino acids. In general, there is conservation of the gene order within phyla but variation between phyla [10,13-17]; the tRNA genes are subject to more change in their position than the genes for proteins and rRNAs. The order of mitochondrial genes has been suggested to be a good phylogenetic marker for studies of relationships [14]. The animal mitochondrial genome is generally very compact with few if any intergenic spaces. Usually there is one (or rarely more) noncoding region, frequently following 12S rDNA. Such a region most often has a reduced G+C content and all or some of the following properties: a) direct repeats, b) inverted repeats, c) stretches of “T”s, “A”s, or “TA”s. By analogy with other well-studied mitochondria, such a region is considered to be a putative origin of DNA replication and a region from which transcription is initiated [10,12]. Variation in mitochondrial size is generally a consequence of variation in the length of the repeats in the noncoding region and not in the number of structural genes. Early studies within the class Insecta suggested conservation of the gene order over a wide range of different organisms indicating an ancestral gene order for this group [10,18]. However, more recent studies have shown that within the Hemipteroid assemblage, there is considerable variation in the order of genes in the orders Phthiraptera, Psocoptera, and Thysanoptera, but no variation in the order Hemiptera (that includes the suborder Sternorrhyncha) [18-21]. The complete sequence of the mitochondria of a representative of the Phthiraptera (wallow louse) and the Thysanoptera (plague thrips) has been obtained [18,20]. The latter shows major differences from the ancestral gene order. The Hemiptera and the Thysanoptera are sister groups and it was consequently of interest to obtain sequences of the mitochondrial genomes of the former. Since the sequence of mitochondrial genomes is poorly conserved, sequence determination of a portion of the genome is useful for the study of closely related species or the population structure within a species [22,23]. The availability of completely sequenced mitochondrial genomes is also an aid to the design of primers for the PCR amplification of the regions selected for population studies.

Results
Evolutionary relationships within the Sternorrhyncha
Table 1 gives the properties and the accession numbers of the mitochondrial DNA sequences determined in this study. An unrooted phylogenetic tree showing the relationships of whiteflies, psyllids and aphids, based on mitochondrial cytB (partial), ND1, and 16S rDNA is presented in Fig. 1. A similar tree is obtained when the amino acid sequence of CytB (partial) and ND1 is used. The sole difference is the position of Neomaskellia andropogonis which becomes part of the cluster containing Bemisia...
tabaci, Tetraleurodes acaeciae, Aleurochiton aceris, and Trialeurodes vaporariorum. Whiteflies, psyllids and aphids have associations with different primary endosymbionts that are transmitted vertically and are essential for the survival of the insect host [2-6]. The time for the establishment of these endosymbiotic associations and the emergence of the composite organism is generally estimated to be between 100 and 200 million years ago [2]. The representative species chosen for study (Fig. 1) probably span the range of diversity within whiteflies, psyllids, and aphids. The maximum % difference in the DNA sequence of these organisms is 33.5% for whiteflies, 29.7% for psyllids and 13.1% for aphids suggesting that the rate of mitochondrial sequence change in aphids is considerably less than that in whiteflies and psyllids. Resolution of the order of these tRNA genes is reversed and this probably constitutes the whitelfly ancestral gene order. In the mitochondria of T. vaporariorum, tRNA-G is transposed from its position between COIII and ND3 to a position between tRNA-W and tRNA-Y (Fig. 1). tRNA-S1 was not detected in the mitochondria of S. graminum; this tRNA and tRNA-Q was not detected in A. dugesi.

**Mitochondria of whiteflies with transposition of COIII-(tRNA-G)-ND3-(tRNAs-A-R-N)**

A number of whitefly mitochondria had transpositions of DNA fragments containing COIII-(tRNA-G)-ND3-(tRNAs-A-R-N). In most cases in which these genes are removed, there is a change in the direction of transcription of the adjacent downstream tRNA-S1 from clockwise to counter clockwise (Fig. 3, 5, 6). There is variation in the mitochondrial position into which these genes are transposed. In addition, there are differences with respect to the retention of the number and the order of the excised tRNA genes at the mitochondrial location in which the genes are inserted. The maximal insertion involves all of the genes from the excised fragment in their original order (Fig. 3) (tRNAs-A-R-N)-ND3-(tRNA-G)-COIII), the minimal insertion involves ND3-(tRNA-G)-COIII (Fig. 4, 5). In all

| Organism                  | Type       | Mitochondrial sequence | Size (bp) | G+C content | GenBank accession number |
|---------------------------|------------|------------------------|-----------|-------------|-------------------------|
| Bemisia tabaci            | whitefly-A | complete               | 15,322    | 25.9        | AY521259                |
| Tetraleurodes acaeciae    | whitefly-B | complete               | 15,080    | 28.0        | AY521626                |
| Neomeanekella andropogonis| whitefly-C | complete               | 14,496    | 18.7        | AY572539                |
| Aleurochiton aceris       | whitefly-D | complete               | 15,388    | 22.1        | AY572538                |
| Trialeurodes vaporariorum| whitefly-Y | complete               | 18,414    | 27.7        | AY521265                |
| Aleurodyscus dugesi       | whitefly-Y | complete               | 15,723    | 13.8        | AY521251                |
| Pachypsylla venusta       | psyllid    | complete               | 14,711    | 26.3        | AY278317                |
| Schizaphis graminum       | aphid      | complete               | 15,721    | 16.1        | AY531391                |
| Bemisia argentifolii      | whitefly-A | cytB-COIII             | 4,796     | 23.2        | AY521257                |
| Bemisia sp.               | whitefly-A | 12S-COIII              | 985       | 19.4        | AY572845                |
| Aleuroplatus sp.          | whitefly-B | cytB-COIII             | 4,540     | 27.4        | AY521256                |
| Tetraleurodes mori        | whitefly-B | cytB-COIII             | 4,416     | 25.3        | AY521263                |
| Vasaevadius concursus     | whitefly-C | cytB-COIII             | 3,374     | 20.0        | AY648941                |
| Siphonius phillyreae      | whitefly-D | cytB-12S               | 4,561     | 22.3        | AY521268                |
| Bactericera cockerellii   | psyllid    | cytB-12S               | 3,077     | 28.0        | AY601890                |
| Calophya schini           | psyllid    | cytB-12S               | 3,044     | 26.3        | AY601891                |
| Glysaspis brindlecombei   | psyllid    | cytB-12S               | 3,081     | 26.8        | AY601899                |
| Diuraphis noxia           | aphid      | cytB-12S               | 3,180     | 15.8        | AY601892                |
| Melaphis rhois            | aphid      | cytB-12S               | 3,184     | 17.0        | AY601894                |
| Schlechtendalia chinensis | aphid      | cytB-12S               | 3,188     | 16.1        | AY601893                |
| Daktulosphaira vitifoliae | phylloxera  | cytB-12S               | 3,215     | 22.6        | AY601895                |

*From [6].

**Table 1: Properties and accession numbers of mitochondrial DNA sequences determined in this study.**
insertions, the transcription direction is altered from that in the original position. Based on the location of the insertions and the adjacent genes, we have subdivided these transpositions into four types (A-D) (Fig. 3, 4, 5, 6). In all cases, it would appear that the excision involved the removal of COIII-(tRNA-G)-ND3-(tRNAs-A-R-N). However, the DNA that is inserted always contains COIII-(tRNA-G)-ND3 and may contain all or only some of the tRNA genes.

Transposition of the A type is shown in Fig. 3. In this case, COIII-(tRNA-G)-ND3-(tRNAs-A-R-N) is removed from the inferred ancestral position and placed between 12S rDNA and tRNA-I. Additional changes involve the position of tRNA-D, tRNA-Q and the direction of transcription of tRNA-S1 and tRNA-E. There is a total of 5 difference between the A type gene arrangement and the ancestral whitefly gene order. Sequence determination of smaller DNA fragments from two related species (cytB, COII) were consistent with the same gene order (Fig. 3).

Transposition of the B type is shown in Fig. 4. In this case, ND3-(tRNA-G)-COIII is inserted into a location downstream of 12S rDNA and is bounded by tRNAs that have also changed locations (tRNAs-Q-V and tRNAs-R-D). In addition, the position of tRNA-A is changed as

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**Figure 1**
Unrooted phylogenetic tree showing the relationships of members of the Sternorrhyncha (whiteflies, aphids, and psyllids). The tree is based on mitochondrial cytB, ND2, and 16S rDNA sequences. Maximum likelihood analysis, values at nodes are for bootstrap percentages from 500 replicates, only nodes supported by 70% or greater are shown. * by species name designates the organisms for which the complete mitochondrial sequence has been determined.
compared to the ancestral position. There were 6 differences from the putative ancestral gene order. No tRNA genes for N, S1, and I were detected. Sequence determination of a smaller DNA fragment (cytB-COIII) from two related species was consistent with the same gene order. Transposition of the C type is shown in Fig. 5. In this case, ND3-(tRNA-G)-COIII is inserted downstream of 16S rDNA between tRNA-P and tRNA-C. Another major difference is the change in the direction of the transcription of tRNA-V and 12S rDNA. Other differences include the change in the order and the position of tRNA-Y and tRNA-
C, the change of position of tRNA-P, and the direction of transcription of tRNA-S1. Putative tRNA-W is transcribed clockwise. A small change in the span of the DNA fragment resulted in a putative tRNA-S2, transcribed counterclockwise. The initially adjacent tRNAs-A-R-N as well as tRNA-I were not detected. There was a total of 7 differences between the whitefly ancestral gene order and the C type gene order. Sequence determination of a fragment of mitochondrial DNA from a related whitefly species was consistent with the C type gene order. The D type gene order is shown in Fig. 6. In this case, t( tRNAs-R-A)-ND3- (tRNA-G)-COIII is found after tRNA-S2 and before tRNA-N. Additional differences from the ancestral gene order involve the change in position of tRNA-N and tRNA-Q and the direction of transcription of tRNA-S1. tRNA-I was not detected. The total number of differences between the ancestral gene order and the D type gene order is 4. The sequence of a mitochondrial DNA fragment from a related species indicated a gene order of the D type (Fig. 6).
D type

WF ancestor

Aleurochiton aceris

Figure 6
Differences of the D type gene order from the postulated whitefly ancestral gene order. Figure legend same as in Fig. 2 and 3.

Figure 7
Agarose gel electrophoresis of PCR products amplified from whole insect DNA using primers complementary to regions encoding COII and ND5. A, B, C, D, refers to different gene arrangement types; Y, ancestral arrangement. Lanes 1 and 9 molecular size markers; lane 2, Bemisia tabaci; lane 3, Tetraleurodes acaciae; lane 4, Neomaskelia andropogonis; lane 5, Aleurochiton aceris; lane 6, Aleyrodes elevatus; lane 7, Trialeurodes vaporariorum; and lane 8, Aleurodicus dugesii.

Figure 8
Agarose gel electrophoresis of PCR products amplified from whole insect DNA using primers complementary to regions encoding COIII and cytB. A, B, C, D, refers to different gene arrangement types; Y, ancestral arrangement. Lanes 1 and 8 molecular size markers; lane 2, Bemisia tabaci; lane 3, Tetraleurodes acaciae; lane 4, Neomaskelia andropogonis; lane 5, Aleurochiton aceris; lane 6, Trialeurodes vaporariorum; and lane 7, Aleurodicus dugesii.
PCR-based screening for excision of COIII-(tRNA-G)-ND3-(tRNAs-A-R-N) and identification of transposition types

We have devised a set of oligonucleotide primers complementary to COII and ND5 that allow the amplification of the DNA between these two genes. The size of the resulting fragments is a potential indication of the presence or absence of COII-(tRNA-G)-ND3-(tRNAs-A-R-N) between COII and ND5 (Fig. 2). Fig. 7 shows the results obtained with insects containing mitochondria that have these genes in the ancestral position (lanes 6–8, bands of 3.7 kb) and those in which they have been excised from this position (lanes 2–5, bands of 2.2 to 2.3 kb).

In addition, we have devised a set of PCR primers that allow the distinction of the four types of transpositions. Using oligonucleotide primers complementary to COIII and cytB, the PCR fragments shown in Fig. 8 were obtained. The sizes characteristic of arrangement types A, B, C and D, were 4.9, 4.5, 3.5, and 1.5 kb, respectively.

Non-coding regions

Mitochondrial genomes of insects are very compact. The principal non-coding segments of the genome are a low G+C content region usually following 12S rDNA [10-12]. The low G+C region usually has stretches of "T"s or "A"s as well as multiples of the sequence "TA." Another feature of this region may be inverted and direct repeats. Fig. 9 presents a diagrammatic summary illustrating some of the properties of the non-coding regions of the mitochondria of the studied insects. Only direct repeats and their sizes are indicated in this figure. No consistent pattern of inverted repeats was found and these are not indicated in the diagrams. All of the non-coding regions in the vicinity of 12S rDNA had a G+C content lower than the G+C content of the full genome (Fig. 9). The decrease ranged from 3.2 to 10.0%. Some of these regions of lower G+C content, adjacent to 12S rDNA, contained direct repeats (Fig. 7, Adu, Tva, Aac). In Bta and Nan (Fig. 9), the direct repeats were in a non-coding region following COII that also had a decrease in the G+C content. The noncoding regions of Tac, containing direct repeats, had a G+C content that was actually higher than that of the full Tac mitochondrial genome. However, the segment before the repeats had regions with a lower G+C content. In Sgr (Fig. 9), the direct repeats were between tRNA-E and tRNA-F and had essentially no decrease in the G+C content. In this organism and Pve (Fig. 9), the region following 12S rDNA had a decrease G+C content but did not contain substantial direct repeats.

tRNA anticodons

In general, the anticodons found in the tRNAs of the mitochondria of whiteflies, psyllids, and aphids were those expected of insect mitochondria [12]. Some exceptions were "TTT" (instead of CTT) for tRNA-K for B. tabaci and A. dugesi, and "TCT" (instead of GCT) for tRNA-S1 for B. tabaci, A. aceris, N. andropogonis and T. vaporariorum. The latter codon maybe the usual tRNA-S1 anticodon in whiteflies; this tRNA was not detected in T. acacae and A. dugesi.

Discussion

The novel aspect of this study is the finding that whitefly mitochondria contain a region of their genome spanning COIII-(tRNA-G)-ND3-(tRNAs-A-R-N) that is prone to excision followed by insertion as a unit or as fragments in different parts of the mitochondrial genome. Based on the collection of whiteflies we have examined, this event occurred three or four different times in the ancestors of the studied species. These conclusions are summarized in Fig. 10 where the phylogeny of the whiteflies is compared to the gene arrangement types. The designation Y refers to the ancestral arrangement established in the species under this designation. Species bracketed under A and B have a similar insertion position for COIII-(tRNA-G)-ND3 (Fig. 3, 4) but differ in adjacent tRNAs, so that it is possible these differences followed the insertion of the transposed fragment in a common ancestor. The positions of the transpositions in species of clusters C and D are very different and are probably the results of independent events. For purposes of this discussion we have chosen the simplest interpretation but this does not exclude other more complex scenarios. Cluster C is of additional interest since it is related to two species of Aleyrodes that have the ancestral (Y) arrangement. From the 16S*-23S* rDNA sequence divergence of Portiera (the primary endosymbiont of whiteflies) and the estimated rate of endosymbiont sequence change [24], it is possible to estimate the time of divergence of cluster C and the two Aleyrodes species. This value corresponds to 30–60 million years ago which is the maximum time for the occurrence of the transposition in an ancestor of cluster C.

The excision of the same mitochondrial fragment at least four times during the evolutionary history of whiteflies suggests that this fragment is prone to transposition. In spite of the apparent similarity of the excisions, we have not been able to find any conserved sequence properties either adjacent to the region of the excised fragment or adjacent to its insertion site. The excision appears to be associated with a change in the direction of transcription of the previously adjacent tRNA-S1 (Fig. 3, 5, 6) and a change in the direction of transcription of the relocated fragments. As previously noted the order of the mitochondrial genes is conserved in most insects [10,14]. The major exceptions are within the three hemipteroid orders Phythiraptera, Psocoptera, and Thysanoptera [18,20,21]. The rearrangements are different within these three orders, being rather extreme in the Thysanoptera. In most
insects, the order of the rRNA genes is 16S-(tRNA-V)-12S and the genes are transcribed in the counterclockwise direction [10,18]. A major exception is in the mitochondrial of *Thrips imagines* where these two genes are distant from each other and transcribed in opposite orientations [18]. In the C type gene order, there is an inversion of 12S-(tRNA-V) that is possibly associated with the insertion of ND3-(tRNA-G)-COIII between 16S and 12S rDNA (Fig. 5,

### Figure 9
**Summary of the properties of non-coding regions found in the mitochondrial genomes of psyllids, aphids and whiteflies.** Letter abbreviations for amino acids, denote tRNAs for the designated amino acid; thin lines, length of non-coding region; thick lines, direct repeats; numerals above line, length of direct repeats, numbers in parentheses at end of fragment denote its length in bp; column of numbers at right represent the difference between the G+C content of the full mitochondrial genome and the considered segment. Pve, psyllid, *Pachypsylla venusta*; Sgr, aphid, *Schizaphis graminum*; Adu, whitefly, *Aleurodiscus dugesi*; Tva, whitefly, *Trialeurodes vaporariorum*; Bta, whitefly, *Bemisia tabaci*; Tac, whitefly, *Tetraleurodes acaciae*; Nan, whitefly, *Neomaskelia andropogonis*; Aac, whitefly, *Aleurochiton aceris*.

| Organism | Region | Length | Direct Repeats | G+C Difference |
|----------|--------|--------|----------------|----------------|
| Pve      | 12S---I | 597    |                | -9.7           |
| Sgr      | 12S---I | 622    |                | -2.9           |
| Sgr      | E-F    | 636    |                | -0.2           |
| Adu      | 12S---I | 1575   |                | -6.6           |
| Tva      | 12S---I | 3729   |                | -6.9           |
| Bta      | 12S---N | 170    |                | -10.0          |
| Bta      | COIII--I | 662    |                | -3.7           |
| Tac      | D---A   | 800    |                | +2.0           |
| Nan      | COIII--C-Y-12S | 426 | | -10.0 |
| Aac      | 12S---M | 1092   |                | -3.2           |
This situation resembles that found in *Thrips imaginis* in that the rRNA genes are transcribed in opposite directions. In whiteflies, besides rearrangements involving COIII-(tRNA-G)-ND3-(tRNAs-A-R-N), there are also substantial rearrangements involving single tRNAs. The physiological significance (if any) of these rearrangements is not known. Genes that are highly expressed (16S, 12S rDNA) when separated and transcribed in opposite orientations would have to become part of different transcription units. In addition, we are not certain of the validity or significance of our inability to find a few of the tRNAs. In some cases, this may stem from our inability to recognize them. In other cases, such as tRNAs-A-R-N that are absent in the type C gene order, there would not appear to be any room for these genes on the mitochondrion and it might be that the tRNAs for these amino acids are provided by the host [25].

The mitochondrion of *A. dugesii* has a G+C content of 13.8 moles % (Table 1). All the other sequenced whitefly mitochondria have G+C contents of 18.7 to 27.7 moles % (Table 1). On the basis of morphological classification, *Aleurodicus* has been placed into a subfamily Aleurodicinae, while the remaining whitefly species listed in Fig. 10 have been placed into the subfamily Aleyrodinae [1]. This separation is supported by a phylogenetic analysis of mitochondrial DNA, host 18S rDNA, as well as Portiera DNA from different whitefly species [6-8]. It is possible that the common ancestor of whiteflies had a higher G+C content in its mitochondria and that in *Aleurodicus* there was a decrease. Alternatively, it is possible that the ancestral G+C content was low and increased in the Aleyrodinae.
Our work points to the uncertainty inherent in making generalizations from one or a few organisms assumed to be representative of a group. We were fortunate that in the whiteflies the first mitochondrion we chose to study was that of *B. tabaci* which had an altered gene order. Had we started with our second or third choice (*T. vaporariorum, A. dugesii*) we would have concluded that the whiteflies have the ancestral mitochondrial gene order and not pursued further studies of mitochondria within this group of insects. Previously, evidence was found of a correlation between the rate of nucleotide sequence change and the rate of gene rearrangement [26]. If this has general applicability one would expect conservation of the mitochondrial gene order in aphids which have a low rate of sequence change (Fig. 1) and perhaps some changes in the gene order of psyllids as has been observed with whiteflies. The relatively localized different changes observed in several whitefly lineages may be of use in the study of the phylogeny and taxonomy of these organisms as is already indicated from the relatively small sample of organism studied in the present work.

Conclusions

Psyllids, aphids, and many whiteflies have mitochondria in which the order of the genes resembles the proposed *Insecta* ancestral gene order. However, in a variety of whitefly species there is a change in the gene order. In these organisms, there is an excision of a DNA segment containing *COIII- (tRNA-G)-ND3- (tRNAs-A-R-N)* from the ancestral position, between *atp6* and *tRNA-S1*, and the insertion of all of these genes or fragments containing *COII- (tRNA-G)-ND* and tRNAs into different locations on the mitochondrial genome. On the basis of the insertion positions, four gene arrangement types were identified. A phylogenetic analysis of 19 whitefly species involving mitochondrial and endosymbiont genes showed that each arrangement type was characteristic of a cluster of related whitefly species indicating that the transposition occurred in a common ancestor of the related species. The reason for the "restlessness" of this DNA segment in whiteflies and the physiological significance of these rearrangements are not known.

Methods

Amplification and sequencing of mitochondrial genomes

In all cases, the starting material was whole insect DNA that was prepared and used in a previous study [6]. In our initial attempts at cloning mitochondrial DNA, we used methods previously developed for obtaining clones of insect endosymbiont DNA that have been described in detail [27]. In outline this involved obtaining a homologous probe for *COI* using previously described primers [28], followed by restriction enzyme and Southern blot analysis of insect DNA. Appropriate sized fragments were electroeluted from agarose gels and cloned into *λ*ZAP (Stratagene, La Jolla, California). Following excision of the insert-containing plasmid, the DNA sequence was determined using a double stranded nested deletion kit (Pharmacia, Piscataway, New Jersey) and where necessary custom-made oligonucleotides. As new sequence data was acquired for the mitochondria of several insect species our ability to design more specific oligonucleotide primers

Table 2: Oligonucleotide primers for PCR amplification of whitefly mitochondrial DNA fragments.*

| Primer | Gene | Position on AY5212566 | Nucleotide sequence of conserved regions (5'->3') |
|--------|------|-----------------------|-----------------------------------------------|
| F-COI-1 | COI  | 172–221               | TCWCATCGCW TTATYATAAT TTTTTYATR ACWATGCTT TDGTWATTGG |
| F-COI-2 | COI  | 673–710               | GAYCYCHATTY TRATCAAAAT YTTDDTDIGATTTTGG |
| R-COI   | COI  | 1133–1069             | ACATAATGAA AATGDGCAAC AACAAAATAAWGTATCATGHA RACAHACATC HACAGAAGAA TTACC |
| F-COI   | COI  | 1845–1876             | CTCCTCTATYC GDATTTTDTA TYAAATRGAT GA |
| R-COI   | COI  | 2093–2067             | AGGAAACHTY CAAGATGHA AACATC |
| F-COII  | COII | 3854–3879             | TTAACWGHT TACGJGNNT HCAGTG |
| R-COII  | COII | 4002–3974             | CARACWAHT CDACRAAATG TCAGATCA |
| F-CYTB  | CYTB| 9169–9215             | GCTTTATR ATBRTATYTT RCTTGRGGY CARATATCTT TTITGRGG |
| R-CYTB  | CYTB| 9566–9544             | GCTATAAAT AATITTTCTGA ATC |
| F-N1    | ND1 | 10275–10314           | ATCTAAATTT AAAWCCWAGGA ATWARYTCTG AYTCTTCTTC |
| R-N1    | ND1 | 10506–10484           | CAAYAATTT CDATAGAAT TAA |
| F-16S-1 | 16S | 11053–11087           | ACPTGGCTT CGCCGGTCTG AACTCAGATC ATGTA |
| F-16S-2 | 16S | 11202–11220           | GCTTTTACCC CTGAGTA |
| R-16S-1 | 16S | 11526–11543           | TAAAATGCT CGGCRWYATT DACTGACTA AGGATCCAATA AATA |
| F-12S-1 | 12S | 12273–12308           | ACTTTCCAGT AADDTTACTT TGTTACGACT TATCTT |
| F-12S-2 | 12S | 12318–12342           | AGAAGTGAGC GCGRATTGTG ACA |
| R-12S-1 | 12S | 12643–12619           | CTTCAGAAT CAAAATTG TGCGG |
| R-12S-2 | 12S | 12861–12843           | GTGGCAGCAG TGWCGCGT |

*Underlined sequences indicate primers used in this study. Mitochondrial genome of *Trialeurodes vaporariorum*. 
was improved. This allowed us to use pairs of primers, in combination with PCR, to obtain the full mitochondrial genome in 2–4 overlapping fragments. Conserved regions of the whitefly mitochondrial genome that are of use for the design of oligonucleotide primers, based on comparisons of six mitochondrial genomes, are given in Table 2. Usually the oligonucleotide primers had added sequences at the 5’-ends for restriction enzymes.

We will illustrate the approach by describing how the full genome of the mitochondrion of *T. acaciae* was obtained in three overlapping fragments. Using primers F-CYTB and R-12S-2 (Table 2) and PCR, a 3.6 kb DNA fragment was obtained. Similarly, using the pairs of primers F-COI-2 and R-CYTB and F-12S-2 and R-COI fragments of 7.3 and 5.5 kb, respectively, were obtained. For fragments of 4 kb or less, the PCR reaction mixture (10 µl) contained 10 ng insect DNA, 1 µg bovine serum albumin, 5 mM MgCl2, 0.2 mM dNTP, 10 pmoles of each primer, 0.6 U Bio-X-Act DNA polymerase, in Opti-Buffer (Bioline, London, United Kingdom). The PCR program was 94°C for 3 min, 30 cycles of, 94°C 30 sec, 55.0–65.0°C (predetermined optimal annealing temperatures) 30 sec, 70° 5 min, followed by 70°C 10 min. For the 5.5 and 7.3 kb DNA fragments, the PCR reaction mixture was modified by the increase of dNTPs to 0.3–0.4 mM and Bio-X-Act to 0.8 U. The PCR program was 94°C for 2 min, 10 cycles of 92°C 20 sec, 55.0–65.0°C (predicted optimal annealing temperatures) 30 sec, 68° 10 min, followed by 20 cycles of 92°C 20 sec, optimal annealing temperature 30 sec, 68°C 10 min with increases of 15 sec each cycle, followed by 68°C 10 min. The DNA fragments were purified by means of the Wizard SV gel and PCR clean-up system (Promega, Madison, Wisconsin) as directed by the manufacturer. Following digestion with restriction enzymes the mitochondrial DNA fragments were cloned into pBluescript (Stratagene). In some cases where difficulty was experienced with using this vector due to possible toxicity of the inserts, the low copy number plasmid pWSK130 was used [29]. The DNA sequence was obtained as described above. Sequences were determined at the University of Arizona (Tucson) LMSE sequencing facility. In some cases, PCR fragments of 1 to 4 kb were directly sequenced after gel purification using custom made oligonucleotide primers.

**PCR amplification of other mitochondrial fragments**

CytB-12S mitochondrial DNA fragments were amplified and cloned into pBluescript as previously described [6]. CytB-COI11 DNA fragments were obtained using oligo WF-CYTB-3 (BamHI, SacI; 5’-GCAGGATCCGGGCCCTWT GRC GHCAAATATC WTTTTRGCGD GC-3’) and WF-COI11-3 (KpnI, 5’-GTGCCGTACCC TCCWATTGTG TATT GRCATTTTGTGAG-3’) and cloned into pBluescript. COII-ND5 DNA fragments (indicative of the presence or absence of COIII-(tRNA-G)-ND3-(tRNAs-A-R-N) were obtained by use of oligo WF-COI1 (5’TGTCCAGAAA YTY GTGGRGT TAATCAYAGR TTTATRCC-3’) and WF-ND5 (5’TACGCMTTAG YTYCATYCTWTC ACAYTATGW ACAC CAGG-3’). CytB-COII1 fragments (size diagnostic of the arrangement type) were obtained by use of WF-CYTB-1 (5’TATTATRGGGT ATATYTTRCC TTRGRC-3’) and WF-COI11-1 (5’TATTCWRTWT GATATTGACA TTTYGT-3’). The PCR reaction mixture (10 µl) differed from those above in containing 0.1 mM dNTP, and 0.8 U Bio-X-Act DNA polymerase. The PCR program was 94°C for 5 min, 30 cycles of, 94°C 30 sec, 56.0–63.1°C (predetermined optimal annealing temperatures) 30 sec, 70° 5 min, followed by 70°C 10 min.

**Identification of genes and phylogenetic analyses**

The protein-coding and rRNA genes were identified by BLAST searches [30] of GenBank. tRNA genes were identified by tRNAscan-SE [31]. DOGMA [32] and in some cases by eye from the anticodons and inferred secondary structures. The methods used for the phylogenetic analyses have been described [6]. In Fig. 1, the phylogenetic analysis of mitochondrial cytB-ND1-16S was based on 2730 characters; the analysis in Fig. 10, which besides cytB-ND1-16S also included cospeciating endosymbiont 16S*-23S* rDNA [6], was based on 6860 characters.

**List of abbreviations used**

**tRNAs**

tRNA-one letter amino acid abbreviation (parenthesis three letter amino acid abbreviation followed by antico- dons): tRNA-A (ala, TGC), tRNA-C (cys, GCA), tRNA-D (asp, GTC), tRNA-E (glu, TIC), tRNA-F (phe, GAA), tRNA-G (gly, TCC); tRNA-H (his, GTG), tRNA-I (ile, GAT), tRNA-K (lys, TTG or CTT), tRNA-L1 (leu, TAG), tRNA-L2 (leu, TAA), tRNA-M (met, CAT), tRNA-N (asn, GTT), tRNA-P (pro, TGG), tRNA-Q (gln, TTG), tRNA-R (arg, TCG), RNA-S1 (ser, TCT or GCT), RNA-S2 (ser, TGA), tRNA-T (thr, TGT), tRNA-V (val, TAC), tRNA-W (trp, TCA), and tRNA-Y (tyr, GTA).

**Other structural genes**

*atp6* (ATP synthase, subunit 6), *atp8* (ATP synthase, subunit 8), *COI* (cytochrome oxidase, subunit I), *COII* (cytochrome oxidase, subunit II), *COIII* (cytochrome oxidase, subunit III), *ND1* (NADH dehydrogenase, subunit 1), *ND2* (NADH dehydrogenase, subunit 2), *ND3* (NADH dehydrogenase, subunit 3), *ND4* (NADH dehydrogenase, subunit 4), *ND4L* (NADH dehydrogenase, subunit 4L), *ND5* (NADH dehydrogenase, subunit 5), *ND6* (NADH dehydrogenase, subunit 6), *12S* (small subunit of mitochondrial ribosomal DNA [rDNA]), 16S (large subunit of mitochondrial rDNA), 16S* (small subunit of primary endosymbiont rDNA), 23S* (large subunit of primary endosymbiont rDNA).
Other abbreviations
%
%G+C (moles percent guanine+cytosine in DNA).

Authors' contributions
MLT cloned and sequenced the mitochondrial genomes of whiteflies. LB cloned and sequenced the mitochondrial genome of a psyllid and an aphid as well as smaller fragments of mitochondrial DNA from psyllids and aphids. PB directed the research and in collaboration with MLT and LB performed the data analysis and wrote the paper.

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