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The mitochondrial genome of the 'twisted-wing parasite' *Mengenilla australiensis* (Insecta, Strepsiptera): a comparative study

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Abstract

Background: Strepsiptera are an unusual group of sexually dimorphic, entomophagous parasitoids whose evolutionary origins remain elusive. The lineage leading to *Mengenilla australiensis* (Family Mengenillidae) is the sister group to all remaining extant strepsipterans. It is unique in that members of this family have retained a less derived condition, where females are free-living from pupation onwards, and are structurally much less simplified. We sequenced almost the entire mitochondrial genome of *M. australiensis* as an important comparative data point to the already available genome of its distant relative *Xenos vesparum* (Family Xenidae). This study represents the first in-depth comparative mitochondrial genomic analysis of Strepsiptera.

Results: The partial genome of *M. australiensis* is presented as a 13421 bp fragment, across which all 13 protein-coding genes (PCGs), 2 ribosomal RNA (rRNA) genes and 18 transfer RNA (tRNA) sequences are identified. Two tRNA translocations disrupt an otherwise ancestral insect mitochondrial genome order. A+T content is measured at 84.3%, C-content is also very skewed. Compared with *M. australiensis*, codon bias in *X. vesparum* is more balanced. Interestingly, the size of the protein coding genome is truncated in both strepsipterans, especially in *X. vesparum* which, uniquely, has 4.3% fewer amino acids than the average holometabolan complement. A revised assessment of mitochondrial rRNA secondary structure based on comparative structural considerations is presented for *M. australiensis* and *X. vesparum*.

Conclusions: The mitochondrial genome of *X. vesparum* has undergone a series of alterations which are probably related to an extremely derived lifestyle. Although *M. australiensis* shares some of these attributes; it has retained greater signal from the hypothetical most recent common ancestor (MRCA) of Strepsiptera, inviting the possibility that a shift in the mitochondrial selective environment might be related to the specialization accompanying the evolution of a small, morphologically simplified completely host-dependent lifestyle. These results provide useful insights into the nature of the evolutionary transitions that accompanied the emergence of Strepsiptera, but we emphasize the need for adequate sampling across the order in future investigations concerning the extraordinary developmental and evolutionary origins of this group.

Background

Strepsiptera are an unusual group of obligate endoparasitoid insects [1]. They occur as a small (approx. 600 spp.) monophyletic insect order with uncertain evolutionary origins, inclusive of any clear understanding over the group's nearest extant relative. Strepsiptera parasitize 7 orders of insects, including silverfish (Thysanura); cockroaches (Blattaria); mantids (Mantodea); crickets and grasshoppers (Orthoptera); bugs (Hemiptera); wasps, ants and bees (Hymenoptera) and flies (Diptera). Understandably, the very uniqueness of Strepsiptera makes their placement within insects using morphological taxonomic methods a contentious task. Strepsiptera have been placed as the sister-group to myriad different insect groups, from beetles to true flies, even being placed outside of Holometabola: each hypothesis being founded on one or two 'key' characteristics that at one time or another have come under question [2-15]. Confident assertions of classification have especially been restricted because intermediate forms have largely gone extinct and are unrecorded in the fossil record (see [16] for a notable exception). Simplification of gross morphology during strepsipteran evolutionary specialization can also be viewed as a significant component of this problem: observable morphological variation is low and unevenly distributed between extremely dimorphic sexes.

Females are especially strongly simplified (lacking in most typical adult characters such as wings, legs or mouthparts), and in most strepsipteran species, they remain in the living host until the end of the reproductive cycle. Conversely, males metamorphose in a typical holometabolan fashion; developing wings and a usual suite of adult insect characters (such as antennae, mouthparts and compound eyes) only to leave the host immediately in search of a mate; usually in the form of a female-containing host [17-19]. Males deliver sperm through a brood canal opening in the cephalothorax (the modified "head") of the female, who as a reproductive adult is only partially exposed to the environment, as an extrusion between the tergites or sternites of living hosts. After fertilization, females are capable of producing many hundreds of thousands of active 1st instar larvae who emerge from the cephalothorax.

Members of the strepsipteran lineage, Mengenillidae, are the sister-group to Stylopodia, a clade that includes all other extant Strepsiptera [[20], unpublished data]. This family represents a transitional phase in the evolutionary specialization of Strepsiptera, whereby both sexes leave the host before pupation, and females do not reproduce or release progeny in the unusual fashion outlined above. The level of simplification in free living mengenillid females is much less extreme than in Stylopodia (e.g. *X. vesparum*); legs, mouthparts and compound eyes are all

still present, although strongly reduced. The invention of a completely endoparasitoid female was probably one of the most important novelties leading to the radiation of this unique group [21]. Evolutionary studies attempting to unravel the developmental and ecological phenomena that make Strepsiptera so biologically interesting therefore require that species from before this important transition occupy a central role in research.

Insect mitochondrial genomes provide a useful medium to deepen and connect comprehension of microevolutionary forces of populations, like neutral drift and selective sweeps, to macroevolutionary events affecting species and/or deeper levels of divergence. The abundance of mitochondria in most metazoan cell and tissue types makes mtDNA an easily obtainable, universally plentiful marker, where a lack of introns or duplicate genes and non-coding variable spacer regions make amplification of mtDNA relatively uncomplicated [22] (although pseudogenes co-opted by the nucleus from the mitochondrial genome can create separate difficulties [23-26]). The near-complete sequence of the mt genome of *Xenos vesparum* is available, and as a member of the more specialized clade Stylopodia, it is important to place it into context. The mitochondrial genome of *Mengenilla australiensis* (Family Mengenillidae) was therefore chosen for sequencing. Genome arrangement, nucleotide content, codon usage and the secondary structure of ribosomal RNA genes are each comparatively assessed between two distant Strepsiptera, and more widely across Holometabola (a major division of insects defined by the process of complete metamorphosis, including the 4 very species-rich orders; Lepidoptera, Diptera, Hymenoptera, Coleoptera and 6 smaller orders).

Results and discussion

Genome composition

The near-entire mt genome fragment of *M. australiensis* is presented as a 13421 bp sequence, across which 13 protein-coding genes (PCGs), a large (16S) and a small (12S) subunit rRNA gene (*rnl* and *rns* respectively) and 18 tRNA sequences can be identified (Figure 1). PCGs are in expected positions, but two tRNA translocations disrupt an otherwise ancestral genome arrangement. Serine₁ (S1: AGN) moves to a position between Alanine (A) and Arginine (R), whereas Valine (V) is either lost, or transferred to a position in a region that spans the flanking region, potentially anywhere between tRNA-Ile and *nad2*. All start codons across the PCGs (*nad2* is only partial) begin with the typical M- or I- residue [27], and end in TAA, TAG, TA, or T. The control region itself, including up to 4 flanking tRNA genes could not be amplified. A variety of approaches were explored, none of which were successful in spanning the presumed A+T region. Similar difficulties were encountered during the amplification of the mt

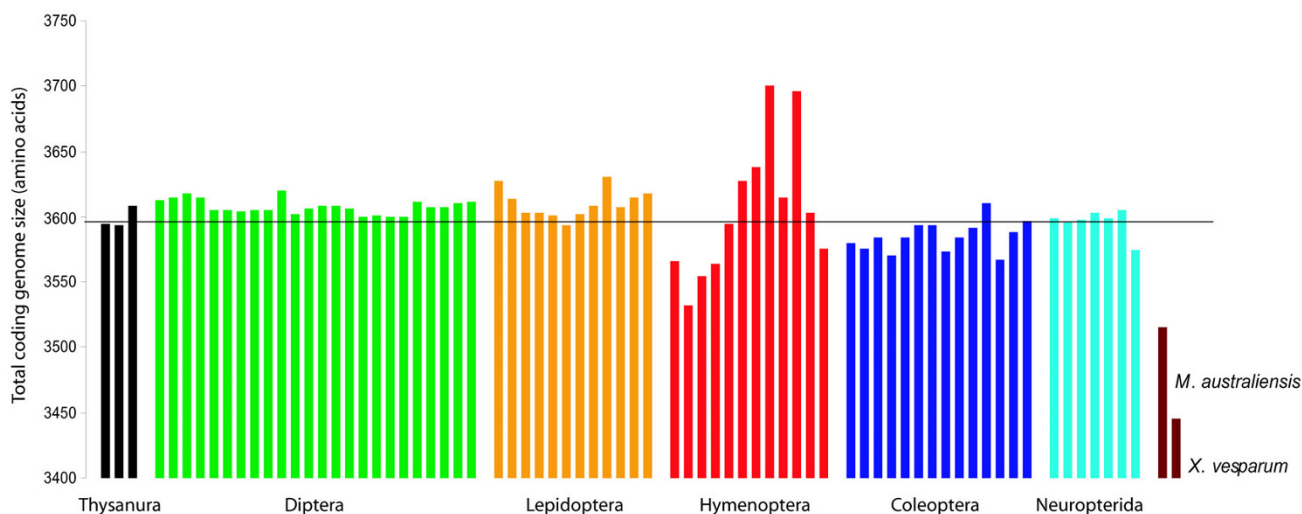


Figure 3
Comparison of the total mt coding genome size (in amino acids) across Holometabola. Black: Thysanura; Green: Diptera; Orange: Lepidoptera; Red: Hymenoptera; Blue: Coleoptera; Turquoise: Neuropterida; Burgundy: Strepsiptera. Each bar represents one species' genome. The superimposed line indicates the mean coding genome size across all 68 taxa.

Overall nucleotide composition is typically A+T rich in *M. australiensis* (84.3%; 6th highest amongst holometabolans insects), and like in *X. vesparum*, C-skew is high (+0.27). A scatter of the variation in skew and A+T% across the 68 holometabolans genomes (plus 3 thysanuran genomes) is shown in Figures 4A and 4B. Notably, in Figure 4B, of the most C-skewed and G+C% poor genomes (occupying the lower right quarter of this graph), 9 (of 12) data points are hymenopteran, 2 (of 2) are strepsipteran and 1 (of 12) is lepidopteran. A similar graph that including hemimetabolous insects [41] shows that only 1 other genome (*Schizaphis graminum*; Aphidoidea, 1 of 11 hemipteran genomes) is so C-skewed and A+T% rich. Conversely, the thysanuran genomes and a subset of Coleoptera are very A-skewed and distinctly more A+T% balanced (Figure 4A). This pattern is discussed in more detail in the following section.

Codon Usage

PCGs from Strepsiptera and the holometabolans dataset were imported into INCA v2.1 [42] to analyze codon usage. *M. australiensis* and *X. vesparum* are compared in Figure 5: codon bias is significantly relaxed in *X. vesparum* (2-tailed paired T-Test of ENC/MILC vectors (see methods); $P < 1 \times 10^{-5}$), and an apparent switch in threonine (T) preference from ACU to ACA has occurred. In Figure 6A, these levels are assessed in context, alongside the holometabolans dataset using the ENC and MILC measures of codon bias for individual PCGs (see methods; [43]). In *X. vesparum*, all 12 PCGs occupy a much more

relaxed, spread apart region of ENC/MILC space (the higher the value, the less codon bias), with average gene ENC and MILC values of 36.26 and 0.71 respectively. In *M. australiensis* these are 29.63 and 0.56, and in Holometabola, they average 32.77 (ENC) and 0.62 (MILC) over all genes. It follows that the values of codon bias among PCGs in *M. australiensis* should be found in the densest cluster of background holometabolans PCGs. The thysanuran genomes (empty black circles), alongside three coleopteran genomes (forming the isolated cluster of empty blue circles with MILC values > 1) have particularly balanced codon usage. Although beetles generally follow this rule; *Pyrophorus divergens*, *Tetraphalerus bruchi* and *Tribolium castaneum* in particular, have unusually high MILC values. Interestingly, unlike the thysanurans, these do not result in correspondingly elevated ENC values, possibly because of differences in the way MILC and ENC estimate bias [43].

These results are consistent with prevailing neutral mutational theories positing that genomic G+C content is the most significant factor in determining codon bias between organisms [44-47]. This may explain why *X. vesparum* has significantly relaxed ENC-MILC values, whose global G+C content is 5% higher than *M. australiensis*. For the thysanuran and beetle genomes, although G+C content in the 3rd codon position of individual PCGs is not necessarily elevated (Figure 6B), it is global G+C content that appears to matter: it has been documented that codon bias can be accurately predicted from inter-

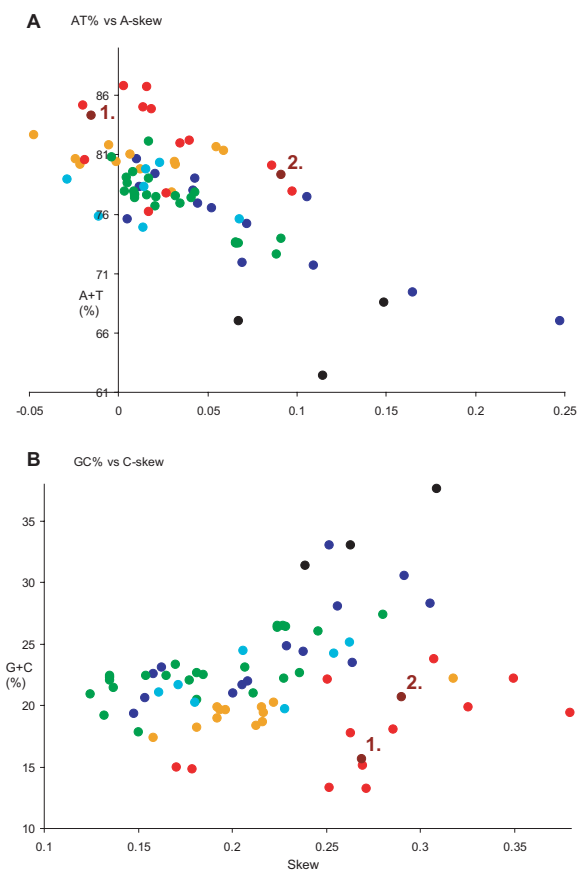


Figure 4
Graphical summary of nucleotide content across 68 holometabolan mt genomes. Measured in bp percentage (Y-axis) and level of nucleotide skew (X-axis) (described in methods, following [41]). Green: Diptera; Orange: Lepidoptera; Red: Hymenoptera; Blue: Coleoptera; Turquoise: Neuropterida; Burgundy: Strepsiptera. 1 and 2 refer to *M. australiensis* and *X. vesparum* respectively. A) A+T% vs A-skew B) G+C% vs C-skew. 3 genomes from Thysanura (in black), the host group for *M. australiensis* are also shown (see methods).

genic regions [44], and it is unsurprising that these genomes should also have the highest genome wide G+C content across the 68 Holometabola dataset (Additional file 2). It is noticeable that the coleopteran *T. bruchi* has a very A-skewed genome (Figure 4A), which could also partly explain some of the discrepancies appearing between the different methods.

Secondary structure of ribosomal and transfer RNAs

With the addition of *M. australiensis*, a comparative revision of strepsipteran mitochondrial rRNA secondary structure was possible. Figures 7 and 8 are complete models for the *rrnL* in *M. australiensis* and *X. vesparum* respec-

tively. The addition of new comparative evidence (from *M. australiensis* and *Apis mellifera* [48]) since Carapelli *et al.* (2006, [28]) enables some improvement over the structural model for *X. vesparum*. These modifications are highlighted in blue. Regions in which sequence variability is still too high for good structural covariation are highlighted in red. Overall, *M. australiensis* predictions are largely consistent with current insect consensus models but substantial portions of secondary structure remain problematic in *X. vesparum*. For example, the helix at the base of domain IV in *M. australiensis* is supported by comparative evidence from *Drosophila melanogaster* and *Apis mellifera*. In *X. vesparum*, most of this primary sequence in this helix is highly divergent and covarying base-pairing cannot be identified. Where this helix terminates in the single-strand bulge however, the base pairing AUG-UAU is supported by *M. australiensis* (AAG-UUU) which provides additional corroborative support for the bps in *D. melanogaster* and *A. mellifera*, which have, AAG-UUU and AGG-UCU respectively.

rrnS secondary structural models for *M. australiensis* and *X. vesparum* are presented in Figure 9. Absence of sequence across domain I precludes a complete analysis, but partial models for domains II and III are generally conserved across Strepsiptera. Regions highlighted in red are more labile, and adequate consensus models for these structures are still lacking. The structural predictions for 18 tRNA genes are given in Figure 10. There is less scope for variation within extremely length-constrained tRNA genes. Despite this, the T Ψ P stem is still too variable to be useful in a deep comparative framework: even between *M. australiensis* and *X. vesparum*, base-pairings demonstrate little covariation. The three remaining stem-loop structures are more conserved across Strepsiptera, and more useful in a comparative structural context. Within these regions, *X. vesparum* demonstrates more substantial modifications to typical insect tRNA structure than *M. australiensis* - consisting largely of single base or base-pair substitutions/compensations. For example, the proximal base-pairs of the acceptor stem of tRNA-Pro (P) in *X. vesparum* appears as UCAG-CUGA. In *M. australiensis*, Lepidoptera (*Ochrogaster lunifer* [41]) and Hymenoptera (*Vanhornia eucnemidarum* [39]) it is CAAA-UUUG. Similarly, the proximal DHU stem consists of a canonical A-U base-pairing in *X. vesparum*, where in *M. australiensis* and other insect groups it appears as a non-canonical G-U. Further, the base-pairing C-G, found across disparate insect lineages is substituted by A-U in the proximal stems of the tRNA-Lys (K) acceptor and the tRNA-Glu (E) DHU helices of *X. vesparum*.

Conclusions

The mitochondrial genome of *X. vesparum* displays a number of characteristics that are not shared by its distant

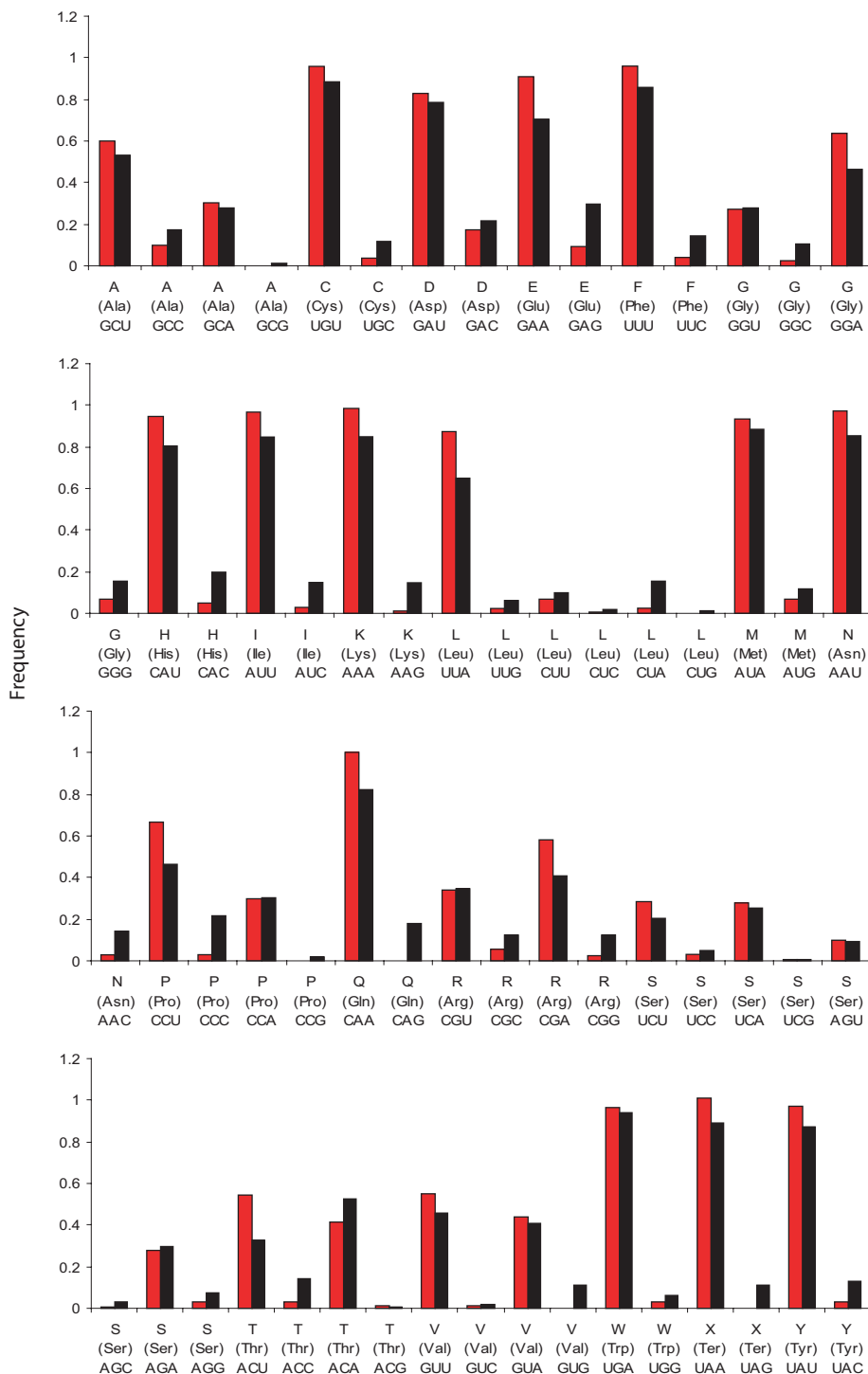


Figure 5
Codon usage in *M. australiensis* (red) and *X. vesparum* (black). Codon frequencies are given (out of 1) for individual amino acids.

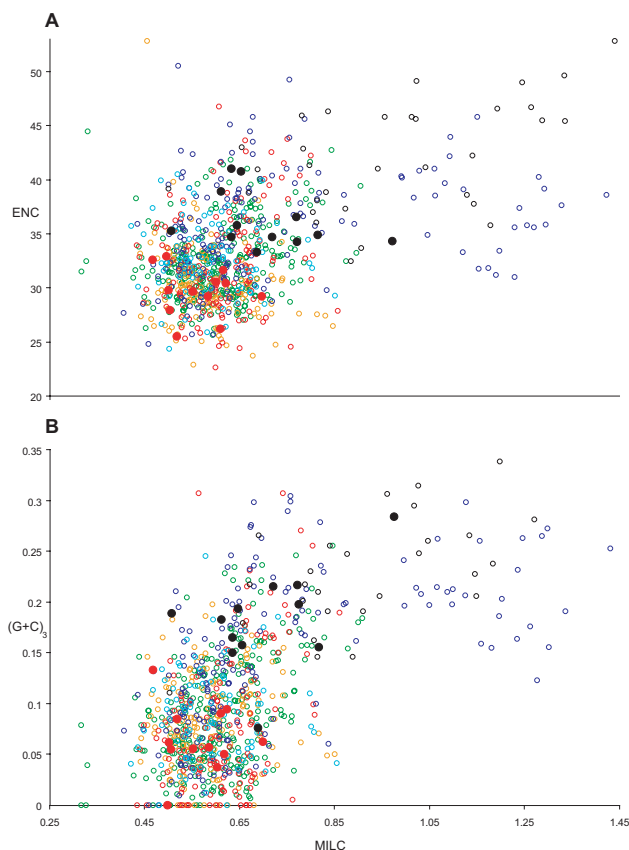


Figure 6
Evaluation of codon bias across Holometabola. A) Codon bias in *M. australiensis* (red filled circle) and *X. vesparum* (black filled circle) measured using ENC and MILC indices (described in methods), are compared against remaining Holometabola, whose data points are given as empty circles: the colour scheme follows taxonomic group as in Figure 3 and 4. Data points refer to individual PCGs. B) MILC values are compared against $(G+C)_3$; which is G+C content at the 3rd codon position of each PCG. This serves as an approximate measure of background nucleotide composition across individual PCGs.

relative *M. australiensis*. In the latter, only 2 tRNA translocations disrupt an otherwise ancestral insect genome organization, whereas in *X. vesparum*, 3 tRNA translocations and at least 2 duplications have occurred (Figure 2). Codon bias in *X. vesparum* is also significantly relaxed: *M. australiensis* occupies a much more typical region of ENC/MILC space (Figures 5 and 6). Further, rRNA secondary structural model predictions in *M. australiensis* are much more consistent with current insect consensus structures (Figures 7, 8, 9 and 10). In *X. vesparum*, increased variability at the primary sequence level precludes the assembly of well corroborated models in several parts of the rRNA

genes. Both strepsipteran genomes are unusually C-skewed and A+T% rich. Interestingly, PCGs are noticeably truncated in both strepsipteran taxa, but especially so in *X. vesparum* which has lost nearly twice as many codons (154) as *M. australiensis* (81), equating to 4.3% and 2.2% fewer codons than the average holometabolan genome respectively.

These results provide useful insight into the evolution of strepsipteran mitochondria spanning the shift from a largely free-living insect into an extremely unique and simplified endoparasitoid, since the emergence of Strepsiptera over 100 million years ago [49]. The modifications to the mitochondrial genomes of *X. vesparum* and *M. australiensis* appear consistent with organisms that have evolved extremely derived lifestyles. *M. australiensis* represents a transitional phase in the evolutionary specialization of Strepsiptera, and the composition, architecture and structure of its genome reflects this. These observations raise important questions about the changing selective environment in (mitochondrial) genomes belonging to small, and almost entirely host-dependent endoparasitoids. Future investigations into the evolutionary and developmental origins of this unique biological system must ensure that strepsipteran species from Mengenillidae are adequately sampled: wide sampling across this group is crucial if the underlying ancestral signal is to be maximized.

Methods

Specimen description

DNA was used from a small set of free-flying males collected in light traps, leaving little possibility for contamination through thysanuran host DNA. Nuclear and mitochondrial gene alignments from a widely sampled Strepsiptera taxon set (unpublished) confirm this. We also include 3 thysanuran genomes in Figure 8 to emphasize the genome composition differences between *M. australiensis* and its target host group. Specimens were collected on the 16th March 2006 in Australia, Queensland, Blackdown Tablelands NP, South Mimosa Creek, 50 m dstr road, 794 mao. Coordinates as follows: S23 47.687'E 149 04. 195'. Light trap loc 34 (Collectors were N. Jönsson, T. Malm & D. Williams). Complete species name: *Mengenilla australiensis* Kifune & Hirashima 1983.

Genome isolation and sequencing

M. australiensis specimens were extracted for PCR either by macerating tissue and digesting overnight at 50°C with Chelex 100 grade resin (Bio-Rad Cat. no 142-1253, Hemel Hempstead, UK) and enzyme Proteinase K (BIO-LINE, Cat. no BIO-37037, London, UK) or by employing a QIAGEN blood and tissue column extraction protocol. Amplification of the *M. australiensis* mitochondrion was carried out by amplifying three ~3 kb fragments, with

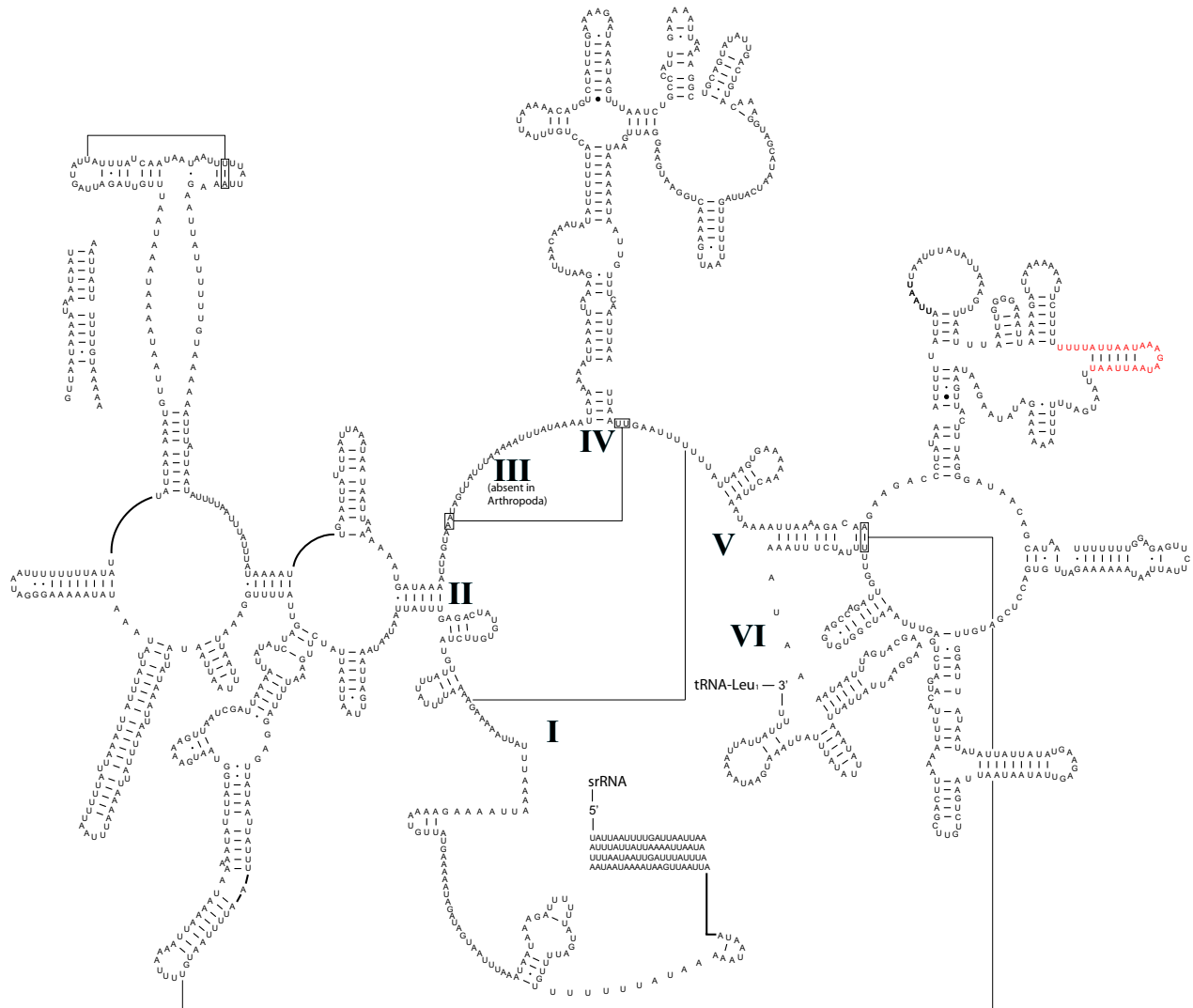


Figure 7
Predicted secondary structure of the *rrnL* in *M. australiensis*. Regions in red receive little comparative support and remain ambiguous. Canonical A-U and G-C base pairings are given as '-'. G-U, G-A, and other non-canonical bp combinations are denoted as, '•', 'm' and 'l' respectively. Predicted tertiary interactions are represented by a line. Missing data are coded as 'N'.

intervening sections that did not overlap being amplified by standard PCR. Long PCR mixes containing 1 µl BIO-X-ACT long DNA polymerase (BIOLINE, Cat. no. BIO-21049), 1 µl dNTPs, 25 µl 2× Polymate additive (BIOLINE, Cat. no. BIO-37041) for A+T rich sequences, 5 µl 10× opti-buffer, 4 µl MgCl₂ solution, 3 µl forward/reverse primers and 5-10 µl of genomic DNA were made up to 50 µl reactions with ultrapure (NANOpure Diamond™) water. Long PCR cycling parameters were as follows: 92 °C for 2 minutes; 10 cycles of: 92 °C for 10 seconds, 53 °C for 30 seconds and 68 °C for 13 minute; 28 cycles of: 92 °C for

15 seconds, 53 °C for 30 seconds and 68 °C for 14 minute; with an extension step of 7 minutes at 68 °C. Long PCR fragments were gel extracted (QIAGEN Cat no. 28704, Hemel Hempstead, UK) and used as templates in subsequent sequencing reactions. Standard PCR mixes contained 0.5 µl *Taq* polymerase, 1 µl dNTPs, 5 µl MgCl₂ free buffer, 4 µl MgCl₂ solution, 3 µl forward/reverse primers and 2 µl of genomic DNA, made up to 50 µl reactions with ultrapure (NANOpure Diamond™) water. Standard PCR cycling parameters were as follows: 94 °C for 2 minutes, then 35 cycles of 94 °C for 1 minute, T °C for 45 seconds

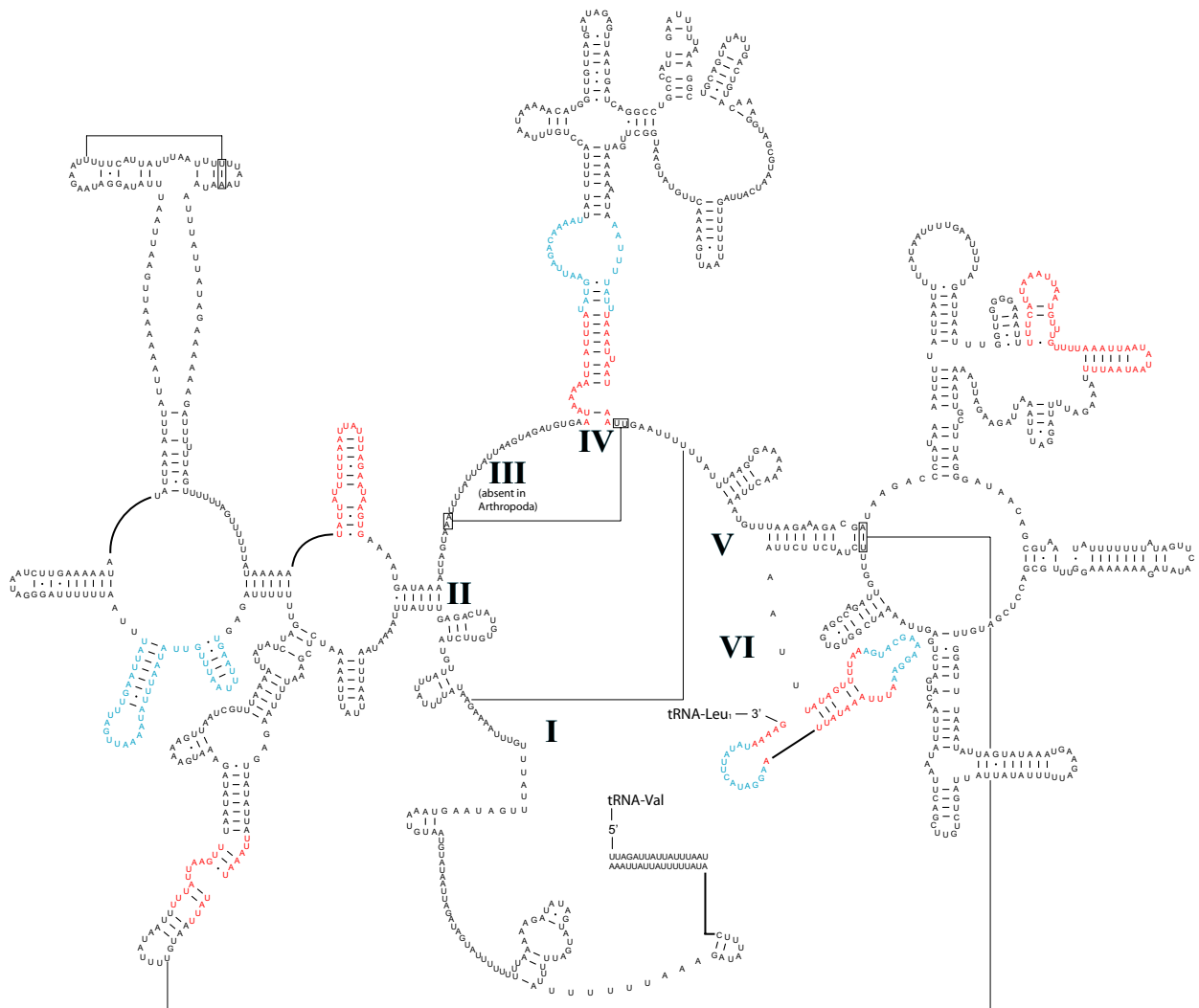


Figure 8
Revised secondary structural prediction of the *rrnL* in *X. vesparum*. Regions in red receive little comparative support and remain ambiguous; regions in blue are structural predictions which have improved with the inclusion of comparative data from *M. australiensis*. Structural annotations follow figure 7.

(*T* varied with primer pair) and 72°C for 1 minute, with an extension step of 5 minutes at 72°C. For difficult regions, Phusion high fidelity *Taq* was utilized, following the manufacturer's instructions. Standard PCR products were cleaned using 2 µl of Shrimp Alkaline Phosphatase (SAP) and 3 µl of Exonuclease I (in a 1:10 SAP buffer dilution) per 50 µl PCR reaction, or fragments were extracted from agarose gels and sequenced directly. Problematic fragments that did not sequence directly were inserted into pGEM-T Easy plasmid vectors (Promega, Southampton, UK), and multiple clones were sequenced for each fragment. Sequencing reactions were performed using the BigDye® Terminator v3.1 cycle sequencing kit with the fol-

lowing modifications: 1 µl of BigDye®, 1.5 µl 5× Buffer, 1 µl primer (3.2 pmol), 1-3 µl genomic DNA made up to 10 µl reactions with ultrapure (NANOpure Diamond™) water. Sequence reads were generated using an Applied Biosystems 3730xl DNA Analyzer. Sequences were imported into GridinSoft Notepad (Lite Edition; <http://notepad.gridinsoft.com>) and BioEdit (version 7.0.5.3; [50]) for analysis. Primer pairs employed are given in Additional file 3. The annotated genome fragment can be found in Genbank under the accession number [GU188852](http://genbank.org/Genbank/entry/188852). Voucher specimens and extractions are deposited in the Hope Entomological Collections, Oxford University Museum of Natural History, Oxford, UK, and

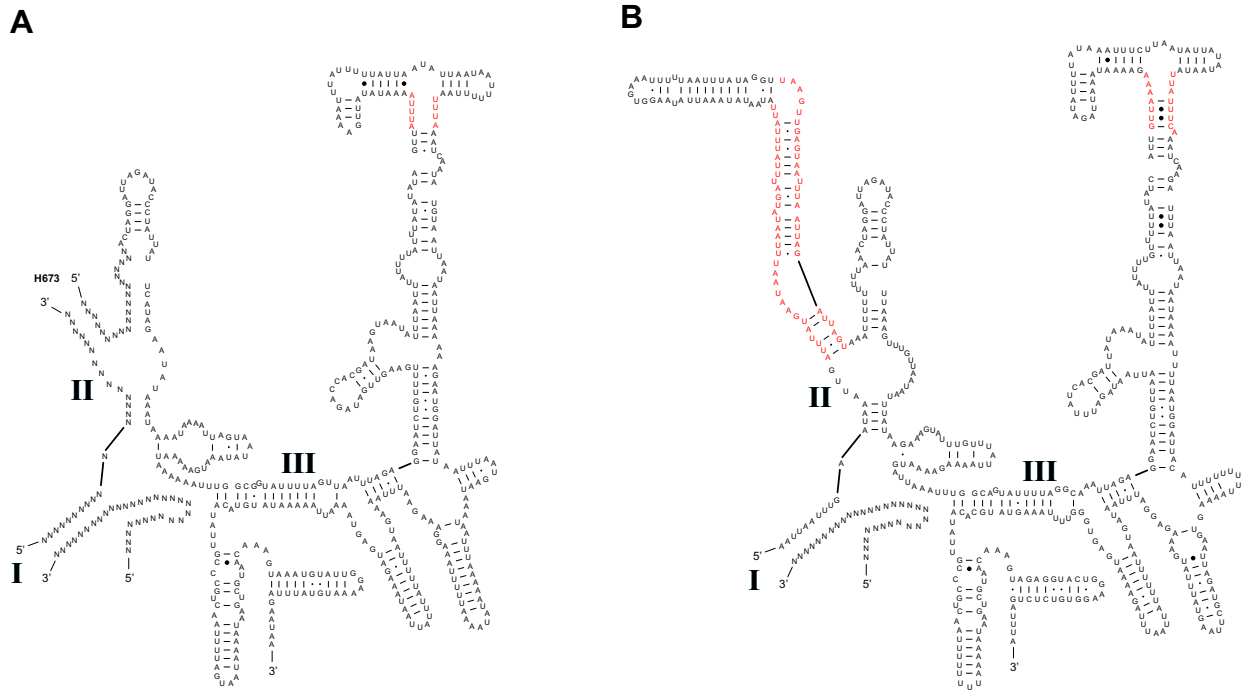


Figure 9
Secondary structural predictions for the *rrnS* in Strepsiptera. A) *M. australiensis*, B) *X. vesparum*. Regions in red receive little comparative support and remain ambiguous. Structural annotations follow figure 7.

the Swedish Museum of Natural History, Stockholm, Sweden.

Genome characterization

PCGs and their boundaries were diagnosed via sequence comparison across alignments derived from the 68 holometabolite genome dataset. Start codons found to be in-frame and not overlapping with upstream genes on the same strand were usually identified as 5' gene boundaries. Stop codons were typically identified as TAA, TAG, TA, or T. A complete annotation of the *M. australiensis* genome is given in Additional file 4. One non-coding fragment, 24 bp in length, was identified between the stop codon of *nad1* and the beginning of tRNA-Ser₂. Secondary structural predictions for ribosomal and transfer RNAs were ascertained by implementing a comparative structural framework [51-55] (for an online tutorial see the jRNA web site: <http://hymenoptera.tamu.edu/rna/>), in conjunction with a thermodynamic-based RNA folding algorithm (*mfold*; [56]). *mfold* gave initial raw estimates of structure: a tool that is especially useful for variable domains with highly divergent primary sequence. In this approach, structures with the lowest thermodynamic stability values were most highly considered (although structures with only marginal thermodynamic differences were also eval-

uated) and robust comparative evidence where available, took priority. The following ribosomal RNA structural templates from the Comparative RNA Web (CRW) site database [57] were used: *Escherichia coli*, *Drosophila melanogaster*, *Drosophila virilis*, *Chorthippus parallelus* and *Apis mellifera* [48]. For tRNA genes, the following models were used as comparative data points: *X. vesparum* (Strepsiptera: [28]), *O. lunifer* (Lepidoptera: [41]), *V. eucnemidarum* (Hymenoptera: [39]) and *Cryptopygus antarcticus* (Collembola: [58]), structures were verified for accuracy in tRNA-Scan [59]. Gene order was assessed across Holometabola using the inferred ancestral insect arrangement as a comparative standard [29,30]. As a measure of gene length fluctuation, total and individual gene length was counted across strepsipteran PCGs, and compared across the dataset of 68 holometabolite genomes. To include *nad2* gene, the 5' portion (approx. 100 amino acids) was excluded from analysis so that *M. australiensis* could be assessed alongside the entire dataset.

The 68 holometabola mitochondrial genome dataset was compiled from previously published data across the 6 major holometabolite divisions: Diptera [60-74], Lepidoptera [33,75-81], Hymenoptera [39,82-87], Coleoptera [88-93], Neuropterida [34,94,95] and Strepsiptera [28].

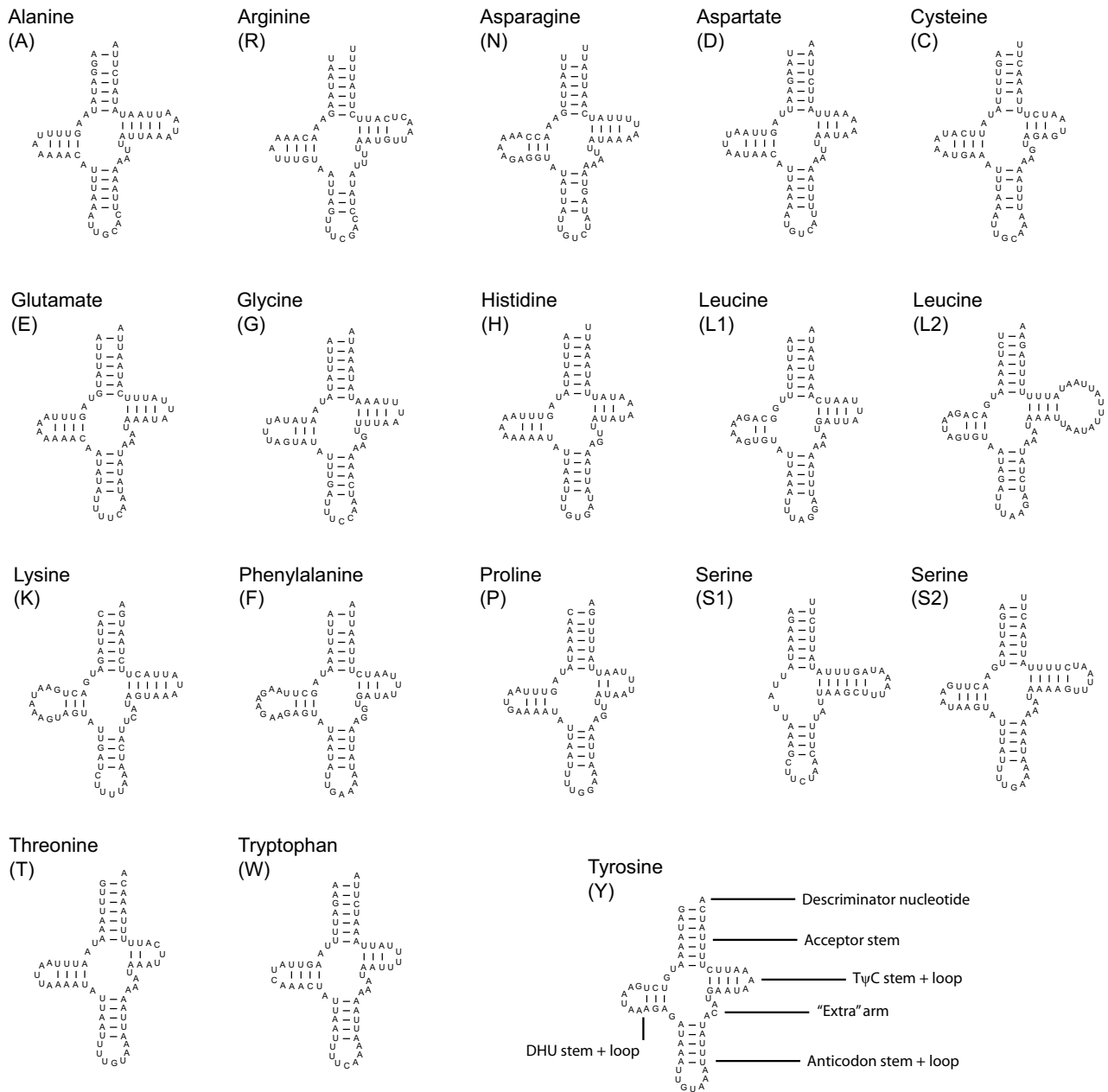


Figure 10
Secondary structural predictions for *M. australiensis* tRNA genes. Pertinent aspects relating to comparative structural differences between *X. vesparum* [28] and other insect lineages [39,41,58] are discussed in the main text.

Nucleotide content and Codon usage

A+T% and G+C% values were calculated for the α strand of the *M. australiensis* genome, which runs clockwise in the direction of transcription from *nad2* (Figure 1). Genomic A- and G-skew measures were calculated using total nucleotide % values in the following manner: $[A-T]/[A+T]$ and $[G-C]/[G+C]$ following Perna & Kocher [96]. A complete list of nucleotide content values for the 68

holometabolan dataset is given in Additional file 3. Codon usage was investigated by importing the complete cDNA datasets of 68 available holometabolan mitochondrial genomes, including *X. vesparum* and the sequenced genome of *M. australiensis* into INCA v2.1 [42]. Two independent measures of codon bias: ENC (Effective Number of Codons used) and MILC (Measure Independent of Length and Composition) are implemented, whose statis-

tical justification and behaviour under simulation differ markedly [43]. These two independent measures were combined as a single vector statistic as follows: $\sqrt{(\text{ENC}^2 + \text{MILC}^2)}$. The shortest gene, *atp8* was also excluded from analyses due to artificially increased ENC/MILC bias in genes shorter than around 100 amino acids. *nad4L* was of borderline length (approx. 90-105 amino acids) and was retained, although *nad4L* from *Drosophila sechellia* and *Melipona bicolor* produced arbitrary ENC 'cut-off' values of 61, because ENC behaves less stably at borderline gene lengths; these data points were removed. Readers are referred to [43] for a detailed explanation.

Abbreviations

mt: mitochondrial; PCG: protein-coding gene; A+T region: the putative control region; α strand: leading strand of transcription; ENC: Effective Number of Codons used; MILC: Measure Independent of Length and Composition; *rrnL* and *rrnS*: large (16S) and small (12S) subunit ribosomal RNA (rRNA) gene; tRNA genes are denoted as single letter amino acid IUPAC-IUB abbreviations; *atp6* and *atp8*: ATP synthase subunits 6 and 8; *cob*: apocytochrome b; *cox1-3*: cytochrome c oxidase subunits 1-3; *nad1-6* and *nad4L*: NADH dehydrogenase subunits 1-6 and 4L; MRCA: Most Recent Common Ancestor.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

DPM and AH devised and completed laboratory work. DPM conducted analyses and early manuscript draughts. All authors contributed to the final version of the manuscript.

Additional material

Additional file 1

Coding gene lengths. Spreadsheet containing coding gene lengths (in amino acids) across the Holometabola dataset.

Click here for file

[<http://www.biomedcentral.com/content/supplementary/1471-2164-10-603-S1.XLS>]

Additional file 2

Nucleotide content. spreadsheet of genomic nucleotide content, A-skew and C-skew across the Holometabola dataset.

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[<http://www.biomedcentral.com/content/supplementary/1471-2164-10-603-S2.XLS>]

Additional file 3

Primers. List of primer pairs used in the study.

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[<http://www.biomedcentral.com/content/supplementary/1471-2164-10-603-S3.XLS>]

Additional file 4

Gene annotation. Detailed summary of genome fragment architecture.

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