Evolutionary Dynamics of a Mitochondrial Rearrangement "Hot Spot" in the Hymenoptera

Mark Dowton†and Andy D. Austin**

*Department of Crop Protection, Adelaide University, Glen Osmond, Australia; and †Department of Biology, Wollongong University, Wollongong, New South Wales, Australia

The arrangement of tRNA genes at the junction of the cytochrome oxidase II and ATPase 8 genes was examined across a broad range of Hymenoptera. Seven distinct arrangements of tRNA genes were identified among a group of wasps that have diverged over the last 180 Myr (suborder Apocrita); many of the rearrangements represent evolutionarily independent events. Approximately equal proportions of local rearrangements, inversions, and translocations were observed, in contrast to vertebrate mitochondria, in which local rearrangements predominate. Surprisingly, homoplasy was evident among certain types of rearrangement; a reversal of the plesiomorphic gene order has arisen on three separate occasions in the Insecta, while the $tRNA^H$ gene has been translocated to this locus on two separate occasions. Phylogenetic analysis indicates that this gene translocation is real and is not an artifactual translocation resulting from the duplication of a resident tRNA gene followed by mutation of the anticodon. The nature of the intergenic sequences surrounding this region does not indicate that it should be especially prone to rearrangement; it does not generally have the tandem or inverted repeats that might facilitate this plasticity. Intriguingly, these findings are consistent with the view that during the evolution of the Hymenoptera, rearrangements increased at the same time that the rate of point mutations and compositional bias also increased. This association may direct investigations into mitochondrial genome plasticity in other invertebrate lineages.

Introduction

The arrangement of mitochondrial genes appears highly conserved when organisms within a phylum are compared (e.g., Jacobs et al. 1989; Boore et al. 1995). The relative positions of protein- and ribosomal RNA-coding genes show little variation, while those of tRNA genes show greater variation (e.g., Pääbo et al. 1991). For this reason, it has been proposed that tRNA gene rearrangements are ideal phylogenetic markers (e.g., Boore et al. 1995; Kumazawa and Nishida 1995; Macey et al. 1997), perhaps representing some of the few retained synapomorphies in the mitochondrial genome useful for retracing ancient evolutionary relationships.

However, determination of the arrangement of tRNA genes across a range of vertebrate mitochondrial genomes suggests that rearrangements occur more frequently in certain lineages. For example, fish and placental mammals have identical genome arrangements, while a number of rearrangements have been characterized for reptiles and birds (e.g., Desjardins and Morais 1990; Kumazawa and Nishida 1995; Quinn and Mindell 1996; Macey et al. 1997; Mindell, Sorenson, and Dimcheff 1998). These observations caution that gene order is not as invariable as previously considered. Characterization of rearrangements in lineages with more plastic gene orders is likely to reveal aspects of the rearrangement mechanism, an important corollary to the appropriate assessment/analysis of rearrangements as phylogenetic characters. For example, a detailed description of the rearrangement mechanism would help assess claims for the extremely low likelihood of homoplastic rearrangement events (Boore et al. 1995). For vertebrates, such assessments have led to the conclusion that rearrangements of tRNA genes are unlikely to evolve in parallel (but see Mindell, Sorenson, and Dimcheff 1998), but that displacement of the origin of light-strand replication (O_L) is prone to homoplasy (Macey et al. 1997).

The rearrangement mechanism has been investigated across a range of vertebrates. These studies suggest that the majority of vertebrate tRNA rearrangements can be explained by a slipped-strand mispairing mechanism (e.g., Pääbo et al. 1991; Macey et al. 1997), wherein slippage of the origin of replication downstream (usually to a tRNA gene; Moritz, Dowling, and Brown 1987) leads to tandem duplication of the intervening sequence. Duplicated genes are then randomly deleted, leading to reorganization of the mitochondrial genome (Moritz, Dowling, and Brown 1987). Such a mechanism may even produce translocations, provided the slippage is to a remote mitochondrial locus (Kumazawa and Nishida 1995).

A corresponding examination of the evolutionary dynamics of invertebrate gene rearrangements has not previously been reported. The dynamics may well be fundamentally different between the two groups. In vertebrates, rearrangements frequently occur close to origins of replication, particularly the O_L (e.g., Pääbo et al. 1991; Macey et al. 1997). However, a region analogous to the O_L is difficult to identify in invertebrates (Clary and Wolstenholme 1984). Furthermore, inversions are rare in vertebrates but are apparent in invertebrates (Jacobs et al. 1989; Crozier and Crozier 1993), making the mechanistic description necessarily more complex. To date, variation in invertebrate gene order has only been reported among relatively distantly related taxa (e.g., Jacobs et al. 1989; Smith et al. 1993; Boore et al. 1995; Boore, Lavrov, and Brown 1998), making inference about the rearrangement mechanism difficult; mechanistic relics of the rearrangement event are likely to have

Key words: transfer RNA, gene organization, invertebrate, mitochondrial DNA, phylogeny, Hymenoptera, inversion, translocation.

Address for correspondence and reprints: Mark Dowton, Department of Biology, Wollongong University, Wollongong, New South Wales, 2522, Australia. E-mail: mark_dowton@uow.edu.au.

Mol. Biol. Evol. 16(2):298–309. 1999

been erased by the long intervening periods of evolution. In the present study, we sequenced a single mitochondrial locus in a broad range of Hymenoptera, surveying 12 of the approximately 18 currently recognized superfamilies. We characterize five apparently independent rearrangements that have occurred at this locus, two of which involve translocations of the $tRNA^H$ gene. These data indicate either that such rearrangements are more common than previously considered or that the Hymenoptera are particularly plastic. In either case, the hymenopteran mitochondrial genome promises to be an excellent model for examining the rearrangement mechanism in invertebrate taxa.

Materials and Methods

DNA Amplification and Sequence Generation

Sequences were generated from the taxa listed in table 1 as previously described (Dowton and Austin 1994*a*). Taxa were identified by A. D. Austin; collection locations can be obtained from the authors on request. Genomic DNA was extracted from insects stored in 100% ethanol. Legs were used for larger wasps (>2 mm long), while the abdomen or thorax was used for smaller wasps (<2 mm long). The head region was excluded, as it frequently contained PCR inhibitors. Ethanol was removed by washing three times (15 min each) in 10 mM Tris-HCl (pH 8) containing 100 mM NaCl and 1 mM $MgCl_2$. Tissue was homogenized in 400 μl of 10 mM Tris-HCl (pH 8), 10 mM EDTA, and 1% SDS containing 100 μg of proteinase K (Boehringer Mannheim) and incubated overnight at room temperature. The homogenate was then extracted with phenol, and genomic DNA was precipitated with 2 volumes of ethanol as described in Sambrook, Fritsch, and Maniatis (1989). DNA was redissolved in 100 μl of sterile water and stored at 4°C. This DNA solution was used directly in the PCR reaction.

A portion of the mitochondrial genome was then amplified, containing the 3′ end of the cytochrome oxidase II (COII) gene, tRNA genes for aspartate (D), lysine (K), and occasionally histidine (H), and the 5′ end of the ATPase 8 gene. PCR reactions were carried out in 50 mM KCl containing 10 mM Tris-HCl (pH 9), 0.1% Triton X-100, 25 μM each dNTP, 1–6 mM $MgCl_2$, 0.4 μM each primer, and 0.75 U *Taq* DNA polymerase (Promega) in a total volume of 20 μl. The primers used were C2-J-4234 (alias MD.KDf; 5′-GCTAATCATAGTTTTATACC-3′) and A8-N-4447 (alias MD.KDr; 5′-CTTATAGGTACTATTTGAGG-3′). Two variants of each primer were synthesized, differing only in the identity of the nucleotide three bases from the 3′ end (G replacing A in C2-J-4234, T replacing A in A8-N-4447). Amplifications with each of the four possible primer combinations were then assessed to determine the appropriate primers for each taxon. The conditions of amplification were (1) 5 cycles of 1 min denaturing at 94°C, 1 min annealing at 45–55°C, and 1 min extension at 72°C, followed by (2) 30 cycles of 1 min denaturing at 94°C, 1 min annealing at 55°C, and 1 min extension at 72°C, followed by (3) 10 min extension at 72°C. Reaction conditions for each template were optimized with respect to $MgCl_2$ concentration, annealing temperature, and primer combination. Double-stranded PCR products were purified using Geneclean Spin Kits (Bio 101) to remove primers and unincorporated dNTPs prior to sequencing. Cycle sequencing reactions were performed with the ABI Prism Dye Terminator Cycle Sequencing Kit (Perkin Elmer) with AmpliTaq FS, using one of the PCR primers to initiate the sequencing reaction. Both strands of the PCR product were sequenced. In some cases, the sequencing reactions were optimized as described in Dowton and Austin (1994*b*).

Identification of tRNA Genes

tRNA genes were identified using tRNAscan-SE (version 1.1, http://genome.wustl.edu/eddy/tRNAscan-SE: Lowe and Eddy 1997). The default search mode was used (cove cutoff score = 15), specifying mitochondrial/chloroplast DNA as the source and using the invertebrate mitochondrial genetic code for tRNA isotype prediction. Where long tracts of noncoding sequence were apparent, the cove cutoff score was reduced to 10 and the search was repeated. This identified some tRNA genes with slightly lower cutoff scores (e.g., $tRNA^D$ from *Dolopsidea* had a score of 11.92, yet it retains all the conserved features of this gene identified from other taxa). In addition, noncoding regions were visually inspected for cloverleaf structures, but this strategy identified no tRNA genes additional to those identified with the strategy outlined above (i.e., using tRNAscan-SE).

Phylogenetic Analysis of $tRNA^D$ and $tRNA^H$ Genes

Unweighted parsimony analysis of $tRNA^H$ and braconid $tRNA^D$ genes was performed using PAUP (4.0d64). Loop regions were excluded from the analysis due to the difficulty in aligning these poorly conserved, length-variable regions. Furthermore, some braconid taxa were excluded from the analysis because they shared 100% homology with other included taxa when these loop regions were excluded. A chi-square test as implemented in Puzzle 4.0 found no evidence of nucleotide composition heterogeneity in these stem regions. Alternately, the *Xorides* (Ichneumonidae) $tRNA^D$ and the dipteran (*Drosophila* and *Anopheles*) $tRNA^H$ genes were used as outgroups. Bootstrapping was not performed, as search times were impractically long, presumably due to the low number of informative sites relative to the number of taxa. A maximum-likelihood analysis was also performed with Puzzle 4.0 (Strimmer and von Haeseler 1996; Strimmer, Goldman, and von Haeseler 1997), using the HKY model of nucleotide substitution (Hasegawa, Kishino, and Yano 1985), with transition/transversion and nucleotide composition parameters estimated from the data. Analyses were performed (1) assuming a uniform rate of evolution at all sites and (2) estimating the gamma distribution rate parameter alpha from the data set with eight rate categories. The relative support for alternate topologies was assessed using the Kishino-Hasegawa test (Kishino and Hasegawa 1989), as implemented in Puzzle 4.0.

Table 1
Genetic Characteristics of the Junction Between the Cytochrome Oxidase II and ATPase 8 Genes for Various Hymenopteran Taxa

		COII—A—tRNA1—B—tRNA2—C—tRNA3—D—ATPase 8					
Taxonomic Affiliation	Taxon	COII (aas; stop codon)	**A** nt	**B** nt	**C** nt	**D** nt	Arrangement ($tRNA^K$ anticodon)
Symphyta							
Xyeloidea							
Macroxyelidae	*Macroxyela ferruginea* (Say)	22 (TAA)	+44	−1 (A)	0	NA	KD (CTT)
Tenthredinoidea							
Pergidae	*Perga condei* (Benson)	18 (TAA)	+18	0	0	NA	KD (CTT)
Orussoidea							
Orrussidae	*Orussus terminalis* (Newman)	21 (TAA)	+1	−2 (T)	0	NA	KD (CTT)
Apocrita							
Ceraphronoidea							
Megaspilidae	*Conostigmus* sp.	22 (TAA)	+4	−1 (A)	+1	NA	KD (CTT)
Proctotrupoidea							
Proctotrupidae	*Disogmus areolator* (Say)	19 (TAA)	+10	−1 (A)	0	NA	KD (TTT)
Cynipoidea							
Figitidae	*Anacharis zealandica* Ashmead	53 (TAA)	+50	−1 (A)	NA	NA	D
Apoidea							
Apidae	*Apis mellifera* (L.)	19 (T)	0	+7	+5	NA	DK (TTT)
	Bombus terrestris (L.)	20 (T)	0	+2	0	NA	DK (TTT)
	Melissodes sp.	24 (TAA)	+35	+5	+5	NA	DK (TTT)
Anthophoridae	*Xylocopa virginica* (L.)	18 (TA)	0	+3	+4	NA	DK (TTT)
Chrysidoidea							
Bethylidae	Not determined	18 (TAA)	+1	−1 (A)	0	NA	KD (TTT)
Vespoidea							
Formicidae	*Myrmecia forficata* (F.)	19 (TAA)	+48	+2	0	NA	KD (TTT)
Chalcidoidea							
Aphelinidae	*Encarsia formosa* (Gahan)	18 (TAA)	+1	+4	+23	NA	**K**D (TTT)
Platygastroidea							
Platygastridae	*Amitus* sp.	18 (TAA)	+1	NA	0	NA	K (TTT)
Scelionidae	*Scelio fulgidus* (Crawford)	18 (T)	0	+8	0	NA	DK (CTT)
	Idris sp.	18 (TAA)	+2	+2	0	NA	DK (CTT)
	Gryon sp.	18 (TAA)	+6	−3 (TAT)	0	NA	DK (CTT)
	Trissolcus basalis (Wollaston)	17 (TAA)	+2	−3 (CAT)	0	NA	DK (CTT)
	Macroteleia sp.	19 (TAA)	+2	−3 (CAT)	+1	NA	DK (CTT)
Ichneumonoidea							
Ichneumonidae	*Xorides praecatorius* (F.)	17 (TAA)	+15	0	+1	NA	KD (CTT)
Braconidae	*Cenocoelius* sp.	18 (TAA)	+138	+2	−1 (A)	NA	KD (TTT)
	Centistes sp.	19 (TAA)	+22	−2 (AA)	0	NA	KD (TTT)
	Diospilus sp.	19 (T)	0	−2 (AA)	0	NA	KD (TTT)
	Blacus sp.	18 (TAA)	+3	0	0	NA	KD (TTT)
	Agathiella sp.	18 (TAA)	0	−1 (A)	0	NA	KD (TTT)
	Ichneutes bicolor Cresson	19 (TAA)	+5	0	−9	NA	KD (TTT)
	Aphidius rosae Haliday	19 (TAA)	+4	−1 (A)	+1	NA	KD (TTT)
(Microgastroids)	*Cardiochiles fascipennis* Szépligeti	21 (TAG)	+4	−2 (AA)		NA	KD (TTT)
	Mirax sp.	20 (TAA)	+1	−2 (AA)	0	NA	KD (TTT)
	Microplitis demolitor Wilkinson	19 (T)	0	+7	0	+8	HDK (TTT)
	Sathon sp.	20 (TAA)	+2	−5 (AGA^3)	−3 (TAT)	+17	HDK (TTT)
(Cyclostomes)	*Jarra phorocantha* Marsh and Austin	19 (TAA)	0	−2 (TA)	0	NA	KD (TTT)
	Heterospilus sp.	19 (TAA)	+2	+8	0	NA	KD (TTT)
	Dolopsidea sp.	19 (TAA)	0	0	+99	NA	**D**K (TTT)
	Histeromerus mystacinus Wesmael	19 (TAA)	−4 (↓)	+4	+45	NA	**D**K (TTT)
	Habrobracon hebetor (Say)	18 (TAA)	−1 (↓)	0	+7	+23	**D**HK (TTT)
	Aleiodes sp.	18 (TAG)	−4 (↓)	+7	0	+6	**D**HK (TTT)
	Spinaria sp.	21 (TAG)	−4 (↓)	+1	+3	+25	**D**HK (TTT)
	Gnamptodon pumilio (Nees)	19 (TAA)	−1 (↓)	+6	+12	+38	**D**HK (TTT)
	Betylobracon waterhousi Tobias	17 (TAA)	−1 (↓)	+7	0	+24	**D**HK (TTT)
	Opius kraussi (Fullaway)	18 (TAA)	−3 (↓)	−1 (A)	+1	+1	**D**HK (TTT)
	Aspilota sp.	18 (TAA)	+19 (↓)	0	−1 (T)	+5	**D**HK (TTT)

Results

Sequence data were obtained from the junctions of the COII and ATPase 8 genes of 42 hymenopteran taxa (table 1). In each case, the 5′ region of the sequenced product showed homology to previously sequenced dipteran (*Drosophila* and *Anopheles*) and hymenopteran COII genes (*Apis* and *Exeristes*; fig. 1). Although the primary homology of the carboxy-terminal region of the COII gene is low among the genera compared, the first three amino acids are hydrophobic residues (I, V, L, M, S, or, rarely, A), the fourth residue is always E, and the 14th residue is an aromatic residue, predominantly W, occasionally F. The length of the carboxy-terminal region is relatively conserved (17–22 amino acid residues among the taxa sequenced in the present study). Although the sequence for *Anacharis* may represent a pseudogene, given its uncharacteristic length (53 residues), it retains all of the conserved features exhibited by the other genera: the first three residues are hydrophobic, the fourth residue is E, and the 14th is W. We conservatively defer judgment regarding the authenticity of the *Anacharis* sequence until additional cynipoid genera are sequenced. The additional length (ca. 23 amino acid residues) corresponds approximately to a tRNA gene (60–70 nt) which might conceivably have been incorporated into the carboxy-terminal region of the COII gene (see Cantatore et al. 1987).

The ATPase 8 primer anneals to this gene immediately 5′ to the start codon, and all sequences contained a start codon (ATN: Crozier and Crozier 1993) immediately 3′ to the primer sequence. Furthermore, the identified tRNA genes in each case could be folded into cloverleaf structures and exhibited conserved features (fig. 2). Taken together, the above evidence suggests that it is unlikely that the reported sequences (excluding perhaps *Anacharis*) represent nuclear or mitochondrial pseudogenes.

Evidence for Multiple Independent Rearrangements

Initially, our purpose was to trace the hymenopteran rearrangement concerning the genes for $tRNA^D$ and $tRNA^K$, evident after Crozier and Crozier (1993) sequenced the entire mitochondrial genome of *Apis.* A similar study was undertaken by Flook, Rowell, and Gellissen (1995*b*) after they sequenced the complete mitochondrial genome of the orthopteran *Locusta* (Flook, Rowell, and Gellissen 1995*a*). These latter authors found that a rearrangement was evident in genera closely related to *Locusta* but not in more basally derived orthopterans (Flook, Rowell, and Gellissen 1995*b*). Apparently, a single rearrangement has occurred at this locus during the evolution of the Orthoptera.

However, our data indicated that a variety of gene arrangements are present at this hymenopteran locus (table 1). Among the 42 hymenopteran genera sequenced, we observed 7 distinct arrangements (ignoring *Anacharis*), 5 of which have not been previously observed for any other animal taxon. Phylogenetic mapping of these arrangements suggests that five of these resulted from independent rearrangement events (fig. 3).

The plesiomorphic arrangement for tRNA genes at this locus is almost certainly KD throughout the Hymenoptera (fig. 3), with both genes encoded on the mitochondrial J-strand (nomenclature after Simon et al. 1994). This arrangement is evident throughout the basally derived members of the order (suborder Symphyta) and in various superfamilies of the suborder Apocrita (such as the Ceraphronoidea, Ichneumonoidea, Chrysidoidea, and Vespoidea). It is also the arrangement in the closely related insect order Diptera (Clary and Wolstenholme 1985*a*; Beard, Hamm, and Collins 1993; Mitchell, Cockburn, and Seawright 1993), in members of basally derived Orthoptera (Flook, Rowell, and Gellissen 1995*b*), and in the crustacean *Artemia* (Perez et al. 1994).

The presence of both the plesiomorphic (KD) and derived arrangements in various monophyletic hymenopteran groups indicates that the rearrangements are independently derived (fig. 3). For example, the Aculeata are a monophyletic assemblage of three superfamilies (Apoidea, Chrysidoidea, and Vespoidea; Brothers and Carpenter 1993). The retention of the plesiomorphic arrangement in representatives from two of these superfamilies (Chrysidoidea and Vespoidea) indicates that the arrangement in *Apis, Bombus, Melissodes,* and *Xylocopa* occurred during the evolution of the Apoidea.

Similarly, both the microgastroid and cyclostome complexes of braconid subfamilies are monophyletic based on both morphological (Quicke and van Achterberg 1990; Wharton et al. 1992) and molecular (Belshaw and Quicke 1997; Belshaw et al. 1998; Dowton, Austin, and Antolin 1998) evidence. Both complexes contain members with the plesiomorphic and derived arrangements, indicating two independent rearrangement events during the evolution of the Braconidae. Within the cyclostomes, two putatively phylogenetically related arrangements are observed (DK and DHK). The monophyly of the taxa that have either of these arrangements is broadly consistent with recent molecular analyses (Belshaw et al. 1998; Dowton, Austin, and Antolin 1998), suggesting that the two arrangements are phylogenetically related. The arrangements differ only by the translocation of $tRNA^H$ into this locus. The different arrangements may thus represent two independent events

←

NOTE.—"aas; stop codon" refers to the number of amino acid residues in the cytochrome oxidase II gene between the primer-binding site and the carboxy terminus, followed by the mitochondrially encoded stop codon in parentheses; (TAA) or (TAG) indicates that complete stop codons can be transcribed directly from the mitochondrial template; (T) or (TA) indicates that a complete stop codon can be produced only after polyadenylation of the transcription product. "A nt" refers to the number of nucleotides between the cytochrome oxidase II gene and the tRNA gene nearest to it, and "B nt" refers to the number of nucleotides between that tRNA gene and the following one, according to the scheme above the column titles. Where numbers are negative, the overlap is indicated in parentheses; "NA" means not applicable. In the "Arrangement" column, lightface letters indicate that the tRNA gene is transcribed from the J-strand, and boldface letters indicate that transcription is from the N-strand. Monophyletic groups are boxed, and independent rearrangements are underlined.

```
Drosophila m.   IVIESVPVNYFIKWISSNNS--------------------------------
Anopheles g.    .....I.M.......T.MTN--------------------------------
Macroxyela      ..V.A..FEIYL..MFEELGVY------------------------------
Perga           ..V.?.FKKFIL..TNM.----------------------------------
Orussus         ..V...KYCK.....N.MLIL--------------------------------
Conostigmus     .S...IKNKN.MNFKNI..KIM-------------------------------
Apis m.         .M...TSFQ..LN.VNKQI----------------------------------
Apis k.         .MV..TSFKF.LN..NKQ.----------------------------------
Bombus          .ML..TSYEM.LN..KKKMMWKIS-----------------------------
Melissodes      .ML..NSIRG.LI.VCYMLKNINF-----------------------------
Xylocopa        ..L..TSYDL..N.MKNKY----------------------------------
Bethylid        .SL.ATSLEN.LN..FNL-----------------------------------
Myrmecia        ....YTNIKI.KN..K.FL----------------------------------
Encarsia        .....TSLVN.LD..KK.-----------------------------------
Amitus          ..V.VTSL.N.L..TNKF-----------------------------------
Scelio          ..M.FISI.N.....KN.-----------------------------------
Idris           ..A.L.SKPL.N..LMNK-----------------------------------
Gryon           .....LTN.L.SI.MKNF-----------------------------------
Trissolcus      .I...INK.K.F...IL------------------------------------
Macroteleia     .M...MLTKN.Y.F.MK.I----------------------------------
Disogmus        .M...TSMTN..N.MKMIK----------------------------------
Anacharis       .M..ATNL..CLS.VNTM.INWSNTTKTSNTKWKMMIKINQLKAPRLMIPYLK
Xorides         .....TSIKN.MN.LKI------------------------------------
Exeristes       .....TSN.N..S.LK.L-----------------------------------
Cenocoelius     ..L...FLDD.FF..TEV-----------------------------------
Centistes       ..L...NIKN.LG.V.KYC----------------------------------
Diospilus       ..L..IKLKN.LI.LKNFK----------------------------------
Blacus          ..L.VIEL.K.FD.LINY-----------------------------------
Agathiella      ..L..IELKK.MN..INF-----------------------------------
Ichneutes       ..L...D.KFYLN.LNNL.----------------------------------
Aphidius        ..L..TNLLF.FN.VKNFE----------------------------------
Cardiochiles    ..L...MWDV.LH.MK.HKKH--------------------------------
Mirax           ..L.IINLDL.LN.LKVMLN---------------------------------
Microplitis     ..L.C.NMKN.LN..I...----------------------------------
Sathon          ..L...KLMN.LN..KL.FN---------------------------------
Jarra           ..L..TSL.S.FN.LKMF.----------------------------------
Heterospilus    ..P...MSLD.FN.LYNF.----------------------------------
Dolopsidea      ..L..TKLYM..E.LKKWF----------------------------------
Histeromerus    ..L..TKLEV.LN.LKLWI----------------------------------
Habrobracon     ..L..IKL.F..E.LKNF-----------------------------------
Aleiodes        .....INMK..L..LK.F-----------------------------------
Spinaria        ..L...DFLMYLN.VMNYYYD--------------------------------
Gnamptodon      ..L...NL.L..N.LKL.F----------------------------------
Betylobracon    ..L..TYL.L.VD.LKL------------------------------------
Opius           ..L...NFDI.LF.LKNF-----------------------------------
Aspilota        ..L...SLKI.MI.LKNF-----------------------------------
```

FIG. 1.—Manual alignment of hymenopteran and dipteran COII amino acid sequences between the primer-binding site of C2-J-4234 and the carboxy-terminus. Identity with the *Drosophila* sequence is indicated by a period. Sequences for some taxa were obtained from the literature: *Drosophila melanogaster* (GenBank U37541; Clary and Wolstenholme 1985*b*), *Anopheles gambiae* (GenBank L20934; Beard, Hamm, and Collins 1993), *Apis mellifera* (GenBank L06178; Crozier and Crozier 1993), *Apis koschevnikovi* (GenBank M77212; Willis, Winston, and Honda 1992), and *Exeristes roborator* (GenBank M83969; Liu and Beckenbach 1992). All other sequences were generated in M.D.'s laboratory. The *Anacharis* sequence may be a pseudogene, considering its uncharacteristic length.

(rearrangement followed by translocation in a subset of cyclostomes) or a simultaneous rearrangement/translocation with subsequent loss of $tRNA^H$ either from its original position (in the DHK taxa) or from its transposed position (DK taxa).

With respect to the microgastroid rearrangement, the only microgastroids with the derived arrangement were from the Microgastrinae, suggesting that the rearrangement occurred during the evolution of this subfamily. Representatives from putative sister groups to the Microgastrinae (Miracinae: Whitfield and Mason 1994; Cardiochilinae: Dowton and Austin 1998) retained the plesiomorphic arrangement.

The remaining two arrangements concern the Platygastroidea and Chalcidoidea. Although these two superfamilies have been tentatively identified as sister groups by both morphological (Rasnitsyn 1988) and molecular (Dowton and Austin 1994*a*; Dowton et al. 1997) analyses, subsequent cladistic analysis of the morphological data (Ronquist et al., unpublished data) fail to recover this relationship, while exclusion of alignment-ambiguous positions from the molecular analysis results in their relationships remaining unresolved (Dowton et al. 1997). Given the very different nature of the arrangements observed in the Chalcidoidea and the Platygastroidea (table 1), we consider these to represent two inde-

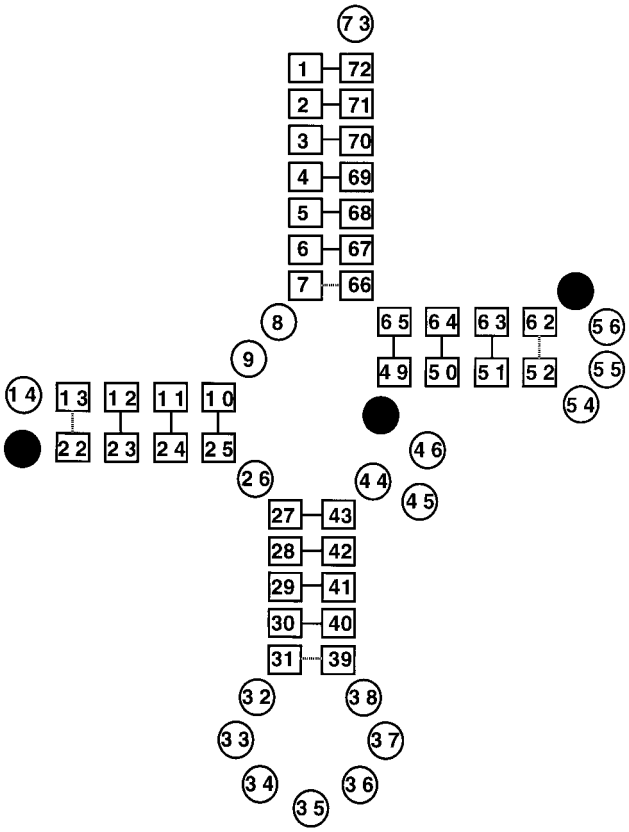

FIG. 2.—Conserved secondary structures of $tRNA^K$, $tRNA^D$, and $tRNA^H$ genes from hymenopteran taxa. Boxes indicate stem positions, open circles indicate loop positions, and closed circles indicate length-variable regions. The dashed lines between some stem positions indicate that base-pairing is not maintained for all taxa.

pendent events. The arrangement observed in the chalcidoid is the only example in which the plesiomorphic gene order is not reversed, the only change being that the $tRNA^K$ gene is now encoded on the N-strand, whereas it is encoded on the J-strand in all other Hymenoptera.

All scelionids sampled had DK arrangements, homoplastic with both the *Apis* (Crozier and Crozier 1993) and *Locusta* (Flook, Rowell, and Gellissen 1995*a*) arrangements, indicating that this arrangement has independently evolved on three occasions during the evolution of the insects. The arrangement observed in the platygastrid (K) may not represent an independent evolutionary event, given that platygastrids and scelionids are generally considered sister groups, and no sampled members of either family retained the plesiomorphic arrangement. For this reason, we conservatively count these two arrangements as representing just one of the five rearrangement events calculated for the Hymenoptera.

Translocations of $tRNA^H$: Real or Apparent?

Our data indicated that during the evolution of the Braconidae, the $tRNA^H$ gene was translocated to this gene junction on two separate occasions. However, it is possible that these translocations are only apparent; the anticodons for $tRNA^D$ and $tRNA^H$ differ by only a single point mutation (GTC and GTG, respectively, cf. $tRNA^K$, which can be either CTT or TTT), such that duplication of a braconid $tRNA^D$ gene followed by a single point mutation in one of the copies could conceivably produce a $tRNA^H$ gene at this locus. To investigate this possibility, we performed phylogenetic analyses of $tRNA^H$ and braconid $tRNA^D$ genes using the relatively con-

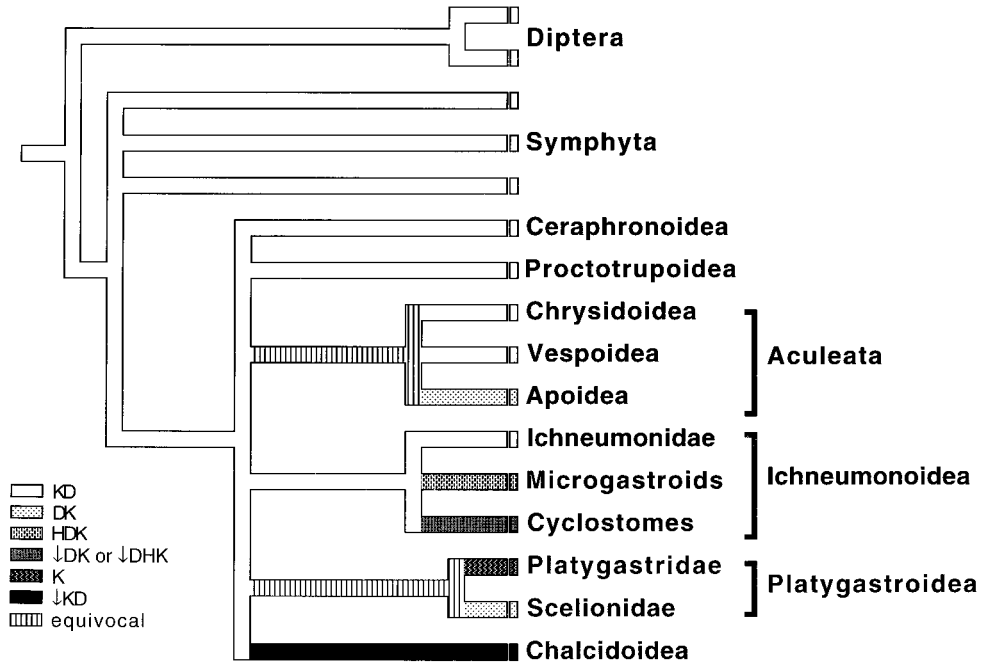

FIG. 3.—Independent tRNA gene rearrangements in the Hymenoptera. Unshaded branches indicate that taxa have the plesiomorphic (KD) arrangement; variously shaded branches indicate that taxa in a group have derived arrangements. The tree is broadly resolved, with the polytomy for the Apocrita indicating that relationships among the superfamilies of this suborder remain speculative; the conclusions for independence do not rely on the phylogenetic resolution of the Apocrita.

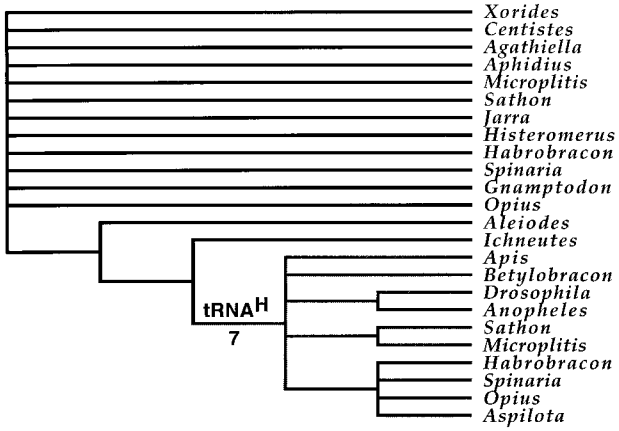

FIG. 4.—Phylogenetic analysis of $tRNA^D$ genes (stem residues only) from the Braconidae (black branches) and all $tRNA^H$ genes from the Hymenoptera and Diptera (shaded branches). Unweighted parsimony analysis found 4,017 shortest trees of length 63 steps (rescaled consistency index [RCI] = 0.53; retention index = 0.86), the strict consensus of which resolved all $tRNA^H$ genes as having a single origin. The shortest tree that did not support this hypothesis was 7 steps longer. The $tRNA^D$ gene from *Xorides* (Ichneumonidae) was used as the outgroup. Alternatively, if the $tRNA^H$ genes from the dipteran representatives were specified as the outgroup, approximately the same number (4,006) of shortest trees were recovered (length = 63 steps), the strict consensus of which resolved all $tRNA^D$ genes as having a single origin. The maximum-likelihood tree also resolved a monophyletic $tRNA^H$ gene (uniform rate model; log L = −332.74), with strong quartet puzzling reliability for this node (98%).

served stem residues of each (boxed residues in fig. 3). Bona fide translocations would be indicated by clustering of the nontranslocated and translocated $tRNA^H$ genes, while apparent translocations would be indicated by clustering of $tRNA^D$ and translocated $tRNA^H$ genes. Parsimony analyses (fig. 4) supported the hypothesis that the translocations are bona fide, with the data indicating a single origin for the translocated and nontranslocated $tRNA^H$ genes. The shortest tree that disrupted this monophyly was seven steps longer and contained just one $tRNA^D$ gene (from *Ichneutes*) clustered among the $tRNA^H$ genes. The maximum-likelihood tree (resolved by quartet puzzling) also recovered a monophyletic $tRNA^H$ gene (whether analyzed under a model of rate uniformity or one of heterogeneity). Ideally, we would have liked to determine the most likely topology in which nonmonophyly of the $tRNA^H$ genes was enforced (to determine if this topology was significantly worse than the monophyletic topology), but constraint searching is not possible with Puzzle 4.0. As an approximation to this test, we compared the maximum-likelihood tree with the set of parsimony trees in which nonmonophyly was enforced (i.e., those trees that were seven steps longer than the most-parsimonious trees, with the *Ichneutes* $tRNA^D$ gene clustering inside the $tRNA^H$ genes). The Kishino-Hasegawa test, as implemented in Puzzle 4.0, indicated that this latter topology was significantly worse than the maximum-likelihood tree.

Mutation of the $tRNA^K$ Anticodon

The $tRNA^K$ gene generally has a CTT anticodon, although TTT is utilized in *Apis* (Crozier and Crozier 1993) and *Caenorhabditis* (Okimoto et al. 1992). Basally derived hymenopterans (suborder Symphyta) utilize CTT (table 1), suggesting that CTT is plesiomorphic for the order. Mutation of the anticodon appears to have occurred a number of times, in the Aculeata (Apoidea + Chrysidoidea + Vespoidea), Chalcidoidea, Platygastroidea, Proctotrupoidea, and Ichneumonoidea. In the latter case, the mutation may mirror changes in compositional bias. Compositional analysis of the mitochondrial 16S rDNA gene (which is encoded on the N-strand) indicated that the A content is significantly higher in the Braconidae than in the Ichneumonidae (Dowton and Austin 1997*b*). If this trend holds across the N-strand, then the T content of the braconid J-strand would be increased relative to that of the Ichneumonidae. Given that the $tRNA^K$ gene is encoded on the J-strand, switching from CTT to TTT would reflect that compositional trend.

Overlapping Genes

The size of the metazoan mitochondrial genome is remarkably conserved, to the extent that, in some cases, genes overlap. In cases in which genes are transcribed from different strands, overlap does not preclude excision of either gene from the primary transcript, but functional constraints presumably limit the amount of overlap to short stretches. Such cases are observed in cyclostome taxa in the present study, where up to 4-nucleotide overlaps occur in genes encoding COII and $tRNA^D$ (table 1). However, in other cases, overlaps occur where genes are transcribed from the same strand. Mature transcripts are presumably only produced after polyadenylation of the excised genes (Ojala, Montoya, and Attardi 1981). Examples of this occur frequently at this hymenopteran locus between the COII and the first tRNA gene, where a functional stop codon in the COII gene can be produced only after polyadenylation of the primary transcript. This occurs in genera that either retain the plesiomorphic arrangement (e.g., *Diospilus*) or have derived arrangements (e.g., *Scelio*). Overlaps are also evident between neighboring tRNA genes. Yokobori and Pääbo (1995*a*, 1995*b*, 1997) have shown that overlapping mitochondrial tRNAs can be cleaved and polyadenylated to facilitate the production of both tRNAs from a single transcript. In the Hymenoptera examined here, overlaps frequently involved just one or two nucleotides that were A anyway (e.g., *Agathiella, Cardiochiles*); polyadenylation simply replaces the overlap with identical nucleotides. However, in some cases, the overlap involves nucleotides other than A (e.g., *Trissolcus*; fig. 5). In these cases, polyadenylation converts a poorly base-paired AA stem into a perfectly base-paired AA stem, as has been observed for land snail mitochondrial tRNA genes (Yokobori and Pääbo 1995*a*). In the present study, there were seven cases of overlap involving bases other than A (see table 1). In each case, the poorly base-paired AA stem is conceptually converted into a perfectly base-paired AA stem by polyadenylation. These overlaps were most frequently, but not exclusively, observed in rearranged genes.

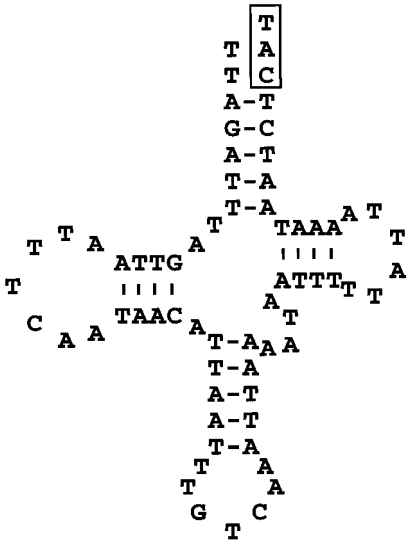

FIG. 5.—tRNA^{D} gene for *Trissolcus*, folded into the inferred tRNA configuration, showing the overlap (boxed) with the tRNA^{K} gene. Excision of the complete tRNA^{K} gene followed by polyadenylation of the tRNA^{D} gene fragment would produce a complete tRNA^{D} gene in which base-pairing of the AA stem is maintained.

Table 2
Sizes, Nucleotide Compositions, and Repeat Characteristics of the Larger Intergenic Regions from the Junction Between the Cytochrome Oxidase II and ATPase 8 Genes

Taxon	Length	%A	%T	%C	%G	Repeats (tandem; inverted)
Macroxyela	44	56.8	36.4	6.8	0	6; 0
Melissodes	35	48.6	45.7	0	5.7	0; 0
Myrmecia	48	56.2	43.8	0	0	12d; 12
Encarsia	23	60.9	39.1	0	0	0; 0
Anacharis	50	41.5	41.5	7.5	9.4	9; 0
Cenocoelius	138	37.7	52.9	0.7	8.7	14 (×3*); 7
Centistes	22	18.2	72.7	0	9.1	8; 0
Dolopsidea	99	39.4	52.5	1.0	7.1	10d; 6
Histeromerus . . .	45	46.7	53.3	0	0	9; 9
Habrobracon . . .	23	43.5	56.5	0	0	10d; 10
Spinaria	25	48.0	52.0	0	0	0; 11*
Gnamptodon	38	36.8	60.5	0	2.6	7d; 0
Betylobracon. . . .	24	29.2	62.5	4.2	4.2	8*; 0

NOTE.—Numbers in the ''Repeats'' column refer to the lengths of the tandem or inverted repeat units; ''d'' indicates that the repeat units are directly adjacent to each other; an asterisk indicates that the repeat is imperfect; ''(× 3*)'' indicates that there are three imperfect copies of the repeat unit. The repeat units for *Myrmecia* and *Habrobracon* are $(AT)_5$ and $(AT)_6$, respectively, such that they can be considered both tandem and inverted repeats.

Noncoding Regions

Noncoding, or intergenic, regions are rare in metazoan mitochondria. For this reason, they are of particular interest when they occur. In most of the genera sequenced here, the various genes are closely arranged, with little intergenic sequence. For example, *Orussus*, *Diospilus*, and *Jarra* have at most a single noncoding nucleotide across the three intergenic regions sequenced (table 1). Intergenic regions do occur in some taxa that retain the plesiomorphic arrangement, always between the COII and first tRNA gene (e.g., *Macroxyela*, *Perga*, and *Myrmecia*). Such intergenic regions also occur in taxa with derived arrangements, but then generally between the last tRNA gene and the ATPase 8 gene (e.g., *Encarsia*, *Sathon*, *Dolopsidea*, and *Gnamptodon*).

Table 2 lists the nucleotide compositions of the larger (>20 nt) intergenic regions. All are AT-biased, with C or G accounting for less than 10% of positions in all taxa. However, there appears to be no systematic bias toward A or T and no association of a particular bias with a particular type of rearrangement. Similarly, in taxa that have not suffered rearrangements (*Macroxyela*, *Myrmecia*, *Cenocoelius*, *Centistes*), there appears to be no trend toward A or T.

Intergenic regions outside of the AT-rich region have been reported in two other hymenopteran groups: various bees (Crozier, Crozier, and Mackinlay 1989; Cornuet, Garnery, and Solignac 1991; Crozier and Crozier 1993), and ants (Crozier, Jermiin, and Chiotis 1997). In isolated taxa, repeat units are observed. For example, Cornuet, Garnery, and Solignac (1991) reported the presence of three exactly and tandemly repeated sequences of 196 nt in *Apis mellifera* but not in closely related species. Similarly, Crozier, Jermiin, and Chiotis (1997) reported a tandemly repeated array (seven copies of a 15-nt unit) in *Camponotus* (GenBank U75351 annotation; L. S. Jermiin, personal communication), but again not in closely related species. We examined the intergenic regions from the taxa listed in table 2 for both tandem and inverted repeats. In no case did we observe long direct repeats such as those reported by Cornuet, Garnery, and Solignac (1991), nor did we see multiple copies of tandemly repeated arrays such as those reported by Crozier, Jermiin, and Chiotis (1997).

Discussion

We traced a relatively large number of rearrangements (at least five) to a single locus of the hymenopteran mitochondrial genome (fig. 3) that must have occurred during a relatively short period of time (ca. 180 Myr based on the earliest fossil records of the Apocrita dating to the Jurassic; Rasnitsyn 1980). There is thus far no evidence of any other invertebrate lineage having experienced such a rapid accumulation of rearrangements, although more extensive sampling of invertebrate lineages may uncover groups with similarly plastic genomes.

In invertebrates, very few rearrangements have been mechanistically assessed; instead, where described, they have been used as phylogenetic markers (e.g., Jacobs et al. 1989; Smith et al. 1993; Boore et al. 1995; Boore, Lavrov, and Brown 1998). For example, an inversion of a large segment of the mitochondrial genome has been described for starfish (encompassing two tRNA genes, the 12S rDNA subunit, and the cytochrome oxidase I gene; Jacobs et al. 1989), but it is unclear whether this represents single or multiple rearrangement events. Among vertebrates, most rearrangements can be explained by a tandem duplication mechanism (e.g., Pääbo et al. 1991; Macey et al. 1997), wherein the com-

position of a tRNA gene cluster does not change—the tRNA genes are simply locally ''shuffled'' relative to each other. Inversions (in which the tRNA gene swaps strands) and translocations (in which tRNA genes move from a remote location into a tRNA gene cluster) are rare. However, in the Hymenoptera, we observed a relatively equal proportion of these three types of rearrangement, suggesting that the evolutionary dynamics of rearrangements may be fundamentally different between vertebrates and invertebrates.

Local Rearrangements (Shuffling)

We saw evidence of this type of rearrangement on two occasions, in the Apoidea and the Scelionidae. As has been suggested for vertebrates, tandem duplication is sufficient to explain the observed rearrangements. However, for this to be the case, the region would need to function as an origin of replication. Identification of the lagging-strand origin of replication has remained elusive in insects, primarily due to the absence of noncoding sequence outside of the AT-rich region. Interestingly, Cornuet, Garnery, and Solignac (1991) identified such an origin of replication in a group of bees, characterized by intergenic sequences close to a tRNA gene ($tRNA^{L}$ [UUR]). The presence of intergenic sequences close to a tRNA gene at the locus sequenced in the present study is thus consistent with the view that this region (perhaps sporadically) functions as an origin of replication. By analogy, the lagging-strand origin of replication for vertebrates has been proposed to be similarly plastic to explain certain gene rearrangements in vertebrates (Macey et al. 1997).

Translocations

It is difficult to speculate on possible mechanisms of translocation until the organization of the mitochondrial genomes in which translocations have occurred have been more completely determined. Nevertheless, tandem duplications may also operate to produce the translocations observed here. In reptiles, tandem duplications of more than 9 kb have been reported (Moritz, Dowling, and Brown 1987), sufficiently long to encompass the DK region and the $tRNA^{H}$ gene, which are separated by ca. 4 kb in *Apis* and *Drosophila* (fig. 6). Duplication of the intervening sequence would lead to a mitochondrial genome containing a tRNA gene cluster containing all three genes (fig. 6*B*). However, random deletion of the various gene duplicates is unlikely to operate, as on two separate occasions each of the genes on the left of the tRNA cluster must have been deleted to produce the contemporary product in which the COII gene lies directly adjacent to the tRNA cluster (fig. 6*C*). Instead, we consider a single large deletion of one of the duplicated regions more likely, possibly involving intramitochondrial recombination. The products of such a recombination event have recently been characterized for the nematode *Meloidogyne javanica* (Lunt and Hyman 1997).

Alternatively, translocations may also be produced through the illicit priming of mitochondrial replication by a tRNA molecule, as originally proposed by Cantatore et al. (1987). After mitochondrial replication is initiated, failure to cleave a tRNA primer from the nascent DNA strand could lead to the ultimate incorporation of a tRNA gene into the mitochondrial genome. In the hymenopteran taxa examined here, the translocated $tRNA^{H}$ gene is in the correct orientation if it was used to prime lagging-strand replication. We consider our data presently too incomplete to distinguish between these two competing translocatory mechanisms.

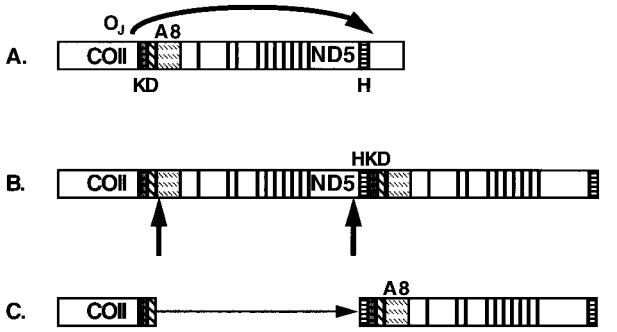

FIG. 6.—Putative translocatory mechanism for the hymenopteran $tRNA^{H}$ gene. *A,* Slipped-strand mispairing during replication leads to duplication of the region spanning the $tRNA^{K,D}$ and $tRNA^{H}$ genes. *B,* All genes between the KD and HKD tRNA genes are deleted, forming (*C*) a genome containing all three tRNA genes at the junction between the COII and ATPase 8 (A8) genes.

Inversions

On two separate occasions, we observed local inversions of tRNA genes (in the cyclostome braconids and Chalcidoidea). Tandem duplications cannot be invoked to explain these. Similarly, illicit priming of DNA replication by a tRNA molecule is an unlikely mechanism, as this would lead to the insertion of a tRNA gene in the same orientation as that of the current $tRNA^{D}$ and $tRNA^{K}$ genes. Instead, the inverted D and K genes may have arisen by local small inversions, necessarily invoking breakage and rejoining of the mitochondrial genome. Recently, evidence for intramitochondrial recombination in a nematode was reported (Lunt and Hyman 1997); breakage and rejoining of mitochondrial DNA strands was indicated by the presence of subgenomic minicircles just 250 bp long. We speculate that inversions could be an alternate end product of intramitochondrial recombination. Following breakage, rejoining could occur in three different ways (fig. 7); (*a*) circularization of the major and minifragments (as observed by Lunt and Hyman 1997), (*b*) reincorporation of the minifragment in the same orientation (resulting in no inversion), or (*c*) reincorporation of the minifragment in the reverse orientation (resulting in inversion). However, it remains to be demonstrated whether intramitochondrial recombination is a general phenomenon.

Conclusions

We demonstrate here that gene rearrangements and translocations are not generally as rare as they were previously considered, with at least five rearrangements occurring during approximately 180 Myr. Furthermore,

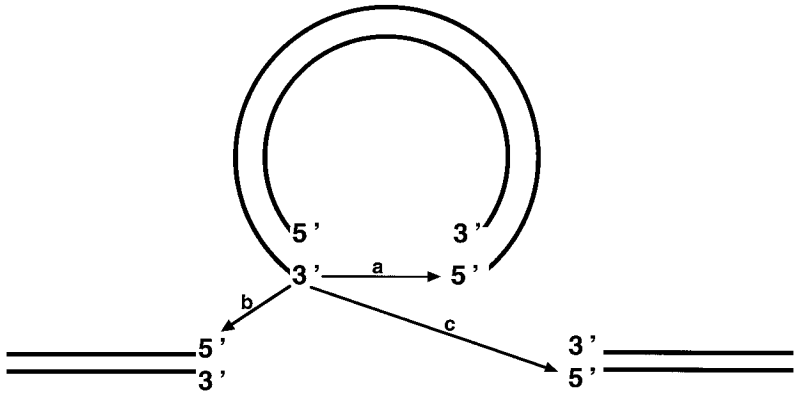

FIG. 7.—Proposed model for the generation of small mitochondrial inversions. After double-strand breakage of the mitochondrial genome at two neighboring loci, rejoining can produce three possible products: (*a*) the major and minicircles observed by Lunt and Hyman (1997), (*b*) an unchanged genome, or (*c*) a genome with a short inverted segment.

certain types of rearrangement appear to be more prone to homoplasy than others, suggesting that aspects of the rearrangement and translocatory mechanism may bias the historical pattern of arrangements observed (similar homoplastic trends were recently reported to occur in birds: Mindell, Sorenson, and Dimcheff 1998). Our data thus indicate that taxonomic sampling is a crucial consideration, even with rearrangement characters. For example, the homoplastic rearrangements in the Apoidea and Scelionidae would have led to spurious phylogenetic conclusions had we not established that some members of the Aculeata (to which the Apoidea belong) retained the plesiomorphic arrangement.

Intriguingly, all rearrangements described here occurred after the adoption of the parasitic lifestyle, which in the Hymenoptera has a single origin, at least for the vast majority (>99%) of parasitic wasps (Rasnitsyn 1988; Wiegmann, Mitter, and Farrell 1993; Dowton and Austin 1997*a*). Although our data are too preliminary to infer an association between parasitic biology and the rate of mitochondrial rearrangements, previous studies of ours indicate that other mitochondrial mutational processes became accelerated at the same time that the parasitic lifestyle first appeared; both the rate and compositional bias (AT content) are increased in wasps descendant from parasitic ancestors (Dowton and Austin 1995; Dowton and Austin 1997b). It will be of interest to examine whether other invertebrate lineages that have accelerated rates of mitochondrial evolution (e.g., certain nematodes; Hugall, Stanton, and Moritz 1997) similarly have experienced more mitochondrial gene rearrangements.

Acknowledgments

For provision of taxa, we thank Eveline Bartowsky, Robert Belshaw, Dave Britton, Vegilio Caleca, Paul Dangerfield, Scott Field, Kris French, Paul Hanson, John Jennings, Patrick Mardulyn, Lubomir Masner, David Murray, Donald Quicke, Scott Shaw, Ross Storey, Gimme Walter, Q. Wang, and Jim Whitfield. Comments by Ross Crozier and two anonymous reviewers greatly improved an earlier version of this manuscript. This work was supported by grants from the Australian Research Council and the Linnean Society of New South Wales. The sequences reported in this paper are deposited in GenBank (accession numbers AF034583–AF034606, AF072122, AF082915–AF082930, and AF099645).

LITERATURE CITED

BEARD, D. B., D. M. HAMM, and F. H. COLLINS. 1993. The mitochondrial genome of the mosquito *Anopholes gambiae*: DNA sequence, genome organization, and comparisons with mitochondrial sequences of other insects. Insect Mol. Biol. **2**:103–124.

BELSHAW, R., M. FITTON, E. HERNIOU, C. GIMENO, and D. L. J. QUICKE. 1998. A phylogenetic reconstruction of the Ichneumonoidea (Hymenoptera) based on the D2 variable region of 28S ribosomal RNA. Syst. Entomol. **23**:109–123.

BELSHAW, R., and D. L. J. QUICKE. 1997. A molecular phylogeny of the Aphidiinae (Hymenoptera: Braconidae). Mol. Phylogenet. Evol. **7**:281–293.

BOORE, J. L., T. M. COLLINS, D. STANTON, L. L. DAEHLER, and W. M. BROWN. 1995. Deducing the pattern of arthropod phylogeny from mitochondrial DNA rearrangements. Nature **376**:163–165.

BOORE, J. L., D. V. LAVROV, and W. M. BROWN. 1998. Gene translocation links insects and crustaceans. Nature **392**:667–668.

BROTHERS, D. J., and J. M. CARPENTER. 1993. Phylogeny of Aculeata: Chrysidoidea and Vespoidea (Hymenoptera). J. Hymenop. Res. **2**:227–304.

CANTATORE, P., M. N. GADALETA, M. ROBERTI, C. SACCONE, and A. C. WILSON. 1987. Duplication and remoulding of tRNA genes during the evolutionary rearrangement of mitochondrial genomes. Nature **329**:853–855.

CLARY, D. O., and D. R. WOLSTENHOLME. 1984. The *Drosophila* mitochondrial genome. Oxf. Surv. Eukaryot. Genes **1**:1–35.

———. 1985*a*. The mitochondrial DNA molecule of *Drosophila yakuba*: nucleotide sequence, gene organization, and genetic code. J. Mol. Evol. **22**:252–271.

———. 1985*b*. The ribosomal RNA genes of Drosophila mitochondrial DNA. Nucleic Acids Res. **13**:4029–4045.

CORNUET, J.-M., L. GARNERY, and M. SOLIGNAC. 1991. Putative origin and function of the intergenic region between COI and COII of *Apis mellifera* L. mitochondrial DNA. Genetics **1128**:393–403.

CROZIER, R. H., and Y. C. CROZIER. 1993. The mitochondrial genome of the honeybee *Apis mellifera*: complete sequence and genome organization. Genetics **133**:97–117.

CROZIER, R. H., Y. C. CROZIER, and A. G. MACKINLAY. 1989. The CO-I and CO-II region of honeybee mitochondrial DNA: evidence for variation in insect mitochondrial evolutionary rates. Mol. Biol. Evol. **6**:399–411.

CROZIER, R. H., L. S. JERMIIN, and M. CHIOTIS. 1997. Molecular evidence for a Jurassic origin of ants. Naturwissenschaften **84**:22–23.

DESJARDINS, P., and R. MORAIS. 1990. Sequence and gene organization of the chicken mitochondrial genome. J. Mol. Biol. **212**:599–634.

DOWTON, M., and A. D. AUSTIN. 1994*a*. Molecular phylogeny of the insect order Hymenoptera: Apocritan relationships. Proc. Natl. Acad. Sci. USA **91**:9911–9915.

———. 1994*b*. A simple method for finding optimal conditions for the direct sequencing of PCR products. BioTechniques **16**:816–817.

———. 1995. Increased genetic diversity in mitochondrial genes is correlated with the evolution of parasitism in the Hymenoptera. J. Mol. Evol. **41**:958–965.

———. 1997*a*. Evidence for AT-transversion bias in wasp (Hymenoptera: Symphyta) mitochondrial genes and its implications for the origin of parasitism. J. Mol. Evol. **44**:398–405.

———. 1997*b*. The evolution of strand-specific compositional bias. A case study in the hymenopteran mitochondrial 16S rRNA gene. Mol. Biol. Evol. **14**:109–112.

———. 1998. Phylogenetic relationships among the microgastroid wasps (Hymenoptera: Braconidae): combined analysis of 16S and 28S rDNA genes, and morphological data. Mol. Phylogenet. Evol. (in press).

DOWTON, M., A. D. AUSTIN, and M. F. ANTOLIN. 1998. Evolutionary relationships among the Braconidae (Hymenoptera: Ichneumonoidea) inferred from partial 16S rDNA gene sequences. Insect Mol. Biol. **7**:129–150.

DOWTON, M., A. D. AUSTIN, N. DILLON, and E. BARTOWSKY. 1997. Molecular phylogeny of the apocritan wasps: the Proctotrupomorpha and Evaniomorpha. Syst. Entomol. **22**: 245–255.

FLOOK, P. K., C. H. F. ROWELL, and G. GELLISSEN. 1995*a*. The sequence, organization, and evolution of the *Locusta migratoria* mitochondrial genome. J. Mol. Evol. **41**:928–941.

———. 1995*b*. Homoplastic rearrangements of insect mitochondrial tRNA genes. Naturwissenschaften **83**:336–337.

HASEGAWA, M., H. KISHINO, and K. YANO. 1985. Dating of the human–ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. **22**:160–174.

HUGALL, A., J. STANTON, and C. MORITZ. 1997. Evolution of the AT-rich mitochondrial DNA of the root knot nematode *Meloidogyne hapla*. Mol. Biol. Evol. **14**:40–48.

JACOBS, H. T., S. ASAKAWA, T. ARAKI, K.-I. MIURA, M. J. SMITH, and K. WATANABE. 1989. Conserved tRNA gene cluster in starfish mitochondrial DNA. Curr. Genet. **15**:193–206.

KISHINO, H., and M. HASEGAWA. 1989. Evaluation of the maximum likelihood estimate of the evolutionary topologies from DNA sequence data, and the branching order in Hominoidea. J. Mol. Evol. **29**:170–179.

KUMAZAWA, Y., and M. NISHIDA. 1995. Variations in mitochondrial tRNA gene organization of reptiles as phylogenetic markers. Mol. Biol. Evol. **12**:759–772.

LIU, H., and A. T. BECKENBACH. 1992. Evolution of the mitochondrial cytochrome oxidase II gene among 10 orders of insects. Mol. Phylogenet. Evol. **1**:41–52.

LOWE, T. M., and S. R. EDDY. 1997. tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. Nucleic Acids Res. **25**:955–964.

LUNT, D. H., and B. C. HYMAN. 1997. Animal mitochondrial DNA recombination. Nature **387**:247.

MACEY, J. R., A. LARSON, N. B. ANANJEVA, Z. FANG, and T. J. PAPENFUSS. 1997. Two novel gene orders and the role of light-strand replication in rearrangement of the vertebrate mitochondrial genome. Mol. Biol. Evol. **14**:91–104.

MINDELL, D. P., M. D. SORENSON, and D. E. DIMCHEFF. 1998. Multiple independent origins of mitochondrial gene order in birds. Proc. Natl. Acad. Sci. USA **95**:10693–10697.

MITCHELL, S. E., A. F. COCKBURN, and J. A. SEAWRIGHT. 1993. The mitochondrial genome of *Anopheles quadrimaculatus*, species A: complete nucleotide sequence and gene organization. Genome **36**:1058–1073.

MORITZ, C., T. E. DOWLING, and W. M. BROWN. 1987. Evolution of animal mitochondrial DNA: relevance for population biology and systematics. Annu. Rev. Ecol. Syst. **18**: 269–292.

OJALA, D., J. MONTOYA, and G. ATTARDI. 1981. tRNA punctuation model of RNA processing in human mitochondria. Nature **290**:470–474.

OKIMOTO, R., J. L. MACFARLANE, D. O. CLARY, and D. R. WOLSTENHOLME. 1992. The mitochondrial genome of two nematodes, *Caenorhabditis elegans* and *Ascaris suum*. Genetics **130**:471–498.

PÄÄBO, S., W. K. THOMAS, K. M. WHITFIELD, Y. KUMAZAWA, and A. C. WILSON. 1991. Rearrangements of mitochondrial transfer RNA genes in marsupials. J. Mol. Evol. **33**:426–430.

PEREZ, M. L., J. R. VALVERDE, B. BATUECAS, F. AMAT, R. MARCO, and R. GARESSE. 1994. Speciation of the *Artemia* genus: mitochondrial DNA analysis of bisexual and parthenogenetic brine shrimps. J. Mol. Evol. **38**:156–168.

QUICKE, D. L. J., and C. VAN ACHTERBERG. 1990. Phylogeny of the subfamilies of the family Braconidae (Hymenoptera: Ichneumonoidea). Zool. Verh. (Leiden) **258**:1–95.

QUINN, T. W., and D. P. MINDELL. 1996. Mitochondrial gene order adjacent to the control region in crocodile, turtle, and tuatara. Mol. Phylogenet. Evol. **5**:344–351.

RASNITSYN, A. P. 1980. The origin and evolution of the Hymenoptera. Tr. Paleontol. Inst. Akad. Nauk SSSR **174**:1–192 (in Russian).

———. 1988. An outline of evolution of the hymenopterous insects (Order Vespida). Orient. Insects **22**:115–145.

SAMBROOK, J., E. G. FRITSCH, and T. MANIATIS. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Press, Plainview, N.Y.

SIMON, C., F. FRATI, A. BECKENBACH, B. CRESPI, H. LIU, and P. FLOOK. 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Ann. Entomol. Soc. Am. **87**:1–51.

SMITH, M. J., A. ARNDT, S. GORSKI, and E. FAJBER. 1993. The phylogeny of echinoderm classes based on mitochondrial gene arrangements. J. Mol. Evol. **36**:545–554.

STRIMMER, K., N. GOLDMAN, and A. VON HAESELER. 1997. Bayesian probabilities and quartet puzzling. Mol. Biol. Evol. **14**:210–211.

STRIMMER, K., and A. VON HAESELER. 1996. Quartet puzzling: a quartet maximum likelihood method for reconstructing tree topologies. Mol. Biol. Evol. **13**:964–969.

WHARTON, R. A., S. R. SHAW, M. J. SHARKEY, D. B. WAHL, J. B. WOOLLEY, J. B. WHITFIELD, P. M. MARSH, and W. JOHNSON. 1992. Phylogeny of the subfamilies of the family

Braconidae (Hymenoptera: Ichneumonoidea): a reassessment. Cladistics **8**:199–235.

WHITFIELD, J. B., and W. R. M. MASON. 1994. Mendesellinae, a new subfamily of braconid wasps (Hymenoptera, Braconidae) with a review of relationships within the microgastroid assemblage. Syst. Entomol. **19**:61–76.

WIEGMANN, B. M., C. MITTER, and B. FARRELL. 1993. Diversification of carnivorous parasitic insects: extraordinary radiation or specialized dead end? Am. Nat. **142**:737–754.

WILLIS, L. G., M. L. WINSTON, and B. M. HONDA. 1992. Phylogenetic relationships in the honeybee (Genus *Apis*) as determined by the sequence of the cytochrome oxidase II region of mitochondrial DNA. Mol. Phylogenet. Evol. **1**:169–178.

YOKOBORI, S., and S. PÄÄBO. 1995*a*. Transfer RNA editing in land snail mitochondria. Proc. Natl. Acad. Sci. USA **92**: 10432–10435.

———. 1995*b*. tRNA editing in metazoans. Nature **377**:490.

———. 1997. Polyadenylation creates the discriminator nucleotide of chicken mitochondral $tRNA^{Tyr}$. J. Mol. Biol. **265**: 95–99.

ROSS H. CROZIER, reviewing editor

Accepted October 29, 1998