Characterization of 67 Mitochondrial tRNA Gene Rearrangements in the Hymenoptera Suggests That Mitochondrial tRNA Gene Position Is Selectively Neutral

Mark Dowton,[1] *Stephen L. Cameron,*†‡[1] *Jessica I. Dowavic,** *Andy D. Austin,*§ *and Michael F. Whiting*‡

*Centre for Biomedical Sciences, School of Biological Sciences, University of Wollongong, NSW, Australia; †Australian National Insect Collection and CSIRO Entomology, Black Mountain Laboratories, P.O. Box 1700, Canberra, ACT, Australia; ‡Department of Biology, Brigham Young University, Provo, UT; and §Australian Center for Evolutionary Biology and Biodiversity, School of Earth and Environmental Sciences, University of Adelaide, SA, Australia

We present entire sequences of two hymenopteran mitochondrial genomes and the major portion of three others. We combined these data with nine previously sequenced hymenopteran mitochondrial genomes. This allowed us to infer and analyze the evolution of the 67 mitochondrial gene rearrangements so far found in this order. All of these involve tRNA genes, whereas four also involve larger (protein-coding or ribosomal RNA) genes. We find that the vast majority of mitochondrial gene rearrangements are independently derived. A maximum of four of these rearrangements represent shared, derived organizations, whereas three are convergently derived. The remaining mitochondrial gene rearrangements represent new mitochondrial genome organizations. These data are consistent with the proposal that there are an enormous number of alternative mitochondrial genome organizations possible and that mitochondrial genome organization is, for the most part, selectively neutral. Nevertheless, some mitochondrial genes appear less mobile than others. Genes close to the noncoding region are generally more mobile but only marginally so. Some mitochondrial genes rearrange in a pattern consistent with the duplication/random loss model, but more mitochondrial genes move in a pattern inconsistent with this model. An increased rate of mitochondrial gene rearrangement is not tightly associated with the evolution of parasitism. Although parasitic lineages tend to have more mitochondrial gene rearrangements than nonparasitic lineages, there are exceptions (e.g., *Orussus* and *Schlettererius*). It is likely that only a small proportion of the total number of mitochondrial gene rearrangements that have occurred during the evolution of the Hymenoptera have been sampled in the present study.

Introduction

Mitochondrial genes are the most intensely used molecule for the investigation of phylogeny (e.g., Milinkovitch et al. 1993; Haring et al. 2001; Grande et al. 2004; Macey et al. 2004; Cameron, Lambkin, et al. 2007; Fenn et al. 2008), population genetics (reviewed in Simon 1991; Cameron and Whiting 2007), and for investigating the mode and mechanism of molecular evolution (e.g., Reyes et al. 1998). An understanding of the ways in which these molecules evolve will facilitate the development of more realistic frameworks for the use of mitochondrial gene sequence data to reconstruct evolutionary relationships, and to discover the historical trends of biological populations.

Increasingly, the molecular evolution of this molecule is being examined using whole mitochondrial genome data (Kondo et al. 1993; Dong and Kumazawa 2005; Sheffield et al. 2008). Mitochondrial genome organization is being used to infer phylogeny (Smith et al. 1993; Boore et al. 1998; Dowton 1999; Haring et al. 2001), whereas the nature and rate of mitochondrial gene rearrangement are being characterized among phylogenetically related organisms (e.g., Kumazawa and Nishida 1995). This latter approach can be particularly powerful, as gene movements can be assessed within a phylogenetic framework established from independent data.

Many mitochondrial genome studies suffer one major shortcoming. Due to the effort required to sequence an entire mitochondrial genome, taxonomic sampling remains relatively poor, such that conclusions are based on relatively few samples (but see Miya et al. 2003). Although we and others have already sequenced nine hymenopteran mitochondrial genomes (table 1), these were not chosen to best represent the phylogenetic diversity of the order. In the present study, we report five newly sequenced hymenopteran mitochondrial genomes, chosen to fill major gaps in our taxonomic sampling of hymenopteran diversity. We consider that the hymenopteran mitochondrial genome represents a useful model system for the study of the mode of mitochondrial gene order evolution, due to its accelerated rate of gene rearrangement (Crozier and Crozier 1993; Dowton and Austin 1999; Dowton et al. 2003). There are not so few mitochondrial gene rearrangements that trends are proposed from isolated observations, whereas there are not so many that the history of each mitochondrial rearrangement becomes blurred by multiple changes (e.g., Shao et al. 2001; Shao and Barker 2002; Cameron, Johnson, and Whiting 2007). Nevertheless, there is already some evidence that different trends will be observed, depending on the animal group studied. Vertebrate mitochondrial gene rearrangements appear to occur less frequently than invertebrate mitochondrial gene rearrangements. For example, only six different mitochondrial genome organizations are evident among 100 entirely sequenced mitochondrial genomes (Miya et al. 2003). Most vertebrate mitochondrial gene rearrangements are consistent with the duplication/random loss model (Moritz et al. 1987; San Mauro et al. 2006), whereas a range of invertebrate mitochondrial rearrangements are inconsistent with this mechanism (Smith et al. 1993; Dowton and Austin 1999; Miller et al. 2004; Shao et al. 2006).

[1] These authors contributed equally to this work.

Key words: duplication/random loss, tRNA gene, selectively neutral, mitochondrial genome, Hymenoptera.

E-mail: mdowton@uow.edu.au.

Mol. Biol. Evol. 26(7):1607–1617. 2009
doi:10.1093/molbev/msp072
Advance Access publication April 9, 2009

Table 1
Hymenopteran Mitochondrial Genome Sequences Employed in the Present Study

Suborder Superfamily: Family	Taxon
Non-apocritans	
Tenthredinoidea: Pergidae	*Perga* (Castro and Dowton 2005)
Cephoidea: Cephidae	*Cephus cinctus*[a]
Orussoidea: Orussidae	*Orussus occidentalis*[a]
Apocrita	
Apoidea: Apidae	*Apis* (Crozier and Crozier 1993), *Bombus* (Cha et al. 2007), *Melipona* (Silvestre et al. 2008)
Chalcidoidea: Pteromalidae	*Nasonia* (Oliveira et al. 2008)
Chrysidoidea: Chrysididae	*Primeuchroeus* (Castro et al. 2006)
Ichneumonoidea: Ichneumonidae	*Venturia canescens*[a], *Enicospilus* sp.[a]
Proctotrupoidea: Vanhorniidae	*Vanhornia* (Castro et al. 2006)
Stephanoidea: Stephanidae	*Schlettererius cinctipes*[a]
Vespoidea: Vespidae	*Abispa*, *Polistes* (Cameron et al. 2008)

[a] Sequenced in the present study.

We divide the Hymenoptera into two groups, the monophyletic Apocrita, and a range of more basally divergent superfamilies, which we refer to here as the "non-apocritans." Previously, the non-apocritans have been grouped together into the suborder Symphyta, but this is clearly not a natural grouping. Two of us previously reported that the rate of both genetic divergence (Dowton and Austin 1995) and gene rearrangement (Dowton and Austin 1999; Dowton et al. 2003; Shao et al. 2003) are accelerated in the mitochondrial genomes of Apocrita (i.e., including the parasitic Hymenoptera), when compared with non-apocritans (predominantly nonparasitic Hymenoptera). Two of the newly reported mitochondrial genomes are from non-apocritans, whereas three are from the Apocrita. In the present paper, we report the sequence and organization of these five mitochondrial genomes and analyze their organization together with the nine previously sequenced hymenopteran mitochondrial genomes. We identify 67 mitochondrial gene rearrangements compared with the ancestral pancrustacean mitochondrial genome organization (Crease 1999; Cook 2005). Most of these rearrangements involve tRNA genes, and all are likely to have occurred during the evolution of the Hymenoptera. We report the underlying molecular evolutionary trends that these analyses reveal.

Materials and Methods

Specimens and DNA Extraction

Cephus cinctus Norton, *Orussus occidentalis* (Cresson), and *Enicospilus* sp. were sequenced in the laboratory of S.L.C. and M.F.W., whereas *Schlettererius cinctipes* (Cresson) and *Venturia canescens* (Gravenhorst) were sequenced in the laboratory of M.D. and A.D.A. DNA was extracted from *Cephus*, *Orussus*, and *Enicospilus* using the DNEasy Tissue Kit (Qiagen, Hilden, Germany) as described (Sheffield et al. 2008). DNA was extracted from ethanol-preserved specimens of *S. cinctipes* and *V. canescens* as previously described (Castro and Dowton 2005), using the method of Sunnucks and Hales (1996).

Amplification and Sequencing of Mitochondrial Genome Fragments

Amplification and sequencing of *Cephus*, *Orussus*, and *Enicospilus* were achieved using methods described by Cameron and others (Cameron, Johnson, and Whiting 2007; Cameron, Lambkin, et al. 2007), whereas those for *Schlettererius* and *Venturia* have also been previously described (Castro et al. 2006). For both sets of taxa, long mitochondrial fragments were sequenced by primer walking; primer sequences can be obtained from the S.C. (*Cephus*, *Orussus*, and *Enicospilus*) or M.D. (*Venturia*, *Schlettererius*) laboratories. *Cephus* and *Orussus* were sequenced entirely, whereas *Enicospilus*, *Schlettererius*, and *Venturia* were incomplete, presumably in the area surrounding the mitochondrial AT-rich region, which has proven difficult to amplify in the Hymenoptera (Castro and Dowton 2005; Castro et al. 2006; Cameron et al. 2008). The sequences are available from GenBank, under accession numbers FJ478173–FJ4781737.

Genome Annotation

Abbreviations for mitochondrial gene names follow Boore (1999). Mitochondrial tRNA genes were identified using tRNA-Scan SE version 1.21 (lowelab.ucsc.edu/tRNAscan-SE) (Lowe and Eddy 1997), specifying mitochondrial/chloroplast DNA as the source and using the invertebrate mitochondrial genetic code for tRNA isotype prediction. The cove cutoff score was set to 1. Some mitochondrial tRNA genes were consistently missed by tRNAScan, such as $trnS_1$ and *trnR*. These were identified by visually inspecting unassigned regions for sequences with similarity to previously identified mitochondrial tRNA genes of these isotypes. In *Cephus*, *Orussus*, and *Enicospilus*, mitochondrial protein-coding genes were identified as described (Sheffield et al. 2008). In *Venturia* and *Schlettererius*, mitochondrial protein–coding genes were identified using open reading frame (ORF) finder (ncbi.nlm.nih.gov), followed by BlastP analysis (implemented from within ORF finder). BlastN was used to identify mitochondrial rRNA genes. The precise ends of the mitochondrial *rnl* and *rns* genes are difficult to define; others have noted this. *rnl* is most generally defined as being bounded by neighboring genes (i.e., with no noncoding sequence between the genes). We have followed this practice here. *rns* is more difficult to define, as one end is usually bounded by the main noncoding region. In those cases, we aligned all previously defined holometabolous *rns* genes with the hymenopteran taxon under investigation, then used the nucleotide that aligned with the *Drosophila rns* noncoding boundary as the cutoff for the *rns* gene. This approximation was considered the most practical.

Results

Sampling Strategy

Previously, nine complete or nearly complete mitochondrial genome sequences had been reported from the Hymenoptera (table 1). However, these are not a representative sample of the order. For example, six of these nine

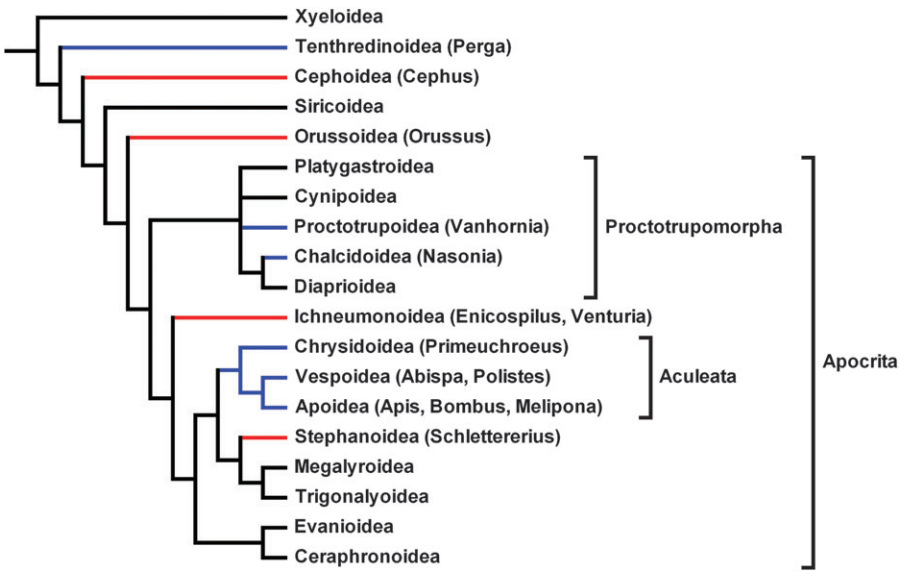

FIG. 1.—Phylogeny of the Hymenoptera, indicating those lineages from which complete or nearly complete mitochondrial genome sequences have been determined. The tree shown is the current best estimate. It is based on separate analyses of non-apocritan (Vilhelmsen 1997, 2001; Schulmeister et al. 2002) and apocritan relationships (Castro and Dowton 2006). Branches in blue indicate lineages with mitochondrial genome sequence reported prior to the present study. Branches in red indicate those branches sampled in the present study.

mitochondrial genomes are from the Aculeata (Apoidea, Chrysidoidea, and Vespoidea in table 1). Thus far, only two nonaculeate apocritans have been sequenced (*Vanhornia* and *Nasonia*), yet this group contains the vast majority of parasitic hymenopterans. Further, these two parasitic wasps are from the Proctotrupomorpha, a monophyletic assemblage identified from our most recent sequence-based analysis (Castro and Dowton 2006). There is a range of parasitic groups that lies outside of this group. Figure 1 shows the hymenopteran phylogeny, as deduced from separate analyses of non-apocritan (Vilhelmsen 1997, 2001; Schulmeister et al. 2002) and apocritan relationships (Castro and Dowton 2006). Blue branches indicate the hymenopteran lineages for which mitochondrial sequence data were available prior to this study.

In order to better represent hymenopteran diversity, we chose to sequence two additional non-apocritans (*Cephus* and *Orussus*) and three additional apocritans (*Schlettererius*, *Enicospilus*, and *Venturia*, the latter two are both from the Ichneumonidae). Figure 1 (red branches) indicates where these taxa lie on the current estimate of the hymenopteran phylogeny.

The Mitochondrial Genomes of *Cephus* and *Orussus*

Just a single (partial) mitochondrial genome from a non-apocritan representative was previously reported, that of *Perga condei* (Castro and Dowton 2005). The organizations of the newly sequenced *Cephus* and *Orussus* are shown in figure 2. The mitochondrial genome organization of the ancestral pancrustacean (Crease 1999; Cook 2005), together with *Perga*, is included to facilitate comparisons. In each of the three non-apocritans, all protein-coding and the two rRNA genes are positioned identically and are transcribed in identical directions. The only differences occur with the relative positions of tRNA genes. An increased plasticity in the relative positions of mitochondrial tRNA genes, as compared with protein-coding and rRNA genes, has been noted in a range of other taxa (Boore 1999; Gissi et al. 2008). Of particular interest are the relative positions of the mitochondrial *trnI*, *trnQ*, and *trnM* genes. These were not determined in *Perga* (Castro and Dowton 2005), but *trnI* and *trnQ* are in identical, derived positions in both *Cephus* and *Orussus*. Similarly, the *trnM* gene in both *Cephus* and *Orussus* is no longer adjacent to the *nad2* gene, but is in different, derived positions. In *Cephus*, it follows the *rns* gene, whereas in *Orussus*, it lies between *cob* and $trnS_2$. In all cases, it is transcribed in the same direction. Indeed, none of the mitochondrial tRNA gene rearrangements are inversions, that is, all mitochondrial genes are transcribed in the ancestral direction.

The Mitochondrial Genome of *S. cinctipes*

Figure 3 shows the organization of the mitochondrial genome of the stephanid, *S. cinctipes*. We chose this taxon because the stephanids have been proposed as an early diverging lineage within the Apocrita (Gibson 1985; Johnson 1988; Whitfield 1992), although this is not supported by molecular data (Castro and Dowton 2006), nor in combined molecular–morphological data analyses (Dowton and Austin 2001). Figure 3 indicates that, similar to the non-apocritans, none of the protein-coding or rRNA genes have changed positions, relative to the ancestral pancrustacean. However, the *trnD* and *trnK* have swapped position, whereas there has been shuffling of the mitochondrial tRNA genes at the *nad3–nad5* junction, with *trnE* moving from its ancestral position (between $trnS_1$ and *trnF*) to a derived position (between *trnA* and *trnR*). This latter mitochondrial gene movement was previously reported but in

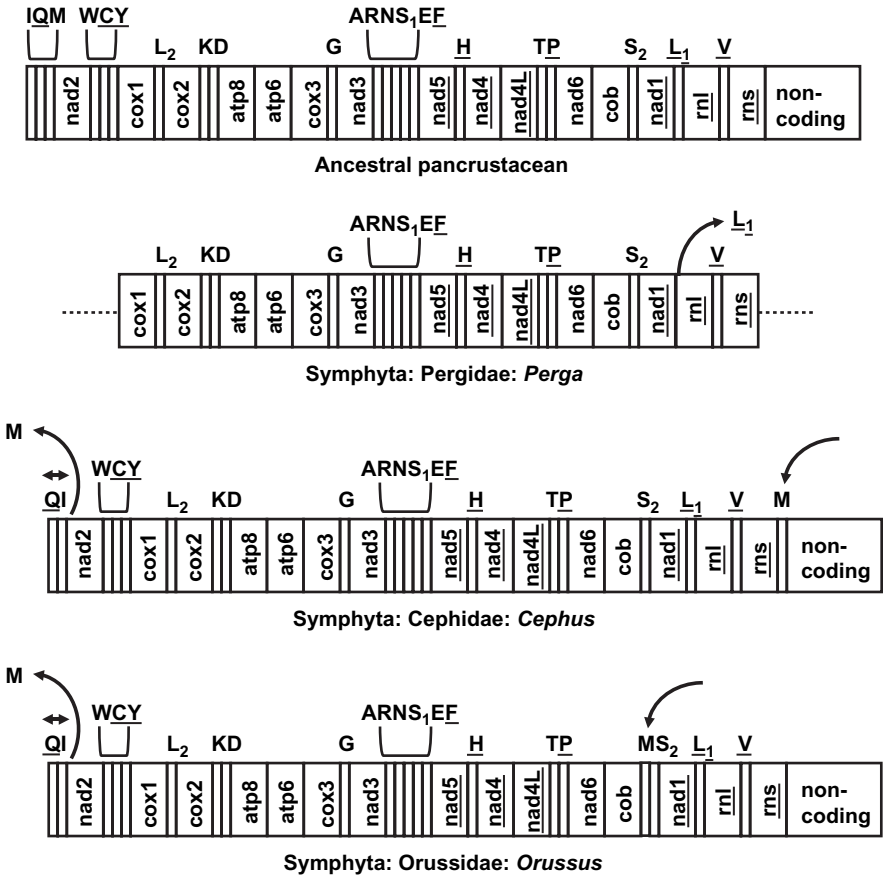

FIG. 2.—Mitochondrial genome organization of non-apocritan representatives, compared with the ancestral pancrustacean mitochondrial genome organization (Crease 1999; Cook 2005). All genes are transcribed from the major strand, except those underlined—these are transcribed from the minor strand. The major strand is that strand that encodes the majority of protein-coding genes. tRNA genes are indicated by the one letter amino acid symbol for which each tRNA encodes. The broken line at the ends of the *Perga* mitochondrial genome indicates that it is not completely sequenced. Both *Cephus* and *Orussus* were determined in the present study. The mitochondrial genomes of the ancestral pancrustacean and *Perga* are shown to facilitate comparisons. Mitochondrial gene rearrangements, relative to the ancestral organization, are indicated with arrows.

a study in which we only sequenced the *nad3–nad5* junction (Dowton et al. 2003) of this wasp.

The Mitochondrial Genomes of Two Ichneumonid Wasps, *Enicospilus* sp. and *V. canescens*

Figure 4 shows the mitochondrial organization of the two ichneumonid wasps sequenced in the present study. *Enicospilus* is from the subfamily Ophioninae, whereas *Venturia* is from the Campopleginae. Both are from the ophionoid group of ichneumonid subfamilies. Some analyses of *28S* data recover these two subfamilies as sister groups (see fig. 3*a* in Belshaw et al. 1998), although with low bootstrap support; the relationship is collapsed in the summary tree presented by these authors (see fig. 5 in Belshaw et al. 1998).

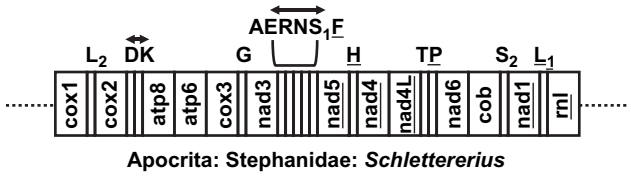

FIG. 3.—Mitochondrial genome organization of a stephanid, *Schlettererius cinctipes*. The broken lines at either end of the mitochondrial genome indicate that it is not completely sequenced. Mitochondrial gene movements, relative to the ancestral organization, are shown with arrows. Gene transcription direction and tRNA abbreviations are as described in figure 2.

Although *Enicospilus* and *Venturia* are the most closely related of those wasps sequenced here, they have the most different mitochondrial genomes, both when compared with each other (fig. 4) and when compared with the ancestral pancrustacean mitochondrial genome organization. Further, no mitochondrial gene rearrangements are shared between these two wasps. Although both have lost the *trnL*$_2$ gene from the *cox1–cox2* gene junction, it has moved to different places in each wasp.

In *Enicospilus*, there are at least six mitochondrial gene rearrangements relative to the pancrustacean mitochondrial gene order; all involve mitochondrial tRNA genes and only one involves a change in transcriptional polarity (i.e., most movements are along the same mitochondrial strand). *trnM* has moved upstream relative to the ancestral pancrustacean (*trnM–trnI–*<u>*trnQ*</u>*–nad2*, compared

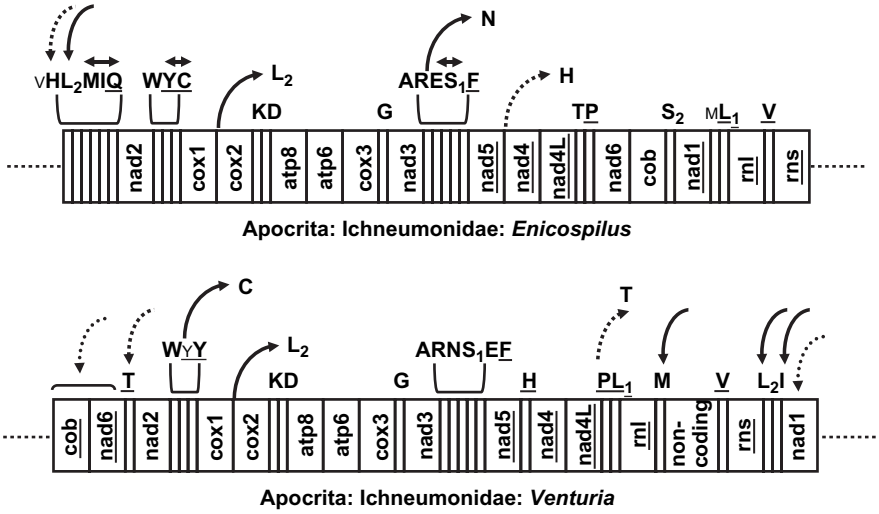

FIG. 4.—Mitochondrial genome organization of two ichneumonids, *Enicospilus* sp. and *Venturia canescens*. Gene transcription direction and tRNA abbreviations are as described in figure 2. Mitochondrial gene movements, relative to the ancestral organization, are indicated with arrows. Solid arrows indicate that the gene movement is along the same mitochondrial strand; broken line arrows indicate that the gene movement is an inversion. The bracket indicates the block movement of a group of mitochondrial genes. The tRNA genes in smaller script are presumed pseudogenes; other copies exist elsewhere in the genome and have higher cove scores.

with *trnI–trnQ–trnM–nad2*). The relative positions of *trnC* and *trnY* are reversed relative to the ancestral pancrustacean, whereas $trnL_2$ has moved out of the *cox1–cox2* junction, to a region containing four other mitochondrial tRNA genes, just upstream of *nad2*. There are two mitochondrial gene rearrangements within the *nad3–nad5* junction. *trnN* has moved out of this junction, although its derived position is not known. $trnS_1$ and *trnE* have swapped positions. *trnH* has moved out of the *nad5–nad4* junction, to the tRNA gene cluster upstream of *nad2*. It is the only inversional mitochondrial gene rearrangement in *Enicospilus*.

In *Venturia*, there are also at least five mitochondrial gene rearrangements relative to the pancrustacean mitochondrial gene order, but at least one of these involves an inversion of multiple protein-coding genes. This is the most drastic mitochondrial gene rearrangement seen in the newly sequenced mitochondrial genomes presented here. The pancrustacean arrangement of *nad6*, *cob*, and *nad1* is *nad6–cob–trnS2–nad1*. In *Venturia*, *cob* is upstream of *nad6*, with both on the opposite mitochondrial strand (*cob–nad6*). Therefore, a single inversion can explain this mitochondrial gene rearrangement. However, *nad1* is also inverted and at the opposite end of the sequenced fragment. Although this may represent two independent mitochondrial gene rearrangements, a more parsimonious explanation is that all three genes were inverted in a single mitochondrial gene rearrangement event, to *nad1–cob–nad6*. The junction between *nad1* and *cob* is not established, as this spans the unsequenced region of this mitochondrial genome. We made several attempts to amplify this mitochondrial gene junction, with primers designed to precisely match *Venturia nad1* and *cob*, but these were unsuccessful. We anticipate that a noncoding region exists between these two mitochondrial genes, as the noncoding region has proved consistently difficult to amplify in the Hymenoptera.

Four other mitochondrial gene rearrangements involve tRNA genes. *trnT* has inverted, and moved from its ancestral position (between *nad4L* and *nad6*) to the *nad6–nad2* gene junction. It is possible that this mitochondrial gene moved along with the protein-coding genes referred to above, as it is in the same relative position to *nad6*. However, the *trnP* gene normally lies between *nad6* and *trnT*, and the *trnP* gene has not moved. This makes it difficult to infer whether the *trnT* inversion is independent of the protein-coding gene inversion.

The *trnC* gene has moved out of the *nad2–cox1* gene junction, but its derived position is unknown. It is one of only three mitochondrial genes not identified in *Venturia* (the others are $trnS_2$ and *trnQ*). However, there appears to be a remnant of the *trnC* gene, in its ancestral position. tRNAScan identified this remnant as a *trnY* gene, and we annotated it as a *trnY* pseudogene due to the presence of another *trnY* gene with a higher cove score (fig. 4). However, when the *trnY* pseudogene is aligned with either the *trnC* or the *trnY* gene from *Enicospilus*, it shares a higher sequence identity with the *trnC* gene (82.3% compared with 62.3% for the *trnY* gene). We therefore tentatively identify this as a remnant of the *trnC* gene.

The mitochondrial $trnL_2$ gene has moved from the *cox1–cox2* gene junction, to a position between the *rns* and *nad1* genes. *trnM* is also in a derived position, at the *rnl* noncoding region junction. *trnI* is in a derived position, at the *rns–nad1* gene junction.

Discussion

The Number and Type of Mitochondrial Gene Rearrangements in the Hymenoptera

The number and type of each mitochondrial gene rearrangement that can be inferred from the nearly complete

or complete sequences from 14 hymenopterans aretabulated in table 2. These numbers are an underestimate for some taxa, due to the failure to obtain sequence in the vicinity of the noncoding region. We expect there to be some, but not substantial hidden change in these regions, given that there is not a large discrepancy when completely sequenced mitochondrial genomes are compared with those missing some sequence data.

Table 2 indicates that individual taxa vary in the number of mitochondrial gene rearrangements, with a low of one rearrangement in *Perga*, to a high of nine in *Apis*. Although we identify 67 mitochondrial genes that are in different relative positions compared with the ancestral pancrustacean mitochondrial genome organization, we consider our sample size small, and more robust trends will be established with observations from additional hymenopteran mitochondrial genomes.

Our data are consistent with each of the approximately 67 mitochondrial gene rearrangements occurring after the Hymenoptera split from the other holometabolous insect orders. This is based on the observation that all of the non-apocritans vary by only one or two mitochondrial gene placements relative to the ancestral pancrustacean, whereas the other holometabolous insect orders share none of the changes identified among the non-apocritans. Further, some of the changes observed within the non-apocritans are not observed in some Apocrita. For example, the one identifiable mitochondrial gene rearrangement in *Perga* is the movement of $trnL_1$ out of the *nad1–rnl* junction (fig. 2). However, the retention of $trnL_1$ in the ancestral position in both *Cephus* and *Orussus* is a strong indication that the inferred mitochondrial gene rearrangement occurred during the divergence of *Perga* from the other non-apocritan lineages, not in the ancestral hymenopteran.

Convergent and Potentially Synapomorphic Mitochondrial Gene Rearrangements Are Found in Relatively Equal Proportions

Of the 67 mitochondrial gene rearrangements described in table 2, just 14 are shared among five derived genome organizations; these are described in table 3. This leaves a minimum of 58 independently derived mitochondrial genome organizations; 53 uniquely derived mitochondrial genome organizations, plus five that are shared. It has been argued that the order of genes in the mitochondrial genome is selectively neutral (Brown 1985), but the observation that gene rearrangement is rare (Rokas and Holland 2000) is inconsistent with neutrality. If the order of genes in the mitochondrial genome really is neutral, then a large number of genome organizations should be observed, unless the rate of rearrangement is very low. The present study establishes that many mitochondrial genome organizations actually occur, consistent with the claim that mitochondrial gene order is selectively neutral (Brown 1985). However, the prediction that seemed to naturally flow from this claim, that convergent mitochondrial gene order would be rare, is not upheld by our study, nor a range of previous studies (Flook et al. 1995; Boore and Brown 1998).

Convergent Mitochondrial Gene Rearrangements

We propose that five of the 14 shared mitochondrial gene organizations are convergences (table 3). We infer these to be convergences on phylogenetic grounds, where two taxa share a mitochondrial gene rearrangement, but a taxon that is uncontroversially more closely related to one of the taxa retains the ancestral mitochondrial gene organization. For example, the *trnK* and the *trnD* genes have reversed in *Schlettererius*, *Apis*, and *Bombus* (rearrangements 6, 40, and 51). *Apis* and *Bombus* may be each other's closest relative—to be conservative for the purpose of the present study, we assume that they are. However, *Schlettererius* is not closely related to either taxon, and a range of aculeates retain the ancestral mitochondrial genome organization (e.g., *Abispa* and *Polistes*). This is strong evidence that the mitochondrial gene rearrangement in *Schlettererius* is independently derived. Similarly, *trnE* and $trnS_1$ are reversed in *Enicospilus*, *Apis*, and *Bombus*. The reversal in *Enicospilus* is almost certainly a convergence, as another representative from the Ichneumonidae (*Venturia*) retains the ancestral pancrustacean mitochondrial genome organization.

Although it could be argued that some of the mitochondrial gene rearrangements that we identify as convergences were synapomorphic, such interpretations are far less parsimonious, as they require a number of reversals. For example, for the *trnK–trnD* reversal to be synapomorphic for *Schlettererius*, *Apis*, and *Bombus*, reversals would have to be proposed for both *Primeuchroeus* and the two vespids (*Abispa* and *Polistes*). Even if these reversals did occur, this does not alter the conclusion that a very large number of mitochondrial genome organizations are possible and are not selected against. Importantly, it is the observation of a large variety of mitochondrial genome organizations that supports our contention that gene position is selectively neutral.

Mitochondrial Gene Rearrangements That May Be Identified as Synapomorphic, After Further Sampling

We propose that nine of the 14 shared mitochondrial gene organizations are synapomorphic (table 3), although these require further sampling to verify. For example, the *trnE–*$trnS_1$ reversal may be a synapomorphy in *Apis* and *Bombus*. These two genes are in identical positions relative to each other in *Apis* and *Bombus*. However, in *Apis*, these two mitochondrial genes are upstream of *nad2*, whereas in *Bombus*, they are at the *nad3–nad5* junction. To be conservative, we count them as a synapomorphy, but they may also be independently derived.

The *trnA* gene has moved just upstream of the *nad2* gene in *Apis*, *Melipona*, and *Bombus*. All of these taxa are from the Apidae, such that this may represent a derived position that is, at least, common to this family. Although the derived position of *trnA* is similar in *Apis* and *Bombus* (*trnM–trnA–trnI* in *Bombus*, *trnM–trnQ–trnA–trnI* in *Apis*), it is not identical. Further, it is distinctly different in *Melipona* (*trnI–trnA–*<u>*trnK*</u>*–trnM*).

The *trnI* and *trnQ* genes have swapped positions in both *Cephus* and *Orussus* (*trnI–*<u>*trnQ*</u> → <u>*trnQ*</u>*–trnI*), so it

Table 2
Number and Type of Mitochondrial Gene Rearrangements among 14 Hymenopterans, Relative to the Pancrustacean Gene Order

Taxon	Rearrangement Description
Perga	1. *trnL*$_1$ moves, derived position unknown
Cephus	2. *trnM* moves out of *trnI–trnQ–trnM* cluster, next to *rns*
	3. *trnI* and *trnQ* swap positions
Orussus	4. *trnM* moves to *cob–nad1* junction
	5. *trnI* and *trnQ* swap positions
Schlettererius	6. *trnK* and *trnD* swap positions
	7. *trnE* moves across three tRNA boundaries
Enicospilus	8. *trnI–trnQ–trnM* becomes *trnM–trnI–trnQ*
	9. *trnC* and *trnY* swap positions
	10. *trnL*$_2$ moves from *cox1-cox2* to *trnI-trnQ-trnM* cluster
	11. *trnN* moves from *trnA–trnR–trnN–trnS–trnE–trnF* cluster, derived position unknown
	12. *trnE* and *trnS*$_1$ swap positions
	13. Long-range inversion of *trnH* from *nad3–nad5*, to *trnI–trnQ–trnM* cluster
Venturia	14. Long-range inversion of *cob*, *nad6*, and *trnT*, upstream of *nad2*
	15. *trnC* moves from *trnW–trnC–trnY* cluster, derived position unknown
	16. *trnL*$_2$ moves from *cox1–cox2* junction, to *rns–nad1* boundary
	17. *trnM* moves from *trnI–trnQ–trnM* cluster, to downstream of *rnl*
	18. *trnI* moves from *trnI–trnQ–trnM* cluster, to *rns–nad1* junction
Primeuchroeus	19. Inversion of *trnM*, moves to upstream of *cox1*
	20. Inversion of *trnI*, moves to upstream of *cox1*
	21. Long-range inversion of *trnQ*, from *trnI–trnQ–trnM* to *trnA–trnR–trnN–trnS–trnE–trnF* cluster
	22. *trnW* moves from *trnW–trnC–trnY* cluster, derived position unknown
	23. *trnL*$_2$ moves from *cox1–cox2* junction, to downstream of *rnl*
	24. Inversion and movement of *trnR* to lie next to *nad3*
	25. Block inversion of *trnL*$_1$*–rnl–trnV–rns*
Abispa	26. *trnI–trnQ–trnM* becomes *trnM–trnQ–trnI*
	27. *trnI* and *trnQ* swap positions
	28. *trnL*$_1$ moves from *nad1–rnl* junction, to *trnI–trnQ–trnM* cluster
Polistes	29. Inversion of *trnI*
	30. *trnQ* and *trnM* move, derived position unknown
	31. *trnY* moves out of *trnW–trnC–trnY* cluster, derived position unknown
	32. *trnL*$_1$ moves from *nad1–rnl* junction, to *cob–nad1* junction
Apis	33. *trnA*, *trnS* and *trnE* move from *trnA-trnR-trnN-trnS-trnE-trnF* to *trnI–trnQ–trnM* cluster
	34. *trnE* and *trnS*$_1$ swap positions
	35. *trnA* changes position relative to *trnS*$_1$ and *trnE*
	36. *trnW* moves across two tRNA boundaries
	37. Inversion of *trnQ*
	38. *trnI–trnQ–trnM* becomes *trnM–trnQ–trnI*
	39. *trnI* moves downstream relative to *trnQ*
	40. *trnK* and *trnD* swap positions
	41. Local inversion of trnR
Melipona	42. Long-range movement of *trnA*, from *trnA–trnR–trnN–trnS–trnE–trnF* upstream of *trnI*
	43. Long-range inversion of *trnK*, from *cox2–atp8* to *trnI–trnQ–trnM* cluster
	44. Local inversion of *trnC*, moves upstream one tRNA gene
	45. Local inversion of *trnR*
	46. Local inversion of *trnT*
Bombus	47. *trnQ* moves out of *trnI–trnQ–trnM* cluster, to downstream of *rns*
	48. *trnI–trnQ–trnM* becomes *trnM–trnI*
	49. *trnA* moves from *trnA–trnR–trnN–trnS–trnE–trnF* cluster, to between *trnM* and *trnI*
	50. *trnW* moves across two tRNA boundaries
	51. *trnK* and *trnD* swap positions
	52. *trnE* and *trnS*$_1$ swap positions
	53. Local inversion of *trnR*
	54. *trnT* and *trnP* swap positions
Nasonia	55. Block inversion of *cox1–trnL*$_2$*–cox2–trnD–trnK–atp8–atp6–cox3–trnG–nad3*
	56. Local inversion of *trnK*
	57. *nad2–trnW–trnC–trnY* moves, to upstream of *nad1*
	58. *trnC* moves from *trnW–trnC–trnY* cluster, derived position unknown
	59. *trnN* moves from *trnA–trnR–trnN–trnS–trnE–trnF* cluster, to upstream of *nad1*
	60. *trnA* moves from *trnA–trnR–trnN–trnS–trnE–trnF* cluster, to *rnl–rns* gene junction
	61. *trnV* moves from *rnl–rns*, derived position unknown
Vanhornia	62. *trnM* inversion, from *trnI–trnQ–trnM* cluster, to *nad1–rnl* junction
	63. *trnL*$_2$ moves from *cox1–cox2*, to downstream of *cox2*
	64. *trnR* moves from *trnA–trnR–trnN–trnS–trnE–trnF* cluster, derived position unknown
	65. *trnA* rearranges within *trnA–trnR–trnN–trnS–trnE–trnF* cluster
	66. *trnN* rearranges within *trnA–trnR–trnN–trnS–trnE–trnF* cluster
	67. *trnL*$_1$ inversion, from *nad1-rnl*, to upstream of *nad2*

Underlined descriptions indicate that an identical gene rearrangement is found elsewhere in the descriptions. Reference numbers facilitate the identification of each rearrangement elsewhere in the study.

Table 3
Synapomorphic and Convergent Mitochondrial Gene Rearrangements

Gene Rearrangement	Category
trnI and *trnQ* swap positions (3, 5, and 27)	C
trnK and *trnD* swap positions (6, 40, and 51)	S (40, 51), C (6)
trnE and $trnS_1$ swap positions (12, 34, and 52)	S (34, 52), C (12)
Local inversion of *trnR* (41, 45, and 53)	S
trnW moves across two tRNA boundaries (36, 50)	S

C, convergent; S, synapomorphic. Numbers in brackets refer to specific rearrangements, as described in table 2.

is tempting to suggest that this may be a derived mitochondrial genome organization that appeared early during the evolution of the order. However, this putatively derived organization is retained only in *Abispa*. The ancestral organization (*trnI–trnQ*) may be retained in *Enicospilus*, but the two genes are separated by a *trnM* pseudogene. The fact that most hymenopterans have the *trnI* and/or *trnQ* genes in different positions makes the location of these two tRNA genes too variable to reach any firm conclusion.

The contention that gene position is neutral led to the prediction that convergent mitochondrial gene order should be rare. However, this prediction is not upheld by our study. As far as our data permit, four of the shared, derived mitochondrial gene organizations are synapomorphic (table 3), whereas three are convergent. However, these numbers are heavily influenced by the sampling strategy. We chose divergent Hymenoptera, in order to maximally capture different mitochondrial genome organizations. Such a strategy is unlikely to find synapomorphies, unless mitochondrial genome organization is relatively stable at higher taxonomic levels (e.g., across hymenopteran superfamilies).

Finally, our study suggests that the few potentially synapomorphic mitochondrial gene rearrangements should be viewed with caution. For example, *Apis* and *Bombus* both share a reversal of the positions of the *trnK* and *trnD* genes (to *trnD–trnK*; table 3), but *Melipona* only has the *trnD* gene present at the *cox1–atp8* junction. A superficial analysis would place *Apis* and *Bombus* together (to the exclusion of *Melipona*), but it is unclear whether the *Melipona* mitochondrial organization represents an independent derivation, or one that occurred after the *trnK–trnD* reversal. The observation that the *trnK–trnD* reversal has independently occurred in *Schlettererius*, the Scelionidae (Dowton and Austin 1999), and the caeliferan grasshoppers (Flook et al. 1995; Fenn et al. 2008) makes us doubly cautious about the phylogenetic utility of this mitochondrial gene rearrangement.

Is Mitochondrial Gene Order Selectively Neutral

Although this is very difficult to test experimentally, we can begin to investigate this claim by examining whether some mitochondrial genes appear less mobile when compared with others; mitochondrial gene boundaries that are retained in the face of accelerated mitochondrial gene rearrangement may indicate some selective constraint on mitochondrial genome organization. For the current discussion, we restrict our commentary to tRNA genes. Although it is clear that protein-coding genes are less mobile than tRNA genes, this could be due to size differences as much as selective differences—the probability of a mitochondrial gene rearrangement being lethal or a selective disadvantage (e.g., by not encompassing the entire gene) may be much greater for a larger gene than it is for a smaller gene. In addition, strand compositional skews may severely restrict the movement of protein-coding genes between strands (Foster et al. 1997; Min and Hickey 2007). Of the 67 mitochondrial gene rearrangements observed, we calculated how many involved each tRNA gene (supplementary table 1, Supplementary Material online). *trnM* was observed to be the most mobile, changing position nine times. Further, mitochondrial genes close to the ancestral position of the noncoding region (the *trnI–trnQ–trnM* cluster; fig. 2) were the most mobile, with *trnI* changing position seven times, *trnQ* changing position six times. Although others have noted that tRNA genes next to the noncoding region are generally more mobile (Duarte et al. 2008), many of the other tRNA genes are nearly as mobile; for example, *trnK* and $trnL_2$ have each moved five times. Indeed, a chi-squared test indicated that the number of rearrangements for each mitochondrial gene was not significantly different from the scenario in which each mitochondrial gene moved with equiprobability (chi-squared $= 31.69$, df $= 21$; $P = 0.06$).

Nevertheless, some tRNA genes have not moved at all; *trnF*, *trnG*, and $trnS_2$ have not moved in any of the sampled hymenopteran mitochondrial genomes. Both *trnF* and $trnS_2$ are on a boundary, where transcriptional polarity changes (see fig. 2). We compared the nonmobile tRNAs with the transcriptional map of *Drosophila*, as reported by Berthier et al. (1986), and summarized by Stewart (see fig. 4.2 in Stewart 2005). There are five main mitochondrial transcripts produced by *Drosophila*, three of which terminate close to the positions of the nonmobile tRNAs. Together, these data suggest that efficient mtDNA transcription and/or processing may require the presence of a tRNA gene as a termination signal, in order to minimize the transcription of noncoding DNA. However, given that there is no statistical difference in the number of movements of each tRNA gene, further evaluation of this hypothesis will require much more data than examined in the present study.

It has been proposed that mitochondrial tRNA genes that are located singly (i.e., between two protein-coding genes) are less mobile (Cha et al. 2007), but we do not generally find this. *trnH*, $trnL_1$, $trnL_2$, and *trnV* are positioned in this way in the ancestral pancrustacean mitochondrial genome, but both $trnL_1$ and $trnL_2$ have been involved in a number of mitochondrial gene rearrangements within Hymenoptera (supplementary table 1, Supplementary Material online).

Mitochondrial Gene Rearrangements Consistent with the Duplication/Random Loss Model Do Not Predominate

We next estimated the proportion of mitochondrial gene rearrangements that were consistent with the

duplication/random loss model (Moritz et al. 1987; Macey et al. 1997), compared with those that were not. Mitochondrial gene rearrangements that are consistent with this model are local rearrangements, where tRNA genes move a small number of gene boundaries (typically within a tRNA gene cluster), but do not move from one mitochondrial strand to the other. Rearrangements that are not consistent with this model include inversions and long-range translocations. One of us (M.D.) has argued previously that long-range translocations, without any additional change in the intervening mitochondrial gene order, is highly unlikely under the duplication/random loss model (Dowton and Austin 1999; Dowton and Campbell 2001). To be conservative, we classified any tRNA gene movement across a single protein-coding gene (or the noncoding region) as a local rearrangement, because the chance of reestablishing the original mitochondrial gene order surrounding the gene movement is not as low. Within this framework, of the 67 mitochondrial gene rearrangements identified in table 2, 27 are local rearrangements consistent with the duplication/random loss model. However, rearrangements inconsistent with this model outnumbered those that did, with 19 inversions and 16 long-range movements (four were unclassified, as the derived position of the mitochondrial gene was unknown, and based on the missing sequence data, it was unclear which category the mitochondrial gene rearrangement belonged to). A chi-square test indicated no significant difference in the proportion of each type of mitochondrial gene rearrangement, when compared with a scenario in which each type of rearrangement occurred at equal frequency (chi-squared = 3.25, df = 2; P = 0.20). The test remained nonsignificant when potential synapomorphies (such as the *trnR* inversion) were only counted a single time.

A number of studies have characterized mitochondrial gene rearrangements that are consistent with the duplication/random loss model (e.g., Macey et al. 1997; Fujita et al. 2007). Strong evidence for duplication/random loss comes from the presence of mitochondrial pseudogenes or intergenic spacers (Macey et al. 1998; San Mauro et al. 2006; Fujita et al. 2007). Although mitochondrial pseudogenes and intergenic spacers are generally rare, their presence close to rearranged mitochondrial genes is highly consistent with the duplication/random loss model. Indeed, we found three such pseudogenes among the two ichneumonid wasps sequenced here.

However, a range of studies have reported mitochondrial gene rearrangements that are more consistent with other mechanisms. In particular, inversions (where a mitochondrial gene moves from one strand to the other) cannot be explained by duplication/random loss (Dowton and Austin 1999; Amer and Kumazawa 2007) but are more consistent with recombination (Dowton and Campbell 2001). A number of recent studies have reported mitochondrial genome organizations that appear to be the products of both duplication/random loss and recombination (Miller et al. 2004; Mueller and Boore 2005; Sun et al. 2005; Mizi et al. 2006; Shao et al. 2006; Kurabayashi et al. 2008). Similarly, we see evidence of both mechanisms in the hymenopteran mitochondrial genomes reported here.

Gene Rearrangements and the Evolution of Parasitism

We previously found that the number of mitochondrial gene rearrangements in hymenopterans that evolved after the evolution of parasitism had consistently higher levels of mitochondrial gene rearrangement when compared with those that diverged prior to the appearance of parasitism (Dowton and Austin 1999). Others have found an association between parasitic insects and the rate of mitochondrial gene rearrangement (Shao et al. 2001; Covacin et al. 2006), although this is not the case for some parasitic groups (Castro et al. 2002). Using the tree in figure 1 as a guide, *Perga* and *Cephus* are the only two hymenopterans sampled prior to the evolution of parasitism. *Orussus* is an important inclusion, as it is the only parasitic non-apocritan and is thought to represent the sister group to the Apocrita. Table 2 indicates that the association between parasitism and an increased rate of mitochondrial gene rearrangement does not appear to be supported in light of the additional data presented here. Although the ancestrally nonparasitic hymenopterans (*Perga* and *Cephus*) have the lowest number of mitochondrial gene rearrangements (one and two, respectively), they are not lower than in *Orussus* (two rearrangements), whereas both *Abispa* and *Polistes* have comparable numbers (three and four, respectively). We consider these comparisons conservative; *Cephus*, *Orussus*, and *Abispa* are completely sequenced, so there are no hidden changes, whereas *Perga* is not completely sequenced, so there may be hidden changes in this genome. Although table 2 indicates that there is clearly a trend toward an increase in both the number and scale of mitochondrial gene rearrangement in some of the hymenopteran parasitic lineages (e.g., the inversion of multiple protein-coding genes in *Venturia* and *Nasonia*), there are no clear trends among phylogenetically related groups.

Supplementary Material

Supplementary table 1 is available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

Acknowledgments

Thanks to Micaela Butler, Otto Schmidt, Suzanne Schulmeister, and Bob Wharton for donating specimens. Thanks also to John LaSalle for identifying *Enicospilus*. This work was supported by a grants from the Australian Research Council (M.D., A.D.A.), the Australian Academy of Science (M.D.), and National Science Foundation grants DEB0120718 (M.F.W.) and DEB0444972 (S.L.C., M.F.W.). Thanks also to Christian Stauffer, who kindly hosted M.D. during a sabbatical in late 2008, during which some of the analyses were done and the manuscript were written.

Literature Cited

Amer SAM, Kumazawa Y. 2007. The mitochondrial genome of the lizard *Calotes versicolor* and a novel gene inversion in

south Asian draconine agamids. Mol Biol Evol. 24: 1330–1339.
Belshaw R, Fitton M, Herniou E, Gimeno C, Quicke DLJ. 1998. A phylogenetic reconstruction of the Ichneumonoidea (Hymenoptera) based on the D2 variable region of 28S ribosomal RNA. Syst Entomol. 23:109–123.
Berthier F, Renaud M, Alziari S, Durand R. 1986. RNA mapping on *Drosophila* mitochondrial DNA: precursors and template strands. Nucl Acids Res. 14:4519–4533.
Boore JL. 1999. Animal mitochondrial genomes. Nucl Acids Res. 27:1767–1780.
Boore JL, Brown WM. 1998. Big trees from little genomes: mitochondrial gene order as a phylogenetic tool. Curr Opin Genet Dev. 8:668–674.
Boore JL, Lavrov DV, Brown WM. 1998. Gene translocation links insects and crustaceans. Nature. 392:667–668.
Brown WM. 1985. The mitochondrial genome of animals. In: MacIntyre RJ, editor. Molecular evolutionary genetics. New York: Plenum. p. 95–130.
Cameron S, Johnson K, Whiting M. 2007. The mitochondrial genome of the screamer louse *Bothriometopus* (Phthiraptera: Ischnocera): effects of extensive gene rearrangements on the evolution of the genome. J Mol Evol. 65:589–604.
Cameron SL, Dowton M, Castro LR, Ruberu K, Whiting MF, Austin AD, Diement K, Stevens J. 2008. Mitochondrial genome organization and phylogeny of two vespid wasps. Genome. 51:800–808.
Cameron SL, Lambkin CL, Barker SC, Whiting MF. 2007. A mitochondrial genome phylogeny of Diptera: whole genome sequence data accurately resolve relationships over broad timescales with high precision. Syst Entomol. 32:40–59.
Cameron SL, Whiting MF. 2007. Mitochondrial genomic comparisons of the subterranean termites from the genus *Reticulitermes* (Insecta: Isoptera: Rhinotermitidae). Genome. 50:188–202.
Castro LR, Austin AD, Dowton M. 2002. Contrasting rates of mitochondrial molecular evolution in parasitic Diptera and Hymenoptera. Mol Biol Evol. 19:1100–1113.
Castro LR, Dowton M. 2005. The position of the Hymenoptera within the Holometabola as inferred from the mitochondrial genome of *Perga condei* (Hymenoptera: Symphyta: Pergidae). Mol Phylogenet Evol. 34:469–479.
Castro LR, Dowton M. 2006. Molecular analyses of the Apocrita (Insecta: Hymenoptera) suggest that the Chalcidoidea are sister to the diaprioid complex. Invert Syst. 20:603–614.
Castro LR, Ruberu K, Dowton M. 2006. Mitochondrial genomes of *Vanhornia eucnemidarum* (Apocrita: Vanhorniidae) and *Primeuchroeus* spp. (Aculeata: Chrysididae): evidence of rearranged mitochondrial genomes within the Apocrita (Insecta: Hymenoptera). Genome. 49:752–766.
Cha SY, Yoon HJ, Lee EM, Yoon MH, Hwang JS, Jin BR, Han YS, Kim I. 2007. The complete nucleotide sequence and gene organization of the mitochondrial genome of the bumblebee, *Bombus ignitus* (Hymenoptera: Apidae). Gene. 392:206–220.
Cook CE. 2005. The complete mitochondrial genome of the stomatopod crustacean *Squilla mantis*. BMC Genomics. 6:105.
Covacin C, Shao R, Cameron S, Barker SC. 2006. Extraordinary number of gene rearrangements in the mitochondrial genomes of lice (Phthiraptera: Insecta). Ins Mol Biol. 15:63–68.
Crease TJ. 1999. The complete sequence of the mitochondrial genome of *Daphnia pulex* (Cladocera: Crustacea). Gene. 233:89–99.
Crozier RH, Crozier YC. 1993. The mitochondrial genome of the honeybee *Apis mellifera*: complete sequence and genome organization. Genetics. 133:97–117.
Dong S, Kumazawa Y. 2005. Complete mitochondrial DNA sequences of six snakes: phylogenetic relationships and molecular evolution of genomic features. J Mol Evol. 61:12–22.
Dowton M. 1999. Relationships among the cyclostome braconid (Hymenoptera: Braconidae) subfamilies inferred from a mitochondrial tRNA gene rearrangement. Mol Phylogenet Evol. 11:283–287.
Dowton M, Austin AD. 1995. Increased genetic diversity in mitochondrial genes is correlated with the evolution of parasitism in the Hymenoptera. J Mol Evol. 41:958–965.
Dowton M, Austin AD. 1999. Evolutionary dynamics of a mitochondrial rearrangement "hotspot" in the Hymenoptera. Mol Biol Evol. 16:298–309.
Dowton M, Austin AD. 2001. Simultaneous analysis of 16S, 28S, COI and morphology in the Hymenoptera: Apocrita—evolutionary transitions among parasitic wasps. Biol J Linn Soc. 74:87–111.
Dowton M, Campbell NJH. 2001. Intramitochondrial recombination—is it why some mitochondrial genes sleep around? Trends Ecol Evol. 16:269–271.
Dowton M, Castro LR, Campbell SL, Bargon SD, Austin AD. 2003. Frequent mitochondrial gene rearrangements at the hymenopteran nad3–nad5 junction. J Mol Evol. 56: 517–526.
Duarte GT, De Azeredo-Espin AML, Junqueira ACM. 2008. The mitochondrial control region of blowflies (Diptera: Calliphoridae): a hot spot for mitochondrial genome rearrangements. J Med Entomol. 45:667–676.
Fenn JD, Song H, Cameron SL, Whiting MF. 2008. A preliminary mitochondrial genome phylogeny of Orthoptera (Insecta) and approaches to maximizing phylogenetic signal found within mitochondrial genome data. Mol Phylogenet Evol. 49:59–68.
Flook P, Rowell H, Gellissen G. 1995. Homoplastic rearrangements of insect mitochondrial tRNA genes. Naturwissenschaften. 83:336–337.
Foster PG, Jermiin LS, Hickey DA. 1997. Nucleotide composition bias affects amino acid content in proteins coded by animal mitochondria. J Mol Evol. 44:282–288.
Fujita MK, Boore JL, Moritz C. 2007. Multiple origins and rapid evolution of duplicated mitochondrial genes in parthenogenetic geckos (*Heteronotia binoei*; Squamata, Gekkonidae). Mol Biol Evol. 24:2775–2786.
Gibson GAP. 1985. Some pro- and mesothoracic structures important for phylogenetic analysis of Hymenoptera, with a review of terms used for the structures. Can Ent. 117: 1395–1443.
Gissi C, Iannelli F, Pesole G. 2008. Evolution of the mitochondrial genome of Metazoa as exemplified by comparison of congeneric species. Heredity. 101:301–320.
Grande C, Templado J, Cervera JL, Zardoya R. 2004. Molecular phylogeny of Euthyneura (Mollusca: Gastropoda). Mol Biol Evol. 21:303–313.
Haring E, Kruckenhauser L, Gamauf A, Riesing MJ, Pinsker W. 2001. The complete sequence of the mitochondrial genome of *Buteo buteo* (Aves, Accipitridae) indicates an early split in the phylogeny of raptors. Mol Biol Evol. 18:1892–1904.
Johnson NF. 1988. Midcoxal articulations and the phylogeny of the order Hymenoptera. Ann Ent Soc Am. 81:870–881.
Kondo R, Oorai S, Satta Y, Takahata N. 1993. Evolution of the hominoid mitochondrial DNA with special reference to the silent substitution rate over the genome. J Mol Evol. 36:517–531.
Kumazawa Y, Nishida M. 1995. Variations in mitochondrial tRNA gene organization of reptiles as phylogenetic markers. Mol Biol Evol. 12:759–772.

Kurabayashi A, Sumida M, Yonekawa H, Glaw F, Vences M, Hasegawa M. 2008. Phylogeny, recombination, and mechanisms of stepwise mitochondrial genome reorganization in mantellid frogs from Madagascar. Mol Biol Evol. 25:874–891.

Lowe TM, Eddy SR. 1997. tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. Nucleic Acids Res. 25:955–964.

Macey JR, Larson A, Ananjeva NB, Fang Z, Papenfuss TJ. 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.

Macey JR, Papenfuss TJ, Kuehl JV, Fourcade HM, Boore JL. 2004. Phylogenetic relationships among amphisbaenian reptiles based on complete mitochondrial genomic sequences. Mol Phylogenet Evol. 33:22–31.

Macey JR, Schulte JA II, Larson A, Papenfuss TJ. 1998. Tandem duplication via light-strand synthesis may provide a precursor for mitochondrial genomic rearrangement. Mol Biol Evol. 15:71–75.

Milinkovitch MC, Orti G, Meyer A. 1993. Revised phylogeny of whales suggested by mitochondrial ribosomal DNA sequences. Nature. 361:346–348.

Miller AD, Nguyen TTT, Burridge CP, Austin CM. 2004. Complete mitochondrial DNA sequence of the Australian freshwater crayfish, Cherax destructor (Crustacea: Decapoda: Parastacidae): a novel gene order revealed. Gene. 331:65–72.

Min XJ, Hickey DA. 2007. DNA asymmetric strand bias affects the amino acid composition of mitochondrial proteins. DNA Res. 14:201–206.

Miya M, Takeshima H, Endo H, et al. (11 co-authors). 2003. Major patterns of higher teleostean phylogenies: a new perspective based on 100 complete mitochondrial DNA sequences. Mol Phylogenet Evol. 26:121–138.

Mizi A, Zouros E, Rodakis GC. 2006. Multiple events are responsible for an insertion in a paternally inherited mitochondrial genome of the mussel *Mytilus galloprovincialis*. Genetics. 172:2695–2698.

Moritz C, Dowling TE, Brown WM. 1987. Evolution of animal mitochondrial DNA: relevance for population biology and systematics. Annu Rev Ecol Syst. 18:269–292.

Mueller RL, Boore JL. 2005. Molecular mechanisms of extensive mitochondrial gene rearrangement in plethodontid salamanders. Mol Biol Evol. 22:2104–2112.

Oliveira DCSG, Raychoudhury R, Lavrov DV, Werren JH. 2008. Rapidly evolving mitochondrial genome and directional selection in mitochondrial genes in the parasitic wasp *Nasonia* (Hymenoptera: Pteromalidae). Mol Biol Evol. 25:2167–2180.

Reyes A, Gissi C, Pesole G, Saccone C. 1998. Asymmetrical directional mutation pressure in the mitochondrial genome of mammals. Mol Biol Evol. 15:957–966.

Rokas A, Holland PWH. 2000. Rare genomic changes as a tool for phylogenetics. Trends Ecol Evol. 15:454–459.

San Mauro D, Gower DJ, Zardoya R, Wilkinson M. 2006. A hotspot of gene order rearrangement by tandem duplication and random loss in the vertebrate mitochondrial genome. Mol Biol Evol. 23:227–234.

Schulmeister S, Wheeler WC, Carpenter JM. 2002. Simultaneous analysis of the basal lineages of Hymenoptera (Insecta) using sensitivity analysis. Cladistics. 18:455–484.

Shao R, Barker SC. 2002. The highly rearranged mitochondrial genome of the plague thrips, *Thrips imaginis* (Insecta: Thysanoptera): convergence of two novel gene boundaries and an extraordinary arrangement of rRNA genes. Mol Biol Evol. 20:362–370.

Shao R, Barker S, Mitani H, Takahashi M, Fukunaga M. 2006. Molecular mechanisms for the variation of mitochondrial gene content and gene arrangement among chigger mites of the genus *Leptotrombidium* (Acari: Acariformes). J Mol Evol. 63:251–261.

Shao R, Campbell NJH, Barker SC. 2001. Numerous gene rearrangements in the mitochondrial genome of the wallaby louse, *Heterodoxus macropus* (Phthiraptera). Mol Biol Evol. 18:858–865.

Shao R, Dowton M, Murrell A, Barker SC. 2003. The rates of gene rearrangement and nucleotide substitution are positively correlated in the mitochondrial genomes of insects. Mol Biol Evol. 20:1612–1619.

Sheffield NC, Song H, Cameron SL, Whiting MF. 2008. A comparative analysis of mitochondrial genomes in Coleoptera (Arthropoda: Insecta) and genome descriptions of six new beetles. Mol Biol Evol. 25:2499–2509.

Silvestre D, Dowton M, Arias MC. 2008. The mitochondrial genome of the stingless bee *Melipona bicolor* (Hymenoptera, Apidae, Meliponini): sequence, gene organization and a unique tRNA translocation event conserved across the tribe Meliponini. Gen Mol Biol. 31:451–460.

Simon C. 1991. Molecular systematics at the species boundary: exploiting conserved and variable regions of the mitochondrial genome of animals via direct sequencing from amplified DNA. In: Hewitt G, Johnston AWB, Young JPW, editors. Molecular techniques in taxonomy. Berlin: Springer-Verlag. p. 33–71.

Smith MJ, Arndt A, Gorski S, Fajber E. 1993. The phylogeny of echinoderm classes based on mitochondrial gene arrangements. J Mol Evol. 36:545–554.

Stewart JB. 2005. The evolution of mitochondrial genome structure and function in insects. Molecular biology and biochemistry. Burnaby: Simon Fraser University. p. 196.

Sun H, Zhou K, Song D. 2005. Mitochondrial genome of the Chinese mitten crab *Eriocheir japonica sinenesis* (Brachyura: Thoracotremata: Grapsoidea) reveals a novel gene order and two target regions of gene rearrangements. Gene. 349: 207–217.

Sunnucks P, Hales DF. 1996. Numerous transposed sequences of mitochondrial cytochrome oxidase I-II in aphids of the genus *Sitobion* (Hemiptera: Aphididae). Mol Biol Evol. 13:510–524.

Vilhelmsen L. 1997. The phylogeny of lower Hymenoptera (Insecta), with a summary of the early evolutionary history of the order. J Zool Syst Evol Res. 35:49–70.

Vilhelmsen L. 2001. Phylogeny and classification of the extant basal lineages of the Hymenoptera (Insecta). Zool J Linn Soc. 131:393–442.

Whitfield JB. 1992. Phylogeny of the non-aculeate Apocrita and the evolution of parasitism in the Hymenoptera. J Hymenop Res. 1:3–14.

Martin Embley, Associate Editor

Accepted April 6, 2009