Letter to the Editor

Increased Rate of Gene Rearrangement in the Mitochondrial Genomes of Three Orders of Hemipteroid Insects

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The mitochondrial (mt) genomes of most animals studied so far are circular, are about 16 kb in size, and have 13 protein-coding genes (*atp6* and *atp8* for ATP synthase subunits 6 and 8, *cox1–cox3* for cytochrome oxidase subunits 1–3, *cob* for cytochrome *b*, *nad1–nad6* and *nad4L* for NADH dehydrogenase subunits 1–6 and 4L), two rRNA genes (*rrnL* and *rrnS* for large and small rRNA subunits), 22 tRNA genes (one for each amino acid except for leucine and serine, which have two genes: $trnL_1$ [anticodon sequence tag], $trnL_2$ [taa], $trnS_1$ [gct], and $trnS_2$ [tga]), and one major noncoding region (Wolstenholme 1992). The arrangement of these 37 genes, especially the arrangement of the large genes that encode proteins and rRNAs, is usually conserved in a phylum but varies substantially among phyla (Boore and Brown 1998). However, the arrangement of protein-coding and rRNA genes has also been found to differ at lower taxonomic levels, e.g., in the family Ixodidae (Campbell and Barker 1998, 1999) and in the genus *Schistosoma* (Le et al. 2000).

The arrangement of genes in mt genomes has been studied in more insects than any other group of invertebrates. So far, 15 species of insects have had their mt genomes sequenced completely: eight flies (order Diptera; Clary and Wolstenholme 1985; Lewis, Farr, and Kaguni 1995; Ballard 2000; Lessinger et al. 2000; Spanos et al. 2000; Junqueira et al., unpublished but available in GenBank, accession number NC_002697); two mosquitoes (Diptera; Beard, Hamm, and Collins 1993; Mitchell, Cockburn, and Seawright 1993); the silkworm *Bombyx mori* (Lepidoptera; Lee et al., unpublished but available in GenBank, accession number AF149768); the honeybee *Apis mellifera* (Hymenoptera; Crozier and Crozier 1993); the locust *Locusta migratoria* (Orthoptera; Flook, Rowell, and Gellissen 1995); the bug *Triatoma dimidiata* (Hemiptera; Dotson and Beard 2001; accession number AF301594); and the wallaby louse *Heterodoxus macropus* (Phthiraptera; Shao, Campbell, and Barker 2001). Furthermore, part of the arrangement of mt genes is known for another 451 species of insects. The species of insects studied prior to this study were from 11 orders, but the vast majority were from four orders: Lepidoptera, Diptera, Hymenoptera, and Hemiptera. The first three orders are in the Endopterygota, whereas the Hemiptera is one of the four orders of the hemipteroid assemblage (Kristensen 1991; fig. 1). The arrangements of genes in the mt genomes of insects studied so far are very conserved since all species, except the wallaby louse, have the same arrangement of protein-coding and rRNA genes and most tRNA genes. Only the positions of a few tRNA genes differ, in particular, those in "hot spot" regions (Dowton and Austin 1999), e.g., near the control region, and in the two clusters of tRNA genes, *trnK-trnD* and *trnA-trnR-trnN-*$trnS_1$*-trnE-*<u>*trnF*</u> (the gene underlined here and those underlined elsewhere in this paper are transcribed from the minority strand; the two strands of the mt genomes of insects are differentiated as "majority strand" and "minority strand" according to the number of genes transcribed from them [Simon et al. 1994]). The most common arrangement of the 37 genes in the mt genome, which is present in the fruit fly *Drosophila yakuba,* the bug *T. dimidiata,* and many other species, is inferred to be ancestral for insects (Boore, Lavrov, and Brown 1998; Crease 1999).

The Hemiptera (bugs, cicadas, whiteflies, aphids, etc.), the Thysanoptera (thrips), the Psocoptera (psocids, book lice, and bark lice), and the Phthiraptera (lice) form the hemipteroid assemblage. In addition to the bug *T. dimidiata,* for which the entire gene arrangement of the mt genome is known, part of the arrangement of mt genes is now known for another 138 species of Hemiptera (GenBank accession numbers can be obtained by searching "Nucleotide" for "Hemiptera AND mitochondri* AND tRNA" on the NCBI web page [http://www.ncbi.nlm.nih.gov/entrez/]). These species represent all three putative major lineages of Hemiptera: Sternorrhyncha, Cicadomorpha, and Neohemiptera (Sorensen et al. 1995). In the lineage Sternorrhyncha, one species has the arrangement *A+T-rich region–trnI–*<u>*trnQ*</u>*–trnM*, 38 species have the arrangement *cox1-*$trnL_2$*-cox2*, and 64 species have the arrangement <u>*rrnL-trnV-rrnS*</u>. In the lineage Cicadomorpha, two species have the arrangement *cox2-trnK*, 16 species have the arrangement *trnD-atp8-atp6*, and 26 species have the arrangement *cob-*$trnS_2$*-*<u>*nad1*</u> (note that the direction of transcription of these $trnS_2$ genes is misannotated in the GenBank records L19837–64 and L19945). The arrangements <u>*rrnL-trnV-rrnS*</u>, *cox1-*$trnL_2$*-cox2,* and *cob-*$trnS_2$*-*<u>*nad1*</u> are very conserved in insects, whereas the *A+T-rich region–trnI–*<u>*trnQ*</u>*–trnM, cox2-trnK,* and *trnD-atp8-atp6* arrangements are in "hot spots" for gene rearrangements in insects (Dowton and Austin 1999). The bug *T. dimidiata,* from the lineage Neohemiptera, has been sequenced for the entire mt genome. This genome has the same gene arrangement that was found in the Sternorrhyncha

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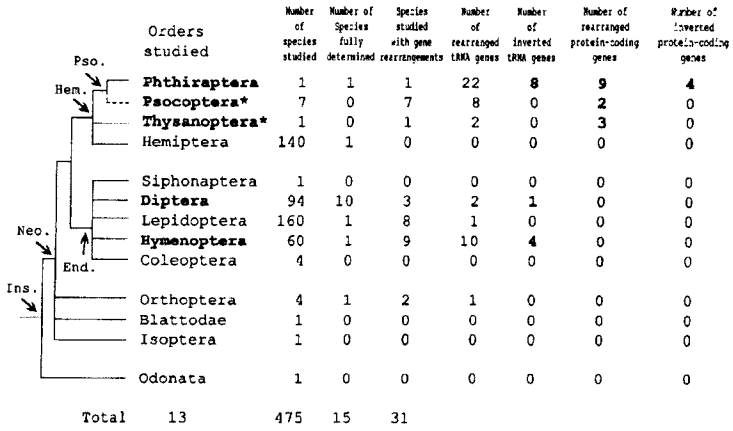

Orders studied	Number of species studied	Number of Species fully determined	Species studied with gene rearrangements	Number of rearranged tRNA genes	Number of inverted tRNA genes	Number of rearranged protein-coding genes	Number of inverted protein-coding genes
Phthiraptera	1	1	1	22	**8**	**9**	**4**
Psocoptera*	7	0	7	8	0	**2**	0
Thysanoptera*	1	0	1	2	0	**3**	0
Hemiptera	140	1	0	0	0	0	0
Siphonaptera	1	0	0	0	0	0	0
Diptera	94	10	3	2	**1**	0	0
Lepidoptera	160	1	8	1	0	0	0
Hymenoptera	60	1	9	10	**4**	0	0
Coleoptera	4	0	0	0	0	0	0
Orthoptera	4	1	2	1	0	0	0
Blattodae	1	0	0	0	0	0	0
Isoptera	1	0	0	0	0	0	0
Odonata	1	0	0	0	0	0	0
Total 13	475	15	31				

FIG. 1.—The 13 orders of insects from which all or part of the arrangement of genes in the mitochondrial genomes has been determined. The phylogenetic tree is that of Kristensen (1991). The orders of insects marked with asterisks are reported for the first time in this study. The orders that have species with gene inversions or rearrangements of protein-coding genes are in boldface type. The broken line leading to the Psocoptera indicates uncertainty about the monophyly of this order (see Lyal 1985). Gene arrangements of all insects studied are compared with that of *Drosophila yakuba,* which is inferred to be ancestral for insects. Gene rearrangements of four of the seven species of Psocoptera were suggested by PCR tests. Abbreviations are as follows: Pso., Psocodea; Hem., hemipteroid assemblage; End., Endopterygota; Neo., Neoptera; Ins., Insecta. The data shown here were summarized mainly from the Mitochondrial Gene Arrangement Source Guide, version 5.0 (available on J. Boore's web page [http://www.biology.lsa.umich.edu/~jboore]), except those for the Hemiptera, which we compiled from up-to-date records in GenBank (accession numbers can be obtained by searching "Nucleotide" for "Hemiptera AND mitochondri* AND tRNA" on the NCBI web page [http://www.ncbi.nlm.nih.gov/entrez/]).

and the Cicadomorpha. In summary, the gene arrangements in the mt genomes of all of the species of Hemiptera studied so far are the same as that of *D. yakuba,* which is thought to have the ancestral arrangement for insects; i.e., gene rearrangements have not been found in the order Hemiptera.

The wallaby louse *H. macropus* is the only species from the order Phthiraptera for which the gene arrangement in the mt genome has been studied. In stark contrast to all other insects studied, *H. macropus* has had many rearrangements of tRNA and protein-coding genes in the mt genome (Shao, Campbell, and Barker 2001). Prior to the present study, nothing was known about the arrangement of genes in the mt genomes of the other two orders of the hemipteroid assemblage, Thysanoptera and Psocoptera. We studied one species of Thysanoptera, seven species of Psocoptera, and another species of Hemiptera. We found that in addition to the numerous gene rearrangements in the mt genome of the wallaby louse, rearrangements of tRNA and protein-coding genes had also occurred in the Thysanoptera and the Psocoptera.

The species we studied were (1) *Tectocoris diophthalmus* (order Hemiptera, suborder Heteroptera, family Scutelleridae); (2) *Thrips imaginis* (Thysanoptera, Terebrantia, Thripidae); (3) *Pteroxanium insularum* (Psocoptera, Trogiomorpha, Lepidopsocidae); (4) an undescribed lepidopsocid species (Psocoptera, Trogiomorpha, Lepidopsocidae); (5) *Caecilius quercus* (Psocoptera, Psocomorpha, Caeciliusidae); (6) *Caecilius concavistigma* (Psocoptera, Psocomorpha, Caeciliusidae); (7) *Clematostigma lunulata* (Psocoptera, Psocomorpha, Psocidae); (8) *Hemipsocus chloroticus* (Psocoptera, Psocomorpha, Hemipsocidae); and (9) *Pseudoscottiella tanei* (Psocoptera, Psocomorpha, Pseudocaeciliidae). The cotton harlequin bug *T. diophthalmus* and the plague thrips *T. imaginis* were identified by Dianne Bell and Greg Daniels, respectively, Department of Zoology and Entomology, University of Queensland. *Pteroxanium insularum* was identified by Courtenay Smithers, Australian Museum, Sydney. One of us (E.R.S.) identified the six other psocopterans.

Live specimens were snap frozen in liquid nitrogen and stored at −70°C until DNA extraction. A CTAB method (Shahjahan et al. 1995) or a DNeasy Tissue Kit (QIAGEN) was used to extract total genomic DNA. Fragments of mtDNA were amplified by PCR with the following primers: (1) C3-J-5476 and N4-N-8924 (3.5 kb), and N5-J-7567 and SR-N-14594 (7 kb) for *T. diophthalmus*; (2) C1-J-1718 and C3-N-5460 (3.2 kb) for *T. imaginis*; (3) C3-J-5476 and C2-N-3133 (5′-GATCATGAAAAAAAATTATTTGTTC-3′) (616 bp) for *P. insularum*; (4) C1-J-2177 and C2-N-3661 (3.8 kb), and C2-J-3400 and N5-N-7736 (5′-TGTAAAATCTTATAATGCTGG-3′) (2.4 kb) for the lepidopsocid species; and (5) C3-J-5357 (5′-TTTATAGCAACAGGTTTTCATGGA-3′) and N5-N-7736 (1.8 kb), and N5-J-7567 and N4-N-8924 (2 kb) for *C. quercus* (only the sequences of primers reported here for the first time are shown; other primers and PCR conditions were reported in Shao, Campbell, and Barker 2001). C1-J-1718, C2-J-3400, C2-N-3661, C3-N-5460, and N4-N-8924 are conserved insect mt primers (Simon et al. 1994); other primers were designed from the sequences we gathered and sequences in GenBank. The sizes of PCR products were estimated by electrophoresis in 1% agarose gel stained with ethidium bromide; a single, clear band was viewed from each PCR product. The PCR products from the bug *T. diophthalmus* were of the size expected for the ancestral arrangement of insects, whereas those from

the thrips and the psocopterans were larger or smaller than expected. PCR products were purified with the QIAquick PCR Purification Kit and sequenced directly with the PCR primers and internal primers. An ABI Prism BigDye Terminator Kit was used for the sequencing reactions; sequencing products were precipitated and washed with sodium acetate and ethanol and resolved by an ABI 377 sequencer. The regions of the mt genomes reported here were sequenced from both strands for the thrips and three psocopterans, and mainly from one strand for the bug. Protein-coding and rRNA genes were identified by BLAST searches (Altschul et al. 1990) of GenBank. tRNA genes were identified by the program tRNAscan-SE (Lowe and Eddy 1997) or by eye. The nucleotide sequences are in GenBank under accession numbers AF335990–AF335997.

Sequence analyses confirmed that the above PCR products were amplified from authentic mt targets, since (1) the base composition and the codon use were consistent with that of characterized insect mt genomes (A+T content ranged from 72% to 79% and codons with C+G at third positions range from 6% to 17%); (2) the lengths of fully sequenced *cox2* and *nad3* were as expected for mt genes; and (3) no frameshifts were found in the protein-coding regions when nucleotides were translated into amino acids.

Fifteen genes were identified for the bug *T. diophthalmus* (fig. 2*A*). The arrangement of these genes is identical to the ancestral arrangement of insects. The thrips *T. imaginis* has the arrangement *cox1-nad3-trnL*$_2$*-cox2-trnG-trnK-cox3*. If *cox1, trnL*$_2$, *cox2, trnK,* and *cox3* are in their ancestral positions, then three protein-coding genes (*nad3, atp8,* and *atp6*) and two tRNA genes (*trnD* and *trnG*) have changed their positions in this thrips relative to the ancestral arrangement of insects.

We found two different gene arrangements in the psocopterans. First, *P. insularum* and the undescribed lepidopsocid species (Trogiomorpha, Lepidopsocidae) share the arrangement *cox3-trnR-trnS*$_1$*-trnE-trnS*$_2$*-trnI-trnM-trnW-cox2*. Furthermore, the undescribed lepidopsocid species has *trnG-nad3* adjacent to the 3′ end of *cox2*. If *cox3, trnG,* and *nad3* are in their ancestral positions in this lepidopsocid species, then *cox2* and seven tRNA genes (*trnR, trnS*$_1$, *trnE, trnS*$_2$, *trnI, trnM,* and *trnW*) have changed their positions relative to the ancestral arrangement of insects. Second, *C. quercus* (Psocomorpha, Caeciliusidae) has the arrangement *cox3-trnG-trnA-trnR-trnF-nad5-nad3-trnN-trnE-trnS*$_1$*-trnH-nad4*. If *cox3, trnG, trnA, trnR, trnF, nad5, trnH,* and *nad4* are in their ancestral positions, then *nad3, trnN, trnE,* and *trnS*$_1$ have changed their positions in *C. quercus*. PCR tests with primers N5-J-7567 and N4-N-8924 or N5-J-7581 (5′-TGTGCACTCCTAGTTATAGCAGC-3′) and N4-N-8924 indicated that the other four species of Psocoptera, from four different families of the suborder Psocomorpha, most likely have these gene rearrangements too (fig. 2*B*, lanes 4–7).

The gene rearrangements in the thrips, the psocopterans, and the wallaby louse suggest that the rate of gene rearrangement has accelerated in the mt genomes of three of the four orders of the hemipteroid insects: Thysanoptera, Psocoptera, and Phthiraptera. Of the 319 species of endopterygote insects that have been studied, only 20 species were found to have rearrangements of tRNA genes, and none had rearrangements of protein-coding or rRNA genes. Rearrangements have not been found in the 140 species of Hemiptera studied. However, we found rearrangements of tRNA and protein-coding genes in all of the Thysanoptera, Psocoptera, and Phthiraptera sequenced, and our PCR tests suggest that four other species of Psocoptera probably have gene rearrangements too. In the order Psocoptera, species from two different suborders have different arrangements of tRNA and protein-coding genes. Note that gene rearrangements have occurred not only in the "hot spot" regions of insects, but also in regions which are thought to be extremely conserved in animals, e.g., the *rrnS-trnV-rrnL, nad4L-nad4-nad5,* and *cox1-cox2-atp8-atp6-cox3-nad3* regions.

Gene rearrangements in the mt genome provide great potential for the phylogenetic studies of the hemipteroid assemblage. Many phylogenetic relationships in the hemipteroid assemblage are contentious, e.g., whether or not the Psocoptera are monophyletic (Lyal 1985) and which group is the sister group of the Thysanoptera (see fig. 1). Some morphological features indicate a sister group relationship between the Thysanoptera and the Psocodea (Psocoptera plus Phthiraptera), whereas others indicate a sister group relationship between the Thysanoptera and the Hemiptera (Kristensen 1991). The most recent analysis of the phylogeny of insect orders, from 18S, 28S, and morphological data, placed the Thysanoptera as the sister group of the Psocodea (Whiting et al. 1997). It is intriguing that all of the Thysanoptera, Psocoptera, and Phthiraptera studied so far have had many gene rearrangements in their mt genomes, whereas the Hemiptera studied so far have the ancestral arrangement.

Why have the insects in the three hemipteroid orders had more gene rearrangements than other groups of insects? How have these rearrangements occurred? We cannot yet answer these questions. Dowton and Austin (1999) noticed that in the Hymenoptera all mt gene rearrangements occurred after the parasitic lifestyle was adopted. The correlation between frequency of gene rearrangement and parasitism is supported by the numerous gene rearrangements in the mt genome of the wallaby louse, which is an obligate ectoparasite. In the well-studied order Diptera, gene rearrangements have been found only in three species of mosquitoes, which are also ectoparasites. Furthermore, inversions of mt genes in insects have been found only in mosquitoes, the wallaby louse, and species of Hymenoptera that evolved after the parasitic lifestyle was adopted. On the other hand, however, rearrangements of tRNA and protein-coding genes have been found in free-living insects like the thrips and the psocopterans studied by us.

Unlike the gene rearrangements of the wallaby louse, in which both translocations and inversions have occurred, only translocations were found in the thrips and the psocopterans studied here. Tandem duplications of a region followed by deletions of redundant copies

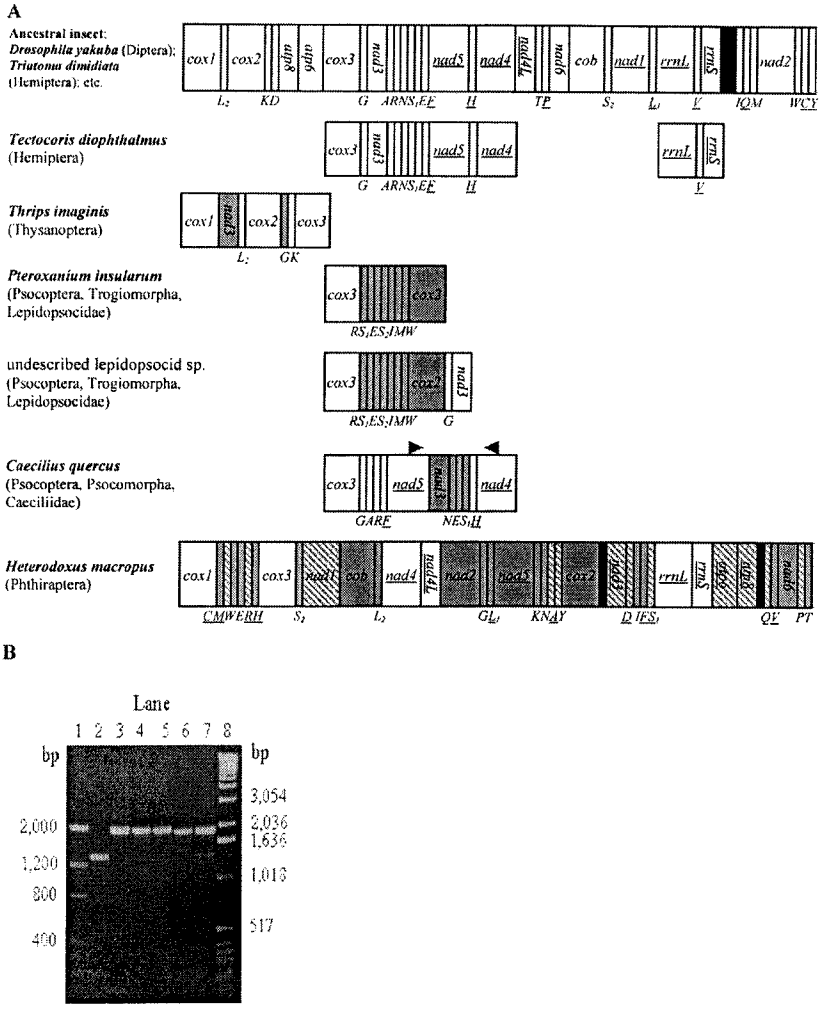

FIG. 2.—*A*, Linearized gene arrangements in the mitochondrial (mt) genomes of some insects of the hemipteroid assemblage. Genes are transcribed from the majority strand except those with names underlined, which are transcribed from the minority strand. The two strands of the mt genomes of insects are designated the majority strand and the minority strand according to the numbers of genes transcribed from them (Simon et al. 1994). tRNA genes are labeled with the single-letter abbreviations of their amino acids, except for those encoding leucine and serine, which are labeled as L_1 (anticodon sequence tag), L_2 (taa), S_1 (gct), and S_2 (tga). Abbreviations of protein-coding and rRNA genes are as follows: *atp6* and *atp8*, ATP synthase subunits 6 and 8; *cox1–cox3*, cytochrome oxidase subunits 1–3; *cob*, cytochrome *b*; *nad1–nad6* and *nad4L*, NADH dehydrogenase subunits 1–6 and 4L; *rrnL* and *rrnS*, large and small rRNA subunits. Genes that have moved from their ancestral positions are gray-shaded. Genes that have inverted, or moved and inverted, are diagonal-shaded. The black area indicates the A+T-rich region or the control region. The black triangles show the positions of primers used in the PCR tests for the gene rearrangements in the suborder Psocomorpha (Psocoptera). *B*, The PCR tests for the gene rearrangements in the suborder Psocomorpha (Psocoptera). Lane 1, Low DNA Mass Ladder marker (GibcoBRL); lane 2, the bug *Tectocoris diophthalmus* (Hemiptera), which has the ancestral arrangement *nad5-trnH-nad4* (the genes underlined are transcribed from the minority strand); lane 3, the psocopteran *Caecilius quercus* (Caeciliusidae), which has the arrangement *nad5-nad3-trnN-trnE-trnS*$_1$*-trnH-nad4*; lane 4, *Caecilius concavistigma* (Caeciliusidae); lane 5, *Clematostigma lunulata* (Psocidae); lane 6, *Hemipsocus chloroticus* (Hemipsocidae); lane 7, *Pseudoscottiella tanei* (Pseudocaeciliidae); lane 8, DNA Molecular Weight Marker X (Boehringer Mannheim). The fragment amplified by the two PCR test primers is about 1,400 bp for insects with the ancestral gene arrangement. Note that the PCR products are about the same size (~2,000 bp) for all five species of psocopterans from the suborder Psocomorpha (lanes 3–7). The PCR products and markers were resolved by electrophoresis in a 1% agarose gel stained with ethidium bromide.

of genes (Macey et al. 1997) is a plausible mechanism for the rearrangements found in the thrips and the psocopterans. However, it is not clear how the translocations and inversions of genes in the mt genome of the wallaby louse occurred. The mechanism of tandem duplication and deletion can hardly account for the drastic translocations of genes and is not applicable to the inversions of genes. Lunt and Hyman (1997) reported an intramitochondrial recombination in a nematode: there was double-strand breakage at both ends of a fragment in the major noncoding region, after which rejoining was indicated by the presence of a subgenomic minicircle about 250 bp long. This mechanism could account for small inversions, especially those that have occurred in the noncoding region. Whether or not fragments in a coding region, particularly large genes like *nad1* (894

bp) and *atp6* (657 bp) of the wallaby louse, could invert in this way awaits further studies. Comparisons between fully characterized mt genomes of a range of hemipterans, thrips, psocopterans, and lice should provide further insight into the mechanisms of gene rearrangement in animal mt genomes.

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