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Mitochondrial Transfer RNA Genes in a Black Fly, *Simulium vittatum* (Diptera: Simuliidae), Indicate Long Divergence from Mosquito (Diptera: Culicidae) and Fruit Fly (Diptera: Drosophilidae)

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Abstract

Sequences are given for nine complete genes and one partial mitochondrial tRNA gene of the black fly, *Simulium vittatum* (Zetterstedt). Sequenced tRNA genes were for alanine, arginine, asparagine, aspartic acid, glutamic acid, glycine, leucine(CUN), lysine, serine(AGN), and valine. Nucleotides were aligned with the same previously sequenced genes in *Aedes albopictus* Skuse and *Drosophila yakuba* Burla. A cluster of six tRNA genes, which differ in arrangement in *Ae. albopictus* and *D. yakuba*, was amplified by PCR and found to have the same position and orientation in *S. vittatum* as in *D. yakuba*. Overall, similarity with either *D. yakuba* or *Ae. albopictus* was 86%. Sequences that were common to the three insects suggest that black flies and mosquitoes are as divergent from each other as either is from *Drosophila*. Sequences for nine species of black flies were obtained for tRNA leucine(CUN) from DNA amplified with another primer set. Little variation occurred within the tRNA gene but, by including the flanking regions to provide 175 base pairs, a phylogeny of the nine species was obtained that was largely consistent with current classification.

Keywords: Insecta, *Simulium vittatum*, mitochondrial DNA, tRNA genes

Mitochondrial tRNA genes in insects are poorly known (Sprinzl et al. 1991). The mitochondrial genome of only a single insect, *Drosophila yakuba* Burla, has been sequenced fully (Clary & Wolstenholme 1985a). Partial sequence data of other insects indicates that insect tRNA genes were rearranged relative to other eukaryotes (Clary et al. 1982, 1983) and that their positions and orientations vary among taxa. Both the migratory locust, *Locusta migratoria* (L.) (Haucke & Gellissen 1988), and the honey bee, *Apis mellifera* L. (Crozier et al. 1989), have tRNAs lysine and asparagine transposed relative to *Drosophila*. The honey bee also has cysteine, tyrosine, and tryptophan tRNAs rearranged. A cluster of six tRNA genes has order and orientation changed in a mosquito, *Aedes albopictus* Skuse, relative to *D. yakuba* (HsuChen & Dubin 1984). Alanine and asparagine are transposed and serine(AGN) is on the opposing strand. Such changes in gene order and orientation have potential phylogenetic significance. By folding sequences into the inferred configuration of tRNAs, most nucleotides can be homologized.

Zhu (1991) cloned and sequenced portions of the mitochondrial genome of S. *vittatum* Zetterstedt sibling IIIL-1 (Rothfels & Featherston 1981). All identified genes occurred in the same order and orientation as in *D. yakuba*. Here, we report the sequences of 10 tRNA genes in S. *vittatum* and compare the sequences with *D. yakuba* and *Ae. albopictus*. The sequences for tRNA leucine(CUN) and flanking regions in nine black fly species are also compared.

Materials and Methods

We utilized mitochondrial DNA from *S. vittatum* IIIL-1 larvae from southeast Nebraska, which was extracted, purified, and cloned by standard methods (Zhu 1991). Two regions of interest were amplified with the following primers by polymerase chain reaction (PCR). Asymmetric PCR was used to produce single-stranded DNA for sequencing (Gyllensten & Erlich 1988). All numbers in parentheses refer to the position of the 3′ end on the *D. yakuba* map:

primer 1, 5′-GGTCCCTTACGAATTTGAAT ATATCCT-3′ (12585);
primer 2, 5′-GAGTTCAAACCGGCGTAAGCCAGGT-3′ (12854);
primer 3, 5′-GGACTATATCATGAATGAAATCAAGG-3′ (5945);
and primer 4, 5′-GCTTATATTTAGAGTATGACACTGAA-3′ (6385)

Primer 1 was based on the large rRNA sequences for mosquito (HsuChen et al. 1984), locust (Uhlenbusch et al. 1987), honey bee (Vlasak et al. 1987), and *D. yakuba.* Primer 2 was based on the ND1 gene for locust (McCracken et al. 1987) and *D. yakuba.* Leucine(CUN) is included between the above genes. This primer set was employed on crude DNA from individual black fly larvae of nine species. DNA was extracted by phenol-chloroform, followed by ethanol precipitation.

Primers 3 and 4 were designed to amplify the cluster of six tRNA genes. Primer 3 used mosquito (Dubin et al. 1986) and *D. yakuba* sequences for the ND3 gene, whereas primer 4 was the anticodon of phenylalanine in *D. yakuba* and *Ae. albopictus*. These primers were

used to amplify the region of interest from a cloned fragment and from purified mtDNA. All other tRNA sequences were from ends of cloned fragments.

Sequences were aligned with those of *D. yakuba* and *Ae. albopictus* (HsuChen et al. 1983a,b, 1984; HsuChen & Dubin 1984; Dubin et al. 1986). Alignment of *S. vittatum* and *D. yakuba* sequences was done with the aid of the Genetics Computer Group Sequence Analysis Software using the WORDSEARCH program to identify regions of similarity.

Phylogenetic analysis using parsimony (PAUP [Swofford 1989]) was used to compare tRNA leucine(CUN) and flanking regions in nine species of black flies with *Ae. albopictus*, *D. yakuba*, and *D. melanogaster* Meigen. *L. migratoria* was used as an outgroup. Shared gaps, inserted for alignment, were given equal weight. Analyses also were conducted in which gaps, if providing the only phylogenetically significant characters, were given a weight of 0.5 and nucleotide changes that would result in amino acid replacement were given a weight of 2.

Results

Sequences for the 10 tRNA genes in *S. vittatum*, folded into the inferred structures of the tRNAs, are shown in Figure 1. Figure 2 is our alignment with the same genes in *Ae. albopictus* and *D. yakuba*. Gaps have been introduced to improve alignment in the D- and TψC loops.

Similarity

In Table 1 nucleotide differences for each gene for the three insects are summarized. We have computed similarity based only on the stems plus anticodon and extra loops, which can be fully homologized, and also on the D- and TψC loops where alignments are more subjective. We used the alignment shown in Figure 2. Watson-Crick pairing was the rule in the arms, and a change in one arm usually was compensated by a matching change in the other arm. However, there were sufficient T-G pairings as well as mismatches, that we scored each nucleotide change as a difference.

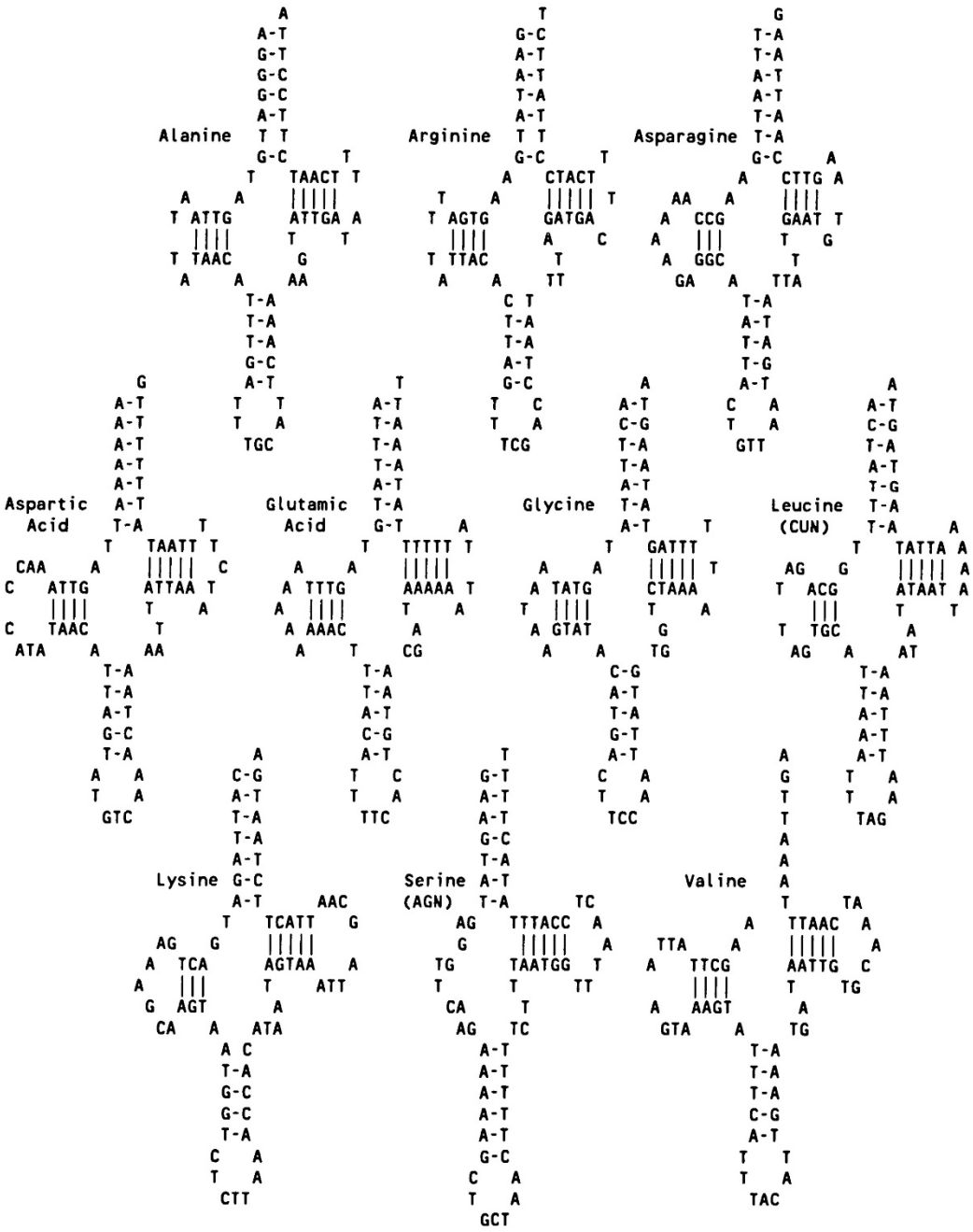

Figure 1. Mitochondrial tRNA genes of *S. vittatum* folded into inferred configurations of tRNAs.

```
     5' Arm      D-loop                  Anticodon          Extra     TψC Loop            3' Arm
__   _______  ______________________  __________________  _____  ____________________  ________
Alanine
S    AGGGATG TAGTTA  ATTA    TAACA  TTTGATTTGCATTCAAA  AAGT   ATTGA TATT---  TCAAT CTTCCTTA
A    ....TAA ......  ....    .....  ...A.......C.T...  ....   ..... AT..AAT  ..... T.A.....
D    ....T.. ......  ....    .....  .................  ....   ..... ATA.---  ..... ..A.....

Arginine
S    GAATATG AAGTGA  TTTA    TTACA  CTTAGTTTCGACCTAAT  TTTA   GATGA CTT      TCATC CTTATTCT
A    A...... ...C..  ....    ..G..  A................  C...   .G... AA.      ...C. .A....T.
D    ....... ...C..  ..A.    ..G..  G...............C  C...   .G.AT TA.      AT.C. ......T.

Asparagine
S    TTAATTG AAGCC   AAAAAGA  GGCA  TATTACTGTTAATGATA  TTATT  GAAT  GTAA--   GTTC CAATTAAG
A    ....... ..A..   .......  ..T.  ...C.............  .A...  ....  A.T.TT   A... ........
D    ....... .....   .......  ...G  ...C............   .A...  ..G.  A...--   AC.. ........

Aspartic Acid
S    AAAAAAT TAGTTA AACCCATA TAACA  TTAGTATGTCAAACTAA  AATT   ATTAA  ATCTT   TTAAT ATTTTTTG
A    ....... .....T ..T.A.-- A...C  .................  ..AA   ....G  ...A.   C.... .......A
D    ....... ...... ..TTATA- .....  .................  ....   .....  ..TA-   ..... .......A

Glutamic Acid
S    ATTTATG TAGTTT  AAAAA-  AAACT  TTACATTTTCACTGTAA   CGAT  AAAAA  ATTA-   TTTTT TATAAATT
A    ......A ......  ...T.-  ....A  .................   AA..  .....  T...-   ..... .......A
D    ......A ......  ....TA  ....C  ...........T.....   TA..  ....T  .A.TT   A.... ........

Glycine
S    ACTTATA TAGTAT  AATAA-  GTATA  CATGACTTCCAATTATG   TGGT  CTAAA   ATT-   TTTAG TATAAGTA
A    .T..... ......  .TA.TT  .....  TG...........C.CA   A..A  .....   TAAT   ..... .....A..
D    .TC.... ......  ..A.--  .....  TT...........C..A   A...  ...TT   .A.A   AA... ....GA..

Leucine (CUN)
S    ACTATTT TGGCA   GATTAG   TGCA  TTAAATTTAGAATTTAA   ATAT  ATAAT -TAAA-A  ATTAT AAGTAGTA
A    ....... .....   ......   ....  G...............T   T...  ..... T...TT.  ..... ..A.....
D    ....... .....   ......   ....  A...............T   ....  G.... T.--TT-  ....C ..A.....

Lysine
S    CATTAGA TGACT   GAAAGCA  .AGTA  ATGGTCTCTTAAACCAC   ATAAT AGTAA ATTAGCAA TTACT TCTAATGA
A    ....... .....   .......   ....  ...A.........T..T   .AT.. ..... .......C ..... ........
D    ....... .....   .......   ....  C...............T   T.T.. ..... .......C ..... ........

Serine (AGN)
S    GAAGTAT         GAGGTTCAAG      AAAAAGCTGCTAACTTTTT  TCTT TAATGG TTTAACT CCATTT ATACTTTT
A    ...A...         .TT.A.....      ...................  .... ...... .....T. ...... ...T..C.
D    .......         .GT.A.....      T..................  .... ...... ..A..T. ...... ......C.

Valine
S              AAGCTT   ATTAAGTA AAGTA TTTCATTTACATTGAAA   TGAT  AATTG TGCAAAT  CAATT TAAATTGA
A    CAATTTA ......   .A.T.... ..... .................   A..A  .T... .......  ..... ........
D    CAATTTA ......   ........ ...C. .................   A...  TT... .......  ....A ........
```

Figure 2. Nucleotide sequences of mitochondrial tRNA genes of *S. vittatum* (S) aligned with sequences of the same genes in *Ae. albopictus* (A) and *D. yakuba* (D). Dots (.) indicate identity with *S. vittatum*. Gaps (–) have been inserted to improve alignment.

Table 1. Substitutions, including deletions or insertions, in stems and loops of 10 mitochondrial tRNA genes in *S. vittatum* (S), *Ae. albopictus* (A), and *D. yakuba* (D)

Comparison	Ala	Arg	Asn	Asp	Glu	Gly	Leu	Lys	Ser	Val	Total
Stems (*N*)	57	57	53	57	57	57	55	57	48	50	578
S vs. A	8	9	5	8	5	9	5	5	3	3	60
S vs. D	2	12	6	1	8	13	5	4	2	5	58
A vs. D	6	8	5	7	7	9	5	5	3	4	59
Loops (*N*)	11	7	13	13	11	10	13	13	17	15	123
S vs. A	5	2	4	5	2	8	3	1	4	2	36
S vs. D	3	3	1	9	5	4	6	1	5	0	37
A vs. D	4	2	3	5	7	6	4	0	2	2	35

All five sequenced genes from the cluster of six genes had the same position and orientation as in *D. yakuba* and, because the primer set provided amplification, we assumed tRNA phenylalanine also was the terminal gene in the sequence. Other than order and orientation, the genes in this cluster were quite similar in the three insects. Of the other tRNA genes, that for glycine was least similar and that for valine was most similar. Although our sequence for valine is incomplete, the 3′ acceptor arm was identical to *Ae. albopictus* and *D. yakuba*, and we suspect the same was true of the 5′ end, where all three insects had a HindIII restriction site.

Stems and other fully homologizable positions in *S. vittatum* were 90% similar to either *Ae. albopictus* or *D. yakuba*; loops were 71 and 70% similar, respectively. Mean total similarity for any comparison was ≈86%. Transitions (56%) predominated in the stems, transversions (83%) in the loops (χ^2 = 32.74, df = 1, $P < 0.01$). Overall, transitions (56%) and transversions (44%) were nearly equal (χ^2 = 2.49, df = 1, $P > 0.1$). S. *vittatum* had a slightly (ns) greater G-C content (22.4%) than *Ae. albopictus* (20.0%) (χ^2 = 1.25, df = 1, $P > 0.25$); *D. yakuba* was 22.2% G-C. Conservation of the 5′ half of tRNA genes (89%) was higher than that of the 3′ half (83%) (χ^2 = 11.91, df = 1, $P < 0.01$); this is also true in mammals (Gadaleta et al. 1989).

Mispairings

Apparent mispairings of nucleotides in at least one insect were noted in 6 of the 10 genes. *D. yakuba* had mispairings in all six and *S. vittatum* had five mispairings in four genes, but *Ae. albopictus* had single mispairings in only two genes. Both *D. yakuba* and *S. vittatum* had T-T in acceptor arm of alanine, but *Ae. albopictus* was A-T. The same mispairing in the acceptor arm of arginine occurred in *D. yakuba* and *S. vittatum* with *Ae. albopictus* again T-A; additionally *S. vittatum* had a C-T mispairing in the anticodon arm where *Ae. albopictus* was A-T and *D. yakuba* was G-C. All three shared the T-T in TψC arm of serine(AGN); *D. yakuba* also had a T-T in anticodon arm but both *Ae. albopictus* and *S. vittatum* were A-T. Only *D. yakuba* had a T-T mispairing in anticodon arm of glycine; *S. vittatum* was A-T and *Ae. albopictus* was G-C. *D. yakuba* had C-T in anticodon arm of lysine, *S. vittatum* was A-C but *Ae. albopictus* had paired A-T. *D. yakuba* and *Ae. albopictus* had T-T in TψC arm of valine whereas *S. vittatum* had T-A.

Intergenic Regions

Intergenic regions for which we have sequence data are shown in Figure 3. Published data for these regions in *Ae. albopictus* are incomplete, and transposition of genes in the cluster of six genes precludes direct comparison. Neither *S. vittatum* nor *Ae. albopictus* had inserts that were as long as inserts in *D. yakuba*.

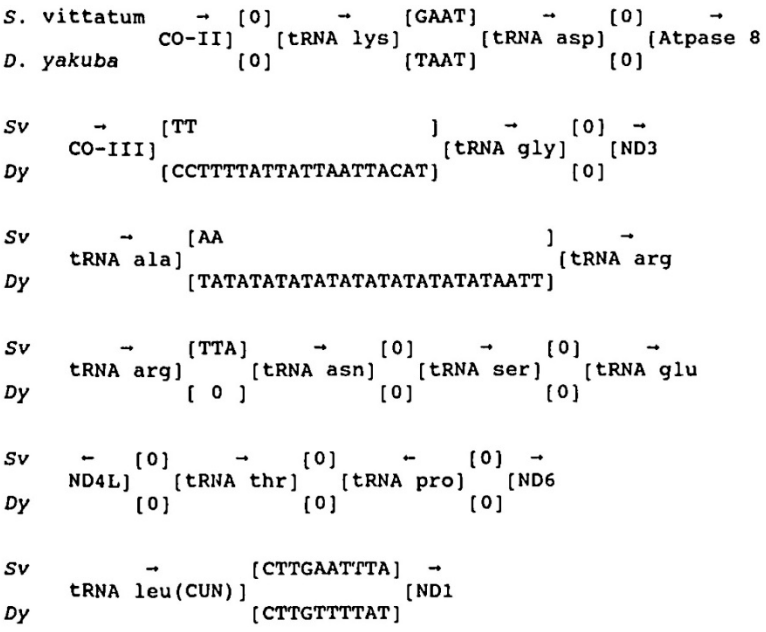

Figure 3. Intergenic nucleotides in *S. vittatum* (*Sv*) and *D. yakuba* (*Dy*). When two genes are contiguous without intervening nucleotides it is indicated with [0].

Black Fly Species Comparisons

The primer set that includes tRNA leucine was used to amplify and sequence ≈175 base pairs from nine species of black flies, corresponding to nucleotides 12600–12777 on the *D. yakuba* map. Results, including the flanking regions and published sequences from *D. yakuba*, *D. melanogaster* (de Bruijn 1983, Garesse 1988), *Ae. albopictus*, and *L. migratoria* are in Figure 4. Gaps in ND1 were from codon insertion or deletion in *L. migratoria* relative to *D. yakuba* (McCracken et al. 1987). Gaps in the 3′ end of the large ribosomal gene were more subjective. The tRNA leucine(CUN) gene was highly conserved, and had too few phylogenetically significant nucleotide differences to permit meaningful conclusions from it alone. Using *L. migratoria* as an outgroup, the entire sequence had 51 phylogenetically significant nucleotides, including shared gaps. Results (Fig. 5) were generally consistent with current opinions except for the clustering of *C. dacotensis* with *Simulium*. This was in contrast to the closer relationship of *Cnephia* and *Prosimulium* inferred from a ribosomal sequence by Xiong & Kocher (1991). Weighting or dropping shared gaps gave anomalous results.

```
                large rRNA →][tRNA leu(CUN) →
SV TAATACTATTAATA-TTT--T---ACTATTTTGGCAGATTAGTGCATTAAATTTAGAATTTAAATA
SJ .....T......---...-AT---.....................................
SL .....T......G--...-AT---.....................................
SN .....T......---...-AT---.....................................
SB .....T......A.A...------.....................................
ST .....T......---...-AT---.....................................
SA ............-.A..-------.....................C.................
CD A...........---...------.....................C.................
PO GTT..A.........-G.-A--AA-.............C......C.................
AA G...TA........-...AATAAA.....................G.................
DY G....T........-.A.AATAA-.....................A...............T...
DM G....T........-.--AA-A--.....................A...............T...
LM G.T..AA.......-...------.............A......A.G...........C.TT..

              tRNA leu(CUN) →][         ][ND1 →
SV TATAAT--TAAAAATTATAAGTAGTACTTGAATTTA---ATA---GAT---TTAATTTTTCCT
SJ ......--.............................---...---...---............
SL ......--.............................---...---...---............
SN ......--.............................---...---...---............
SB ......--.............................---...---...---............
ST ......--.............................---...---...---..G.........
SA ......--.............................---...---...---..G.........
CD ......-T..T..........................---...---...---..G.........
PO ......-T.T..........A................---...---...---...G.........
AA ......TTA.TT........A.....-...TT...T---...---...---.ATT.A..AT.A
DY .G....TT--TT-....C............TT..AT---...---..A---..T.....AT.A
DM .G.G..TT--TT-....C............TT..AT---...---..A---..T.....AT.A
LM .G..GATT.TTT-TC.AC..A.....-...------ATA...TAT...TTA..T..G...GT.

                                                              ND1 →
SV TTAGTTGGCAGATTGTTGTTAGTTATTTGTGTAATAGTTGGGGTTGCTTTTTTAACCTTGTTA
SJ ...A....T.....A..A.....................A..A...............A...
SL ...A....T.....A.......................A.................T..A...
SN ...A..........A..A.....................G..A..............T..A...
SB ...A....T..T..A..A.....A................................T..A...
ST ...A....T........A.....A........G........A............G..T..A...
SA ...A....T..T..A..A.....................A.....A...........T..A...
CD ...A....T.....A..ACTT..............G.....A..............T..A...
PO ...A....G.AT..A..A.....AG.......T.....................G..TC.TC.T
AA ...A....A..T..A..A.....AG.............A..A..A........G..T..A...
DY ...A....A..T..A..A...A...........T....AA.T..A..........T..A...
DM ...A....T..T..A..A...A...........T....AA.T..A..........T..A...
LM ....A.---TTT..A..A...A..........TT..A..A.A.............T..AA.G
```

Figure 4. Nucleotide sequence of tRNA leucine(CUN) and flanking regions in nine species of black flies aligned with the same region in *Ae. albopictus* (Aa), *D. yakuba* (Dy), *D. melanogaster* (Dm), and *L. migratoria* (Lm). Dots (.) indicate identity with *S. vittatum* (Sv). Gaps (–) have been inserted to improve alignment. Sj, *S. jenningsi* Malloch; St, *S. luggeri* Nicholson & Mickel; Sn, *S. nyssa* Stone & Snoddy; Sb, *S. bivittatum* Malloch; St, *S. tuberosum* (Lundstrom); Sa, *S. aureum* Fries; Cd, *Cnephia dacotensis* Dyar & Shannon; Po, *Prosimulium onychodactylum* Dyar & Shannon. This sequence corresponds with nucleotides 12600–12777 on the *D. yakuba* map but is written 5′→3′.

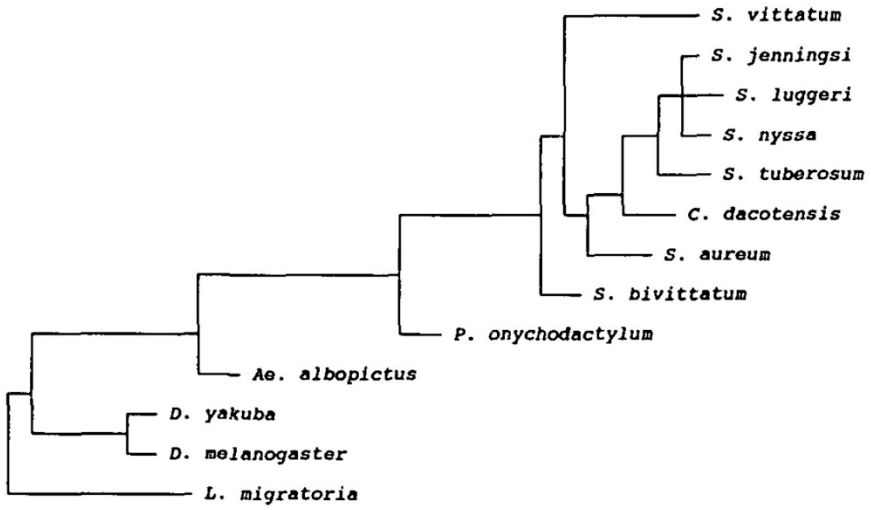

Figure 5. Phylogeny of Diptera computed using PAUP (Swofford 1989) from data in Figure 4 with *L. migratoria* as an outgroup. Branch lengths are drawn proportionally to the number of changes assigned to the branch (total $n = 117$).

Discussion

Based on tRNA comparisons, black flies and mosquitoes appear as divergent from each other as either is from *Drosophila*. Dubin et al. (1986) suggested that the rearrangement of a cluster of six tRNA genes in *Ae. albopictus* was caused by an inversion, analogous to the chromosomal inversions so frequent in Diptera. It is unknown whether this arrangement is present in other mosquitoes.

Serine (AGN) is unusual in that it lacks a defined D-stem. HsuChen & Dubin (1984) and Clary & Wolstenholme (1984) suggested a tertiary folding similar to that proposed for mammals (de Bruijn & Klug 1983) which also lack the D-loop. However, Dubin et al. (1984) proposed that a primitive stem is present in *Ae. albopictus*. Sequences subsequently obtained from an echinoderm (Cantatore et al. 1989) and nematode (Wolstenholme et al. 1987), both of which lack a stem, do not support this suggestion.

In *Drosophila* (Wolstenholme & Clary 1985a, Satta et al. 1987, Garesse 1988), greatest differences between species occur in the D- and TψC loops. This was also the case in our comparison of the three more distantly related lineages and made exact homologies difficult. Even closely related species may differ in the loops, but variation was less between related species of *Drosophila* than we found among the more distantly related Diptera lineages. Within black flies, the loops were identical in leucine(CUN) in all *Simulium*, but the TψC loop differed slightly in *Cnephia* and *Prosimulium*. The intergenic region between ND1 and leucine(CUN) was identical in all black flies but differed from *Ae. albopictus* and the two *Drosophila* species.

Our phylogeny was strongly influenced by a somewhat questionable alignment of nucleotides at the 3′ end of the large ribosomal gene. We have longer sequences from two black flies in which nucleotides 12808–12821 (*D. yakuba* map) are conserved fully as are the

same nucleotides in *D. yakuba*, *Ae. albopictus*, and *L. migratoria*. Nucleotides more 3′ to this region may provide excellent phylogenetic characters if they can be homologized with secondary structure models proposed for *Ae. albopictus* (HsuChen et al. 1984) and *D. yakuba* (Clary & Wolstenholme 1985b). Nucleotide substitutions that would result in amino acid replacement in ND1 often lacked phylogenetic significance because they were not shared with other species.

In total nucleotide similarity, *Ae. albopictus* was more similar to *D. yakuba* (86%) than to *P. onychodactylum* (80%), and *P. onychodactylum* and *D. yakuba* were least similar (75%). *L. migratoria* was a poor choice for use as an outgroup, but data are unavailable for a more reasonable sister group to the Diptera. Longer sequences, inclusion of more species within each group, better alignment at the 3′ end of large ribosomal through secondary structure models, and inclusion of one or more sister groups are prerequisites for use of this sequence in resolving relationships among the Diptera (Swofford & Olsen 1990).

Because mosquitoes and black flies are grouped in the more primitive Nematocera and have diverged over a long time period, we would not necessarily interpret our data as contradictory to the current classification. But the rearrangement of some tRNA genes, as well as the relatively large divergence, suggest that mosquitoes belong to a distinct lineage. Because tRNA genes are more highly conserved in sequence than protein genes, conventional estimates of time of divergence based on sequence divergence are not appropriate for this study. On the basis of a fossil pupa not distinguishable from modern *Prosimulium*, Crosskey (1991) suggests that origins of the Simuliidae go back at least to Lower Jurassic times.

Because of their medical and economic importance, and the sibling species problems that exist in black flies and mosquitoes, it would be desirable to develop universal primers to amplify regions that provide reliable species discrimination. This goal will not be easily achieved without comparative sequence data from numerous species within each group. Efforts are underway to expand the mitochondrial sequence data base for mosquitoes (Cockburn et al. 1990). Such data should also provide phylogenetic characters that are appropriate at different taxonomic levels.

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