2010

Extensive and evolutionary persistent mitochondrial tRNA editing in velvet worms (Phylum Onychophora)

Romulo Segovia
Iowa State University

Extensive and evolutionary persistent mitochondrial tRNA editing in velvet worms (Phylum Onychophora)

by

Romulo Segovia

A thesis submitted to the graduate faculty in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Genetics

Program of Study Committee:
Dennis Lavrov, Major Professor
Lyric Bartholomay
Bing Yang

Iowa State University

Ames, Iowa

2010

Dedicated to

My parents, Romulo Segovia and Alejandrina Ugarte, my family, my friends, and my support

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

It was determined the complete mitochondrial DNA sequences of the velvet worms (Phylum Onychophora) *Oroperipatus* sp. (Peripatidae) and *Peripatoides sympatrica* (Peripatopsidae), and showed that they encode highly reduced tRNA genes. Because a well-paired acceptor stem is essential for tRNA recognition by its cognate aminoacyl-tRNA synthetase, it was suspected that their products are restored by RNA editing. Here it is showed that many reduced mt-tRNA molecules in the onychophorans *Oroperipatus* sp. and *Peripatoides sympatrica* are post-transcriptionally edited by what appears to be a novel RNA editing mechanism. During this process, up to 34 nucleotides are added to the primary truncated transcripts, restoring the amino-acyl acceptor stem, the TΨC arm, and in some cases the variable loop and nucleotide in positions 42 and 43 in a standard tRNA structure, which represent the most extreme tRNA editing event reported in animal mitochondria to date. In addition, it was found sequences that form two stem loop structures in *Oroperipatus* sp. mtDNA, resembling the human stem loop structure associated with the origin of L-strand replication. This suggests that an analogue replication mechanism may exist in *Oroperipatus* sp. mtDNA. It is also reported that the *Peripatoides sympatrica* gene arrangement represents the ancestral Onychophoran gene order.

CHAPTER 1. GENERAL INTRODUCTION

Introduction

Onychophorans, also known as "velvet worms" or "claw bearers," are a group of small terrestrial animals found in some tropical and temperate zones of all continents with the exception of Europe. All extant species are classified into two families, the Peripatidae and Peripatopsidae. Members of the Peripatidae family inhabit tropical zones of the globe. They are found in Central America, the Caribbean islands, the rain forest of South America, Equatorial West Africa, and South East Asia. By contrast, members of the Peripatopsidae group are found in more temperate zones of the planet, including Chile, South Africa, Australia including Tasmania, New Guinea, and New Zealand. Onychophora is an ancient group of animals, possibly related to marine Lobopoda, known from the Early Cambrian (Bergström and Hou, 2001; Monge-Nájera and Xianguang, 2002). Few Onychophoran complete mitochondrial genomes were sequenced and made available for analysis in the last three years. Only four complete mitochondrial genomes are currently available corresponding to the *Epiperipatus biolleyi* (Podsiadlowski et al., 2008; Rota-Stabelli et al., 2010), *Peripatoides sp.* (Rota-Stabelli et al., 2010), *Metaperipatus inae* (Braband et al., 2010b), and *Opisthopatus cinctipes* (Braband et al., 2010a) species from approximately 50 genera to over 150 species reported to date. The need for sequencing more complete mitochondrial genomes from individuals representing genera that are not yet determined is indispensable for clarification of the Onychophoran phylogeny and biogeography.

After the first mitochondrial genomes were made available for analysis, it was realized that the onychophora mtDNA was characterized by numerous gene order

arrangements, a compact genome, and above all severe reduced tRNA genes, reduction of tRNA genes that was hypothesized to occur in mitochondrial genomes by the Muller's Ratchet effect (Muller, 1964). These incomplete tRNAs should be unable to participate in the translational machinery since they were found to lack the 3' side of the acceptor stem which is required for the tRNA recognition by aminoacyl-tRNA synthetases (Schimmel, 2008) and tertiary structure definition (Dirheimer et al., 1995; Martin, 1995). Hence, some type of RNA editing might be turning truncated tRNAs back into functional standard structure. Performing further studies on these genes' transcripts was imperative in order to confirm the editing reaction and the sequences used in the generation of mature tRNAs.

Transfer RNA editing was initially discovered in mtDNA of the amoeboid protist *Acanthamoeba castellanii* (Lonergan and Gray, 1993), where many tRNAs showing the presence of one or more mismatches within the first three base pairs of the acceptor stem were corrected by nucleotide replacement at the 5' end. Since the time of that discovery, tRNA editing has been reported in the mitochondria of a variety of organisms, where it appears to have evolved independently multiple times using various mechanisms (Brennicke et al., 1999). However in the majority of reported cases, the editing is limited from one to three nucleotides, usually at either the 3' or the 5' end of the acceptor stem. In marsupials a C nucleotide in the anticodon of *trnN* is deaminated to a U, changing its identity to a *trnG* (Borner et al., 1996). In the slime mold *Physarum polycephalum,* it was recently demonstrated that two forms of tRNA editing are required for tRNA maturation when tRNAs are edited both co- and post-transcriptionally (Gott et al., 2010). The most extensive tRNA editing has been found in mitochondrial tRNAs of the centipede *Lithobius forficatus* (Lavrov

et al., 2000), where editing of up to five nucleotides at the 3' ends of the acceptor stem of many tRNAs reestablishes the anomalous acceptor stems in a 5' template dependent mechanism.

In order to confirm the presence of tRNA editing in mtDNAs of the Onychophora phylum, we sequenced the complete mitochondrial genomes of two velvet worms belonging to the two recognized families, Peripatidae and Peripatopsidae that diverged 175-140 million years ago (Monge-Najera, 1995), and are significantly and geographically separated. In chapter two of this thesis, it is reported the presence of a novel and extensive type of editing system that has never been described before, which restores the 3' half of the amino-acyl acceptor stems, entire TYC arms, and even variable arms and anticodon stems of several mt-tRNAs from both systems. This extreme tRNA editing has most likely been preserved for millions of years as it is found in both extant families, and it was probably already present in the onychophora common ancestor and retained thereafter. In chapter three, it is described the *Oroperipatus* sp. and *P. sympatrica* mitochondrial genomes and compare them with those of other Onychophora, as well as their putative origin of replication structures. It was also found that the *P. sympatrica* gene organization represents the ancestral gene order of Onychophora.

Although the tRNA editing described in onychophoran mtDNA is undoubtedly extreme, it might not be unique in animals. Recently, the presence of severely truncated mt-tRNA genes has been reported in arachnids (Masta and Boore, 2008), and in gall midges (Beckenbach and Joy, 2009).

Thesis Organization

This thesis consists of four chapters: a general introduction, two manuscripts to be submitted to different journals, and a general conclusion. Chapter two considers the extensive and evolutionary persistent mitochondrial tRNA editing in Onychophora, and chapter three the complete mitochondrial DNA sequence of the onychophorans *Oroperipatus* sp. and *Peripatoides sympatrica*. Chapters two and three were formatted in the style required for submission to the *Proceedings of the National Academy of the United States of America (PNAS)* and the *Mitochondrion* respectively.

References

Beckenbach, A.T. and Joy, J.B.: Evolution of the mitochondrial genomes of gall midges (Diptera: Cecidomyiidae): rearrangement and severe truncation of tRNA genes. Genome Biol Evol 2009 (2009) 278-287.

Bergström, J. and Hou, X.G.: Cambrian Onychophora or xenusians. Zoologischer Anzeiger-A Journal of Comparative Zoology 240 (2001) 237-245.

Borner, G.V., Morl, M., Janke, A. and Paabo, S.: RNA editing changes the identity of a mitochondrial tRNA in marsupials. EMBO J 15 (1996) 5949-5957.

Braband, A., Cameron, S.L., Podsiadlowski, L., Daniels, S.R. and Mayer, G.: The mitochondrial genome of the onychophoran Opisthopatus cinctipes (Peripatopsidae) reflects the ancestral mitochondrial gene arrangement of Panarthropoda and Ecdysozoa. Mol Phylogenet Evol (2010a)

Braband, A., Podsiadlowski, L., Cameron, S.L., Daniels, S. and Mayer, G.: Extensive duplication events account for multiple control regions and pseudo-genes in the mitochondrial genome of the velvet worm Metaperipatus inae (Onychophora, Peripatopsidae). Mol Phylogenet Evol (2010b)

Brennicke, A., Marchfelder, A. and Binder, S.: RNA editing. FEMS Microbiol Rev 23 (1999) 297-316.

Dirheimer, G., Keith, G., Dumas, P. and Westhof, E.: Primary, Secondary and Tertiary Structures of tRNAs. In: Söll, D. and RajBhandary, U. (Eds.), tRNA: Structure, Biosynthesis, and Function. American Society for Microbiology, Washington, DC, 1995, pp. 93-126.

Gott, J.M., Somerlot, B.H. and Gray, M.W.: Two forms of RNA editing are required for tRNA maturation in Physarum mitochondria. RNA 16 (2010) 482-488.

Lavrov, D.V., Brown, W.M. and Boore, J.L.: A novel type of RNA editing occurs in the mitochondrial tRNAs of the centipede *Lithobius forficatus*. Proc Natl Acad Sci U S A 97 (2000) 13738-13742.

Lonergan, K.M. and Gray, M.W.: Editing of transfer RNAs in Acanthamoeba castellanii mitochondria. Science 259 (1993) 812-816.

Martin, N.C.: Organellar tRNAs: Biosynthesis and Function. In: Söll, D. and RajBhandary, U. (Eds.), tRNA: Structure, Biosynthesis, and Function. American Society for Microbiology, Washington, DC, 1995, pp. 127-140.

Masta, S.E. and Boore, J.L.: Parallel evolution of truncated transfer RNA genes in arachnid mitochondrial genomes. Mol Biol Evol 25 (2008) 949-959.

Monge-Najera, J.: Phylogeny, biogeography and reproductive trends in the Onychophora. Zoological Journal of the Linnean Society 114 (1995) 21-60.

Monge-Nájera, J. and Xianguang, H.: Experimental taphonomy of velvet worms (Onychophora) and implications for the Cambrian" explosion, disparity and decimation" model. Revista de biología tropical 50 (2002) 1133-1138.

Muller, H.J.: The relation of recombination to mutational advance. Mutat Res 106 (1964) 2-9.

Podsiadlowski, L., Braband, A. and Mayer, G.: The complete mitochondrial genome of the onychophoran Epiperipatus biolleyi reveals a unique transfer RNA set and provides further support for the ecdysozoa hypothesis. Mol Biol Evol 25 (2008) 42-51.

Rota-Stabelli, O., Kayal, E., Gleeson, D., Daub, J., Boore, J.L., Telford, M.J., Pisani, D., Blaxter, M. and Lavrov, D.V.: Ecdysozoan mitogenomics: evidence for a common origin of the legged invertebrates, the panarthropoda. Genome Biol Evol 2 (2010) 425-440.

Schimmel, P.: Development of tRNA synthetases and connection to genetic code and disease. Protein Sci 17 (2008) 1643-1652.

CHAPTER 2: EXTENSIVE AND EVOLUTIONARY PERSISTENT MITOCHONDRIAL tRNA EDITING IN VELVET WORMS (PHYLUM ONYCHOPHORA)

A paper to be submitted to the *Proceedings of the National Academy of the United States of America (PNAS)*

Romulo Segovia[1], Walker Pett[2], Steve Trewick[3], and Dennis V. Lavrov[4]

Abstract

Mitochondrial genomes of onychophorans (velvet worms) are unusual: some previous studies reported them lacking several transfer RNA (tRNA) genes while others found that all of their tRNA genes were present but severely reduced. To resolve this discrepancy we determined complete mitochondrial DNA (mtDNA) sequences of the onychophorans *Oroperipatus* sp. and *Peripatoides sympatrica* as well as cDNA sequences from 14 and 10 of their tRNAs, respectively. We show that tRNA genes in these genomes are indeed highly reduced and encode truncated molecules, which are restored to more conventional tRNA by extensive tRNA editing. During this editing process, up to 34 nucleotides are added to the

---

1 Graduate Student and primary author, Department of Ecology, Evolution, and Organismal Biology, Iowa State University.

2 Graduate Student, Department of Ecology, Evolution, and Organismal Biology, Iowa State University.

3 Researcher, Ecology Group, Massey University, New Zealand.

4 Professor and author for correspondence, Department of Ecology, Evolution, and Organismal Biology, Iowa State University, dlavrov@iastate.edu

tRNA sequences encoded in mtDNA, rebuilding the amino-acyl acceptor stem, the TΨC arm, and, in some extreme cases, the variable arm and even a part of the anticodon stem. When the entire TΨC arm is added *de novo*, the sequence of this arm is either identical or similar among different tRNA species, yet the sequences show some variation for each tRNA. These and some other observations contradict the involvement of guide RNAs in the editing process and argue against the import of tRNA genes or their parts from the nucleus. The extreme tRNA editing reported here has likely been preserved for >140 million years as it was found in both extant families of onychophorans. Furthermore, a similar type of tRNA editing may be present in several other groups of arthropods, which show a high degree of tRNA gene reduction in their mtDNA.

Introduction

Mitochondrial DNA of bilaterian animals is typically a circular molecule ~16 kbp in size with a well-conserved gene content of 37 genes: 2 for the small and large subunit ribosomal RNA (*rns* and *rnl*), 22 for transfer RNAs (tRNAs), and 13 for protein subunits of complexes I, III, IV, and V involved in oxidative phosphorylation (NADH dehydrogenase subunits 1-6, *nad1-6*; cytochrome b, *cob*; cytochrome c oxidase subunits I-III, *cox1-3*; and ATP synthase subunits 6 and 8, *atp6* and *atp8*). Recently, the complete mtDNA of the onychophoran *Epiperipatus biolleyi* has been published and inferred to lack nine tRNA genes (1). The authors asserted that all of these missing tRNA genes translate 4-fold degenerated codons and suggested that these genes are replaced by their nuclear counterparts via the tRNA import process from the cytosol into the mitochondria. Earlier, one of us had determined the complete mitochondrial genome from another specimen of *Epiperipatus*

biolleyi and came to a different assignment of genes (2). In particular, 21 tRNA genes were found in the genome, most of them in different positions than those inferred by Podsiadlowski (fig. 1). These discrepancies between the results of the two studies led us to question mt-tRNA annotations in *E. biolleyi* and encouraged us to look more closely at onychophoran mitochondrial tRNA biology.

The majority of the tRNA genes annotated in our previous study of *E. biolleyi* mtDNA were inferred to encode highly unusual tRNA structures often lacking a portion of the TΨC arms and 3' side of the aminoacyl acceptor stems. We also noticed that many of these abbreviated genes were immediately followed by their downstream neighbors. Because a well-paired acceptor stem is essential for tRNA recognition by its cognate aminoacyl-tRNA synthetase (3, 4), we suspected that tRNA structures are restored by a novel type of tRNA editing (5). The term RNA editing refers to any programmed alteration of RNA primary structures to generate a sequence that could have been directly encoded at the DNA level (6). To date RNA editing has been discovered in a broad spectrum of RNAs, in genetic systems that have been widely separated by evolution in the living world including plants, animals, fungi, protists, archaea, and viruses. It has been identified in the nucleus of animals (7-10), in mitochondria of trypanosomes (11, 12) and of the plasmodial *Physarum polychephalum,* affecting not only mRNAs but also rRNAs and tRNAs (13-15), in mitochondria and plastids of land plants (16-19), in viruses (20-22), and more recently in archaeal tRNAs (23). While many RNA editing systems appear to repair defective genes or modulate gene differentiation, others have been implicated in the regulation of gene expression: RNA editing has been found to affect microRNAs (miRNAs) (24, 25), as well as the primate-specific *Alu* repeats

(26, 27). Most of cases of RNA editing appear to be recent acquisitions that arose independently in various lineages (28). Transfer RNA editing in eukaryotes has been found to occur at the 5'-end of the molecule (29-31), the 3'-end of the molecule (5, 32-35), its anticodon (36) or in any of the four stems (15, 37). Thus different mechanisms are likely involved in this process as well as the possibility of a combination of such mechanisms.

Here, we present the complete mitochondrial genomes of the onychophorans *Oroperipatus* sp. and *Peripatoides sympatrica* (38), and report the presence of a novel and more extensive type of RNA editing that restores the 3' half of the aminoacyl acceptor stem, the TΨC arm, and, in a few cases, even variable arms and anticodon stems of most mt-tRNAs. This extreme tRNA editing has likely been preserved for >140 million years, since the divergence between two extant families of onychophorans sampled for this study.

Results

Onychophoran mitochondrial genomes

The newly determined mitochondrial genomes of *Oroperipatus* sp. and *Peripatoides sympatrica* are 14801 bp and ~ 14.2 kb in sizes and contain a conserved set of genes for 13 proteins and two rRNA typical for animal mtDNA (Fig. 1). These sequences contain 22 expected tRNA genes in *P. sympatrica* mtDNA, and 21 putative tRNA genes in *Oroperipatus* sp (all except *trnC(gca)*). The mitochondrial genome of *Oroperipatus* sp. has an identical gene arrangement to that of *Epiperipatus biolleyi*, reported in our previous study (39) with the tRNA sequences in the two genomes being ~80% identical (range 61-100%). The mitochondrial genome of *P. sympatrica* has also an identical gene order to that of

Peripatoides sp. (39) and their tRNA sequences are ~85% identical (range 66-100%). The tRNA sequences are only ~56% identical when compared between *Oroperipatus* sp. and *P. sympatrica*. Previous reports (1, 39-41) described several interesting features of onychophoran mitochondrial genomes. However, their most outstanding feature – the severe reduction of nearly all tRNA genes – remained unrecognized/unreported and is investigated in this study.

Truncated tRNA genes

The majority of tRNA genes identified in both mitochondrial genomes appear to be truncated and encode incomplete tRNA structures (Fig. 2. Supplementary Fig. 1 and 2, Supplementary Material). The extent of reduction is more extreme in *Oroperipatus* sp. tRNA genes, all of which code for tRNA molecules lacking the entire 3' side of the acceptor stem, at least part of the TΨC arm, and in some cases, the variable loop. Furthermore, in two cases (*trnT(ugu)* and *trnN(guu)*) the last two nucleotides of the predicted anticodon stem (positions 42 and 43 in a standard tRNA structure) also appear to be missing, with position 41 abutting the 5' ends of the downstream tRNA genes (*trnV(uac)* and *trnI(gau)*, respectively). By contrast, tRNA genes in *P. sympatrica* mtDNA are less reduced, with the extent of reduction ranging from a few nucleotides to the entire 3' side of the acceptor stem plus a part of the TΨC arm. None of the tRNAs encoded in *P. sympatrica* lack the entire TΨC arm and one tRNA gene (*trnF(gaa)*) appears to encode an intact tRNA molecule. When comparing the structural sequences of cognate tRNA genes between the two species we found that the anticodon arms are generally well conserved, while DHU arms are poorly conserved. Four tRNA genes are encoded with a D-replacement loop instead of a standard DHU arm. This

feature was observed in the *trnS(ucu)* gene in both genomes (also characteristic to all bilaterian animals) as well as *Oroperipatus* sp. *trnF(gaa)* and *trnN(guu)*. All D-replacement loop sequences are 11 nucleotides in length, except for the 9-nucleotide D-replacement loop in *Oroperipatus* sp. *trnF(gaa)*.

Mature tRNAs have conventional structures

Although the finding of tRNA-like sequences with a complete or nearly complete set of anticodons needed for mitochondrial translation in both onychophorans provides a strong indication that these genes are functional, their degenerate structures would make it impossible for the encoded tRNAs to participate in protein synthesis without extensive editing. To check for the presence of such editing, we determined cDNA sequences for 14 out of 21 inferred tRNAs in *Oroperipatus* sp. and for 10 inferred tRNAs in *P. sympatrica* (Fig. 2; Supplementary Figs. 1 and 2). In all cases, we discovered editing in tRNA sequences that restored their potential to form a standard cloverleaf secondary structure. In addition, the discriminator nucleotide (always A) and, the trinucleotide CCA has been added to the 3' end of the tRNA, indicating that these cDNA sequences represent mature tRNAs (42). The extent of tRNA editing is unprecedented: on average, 26 nucleotides per studied tRNA molecule are added posttranscriptionally in *Oroperipatus* sp, and 14 nucleotides in *P. sympatrica*.

Conservation and variation in the TΨC arm

Among the inferred tRNA genes in *Oroperipatus* sp. at least seven appear to encode molecules lacking the entire TΨC arm (Supplementary Fig. 1). Because no template exists within a tRNA molecule to rebuild it, the editing of the TΨC arm in such tRNAs is

particularly interesting. Among the seven tRNAs, most cDNA sequences corresponding to mature *Oroperipatus* sp. $tRNA^{Met}_{CAU}$, $tRNA^{Asn}_{GUU}$, and $tRNA^{Val}_{UAC}$, and $tRNA^{Leu}_{UAA}$ have identical TΨC arm sequences, viz., 5'-GGA_8T_5CC-3'. In addition, the most common cDNA sequences of the remaining three tRNAs contain highly similar TΨC arm sequences (AGA_8T_5CT in $tRNA^{Arg}_{UCG}$, GGA_8T_4CC in $tRNA^{Phe}_{GAA}$, and GGA_8T_3CC in $tRNA^{His}_{GUG}$) (Supplementary Fig. 1). Interestingly, no such similarity in TΨC arm sequences was found among *P. sympatrica* tRNAs, in which at least a part of the TΨC arm is always DNA-encoded (Supplementary Fig. 2). Furthermore, some variation was found among individual cDNA clones from the same tRNA in both species of onychophorans. To investigate the extent of this variation, we determined sequences from additional clones of *Oroperipatus* sp. $tRNA^{Leu}_{UAA}$ (16 in total) and *P. sympatrica* $tRNA^{Val}_{UAC}$ (18 in total) (Fig. 3). Seven $tRNA^{Leu}_{UAA}$ clones had the TΨC arm sequence GGA_8T_4CC, and five the sequence GGA_8T_5CC. Similarly, four $tRNA^{Val}_{UAC}$ clones had the sequence $TAGTA_5TACTA$ and another four the sequence $TAGTATA_6TACTA$. Some variation in other regions of the *Oroperipatus* sp $tRNA^{Leu}_{UAA}$ also were observed, including two of $tRNA^{Leu}_{UAA}$ with an aberrant GGA_9T_5CTT TΨC arm that where missing the CCA triplet.

Discussion

The most extensive tRNA editing reported to date

After the initial discovery in the amoeboid protozoon *Acanthamoeba castellanii* (43), tRNA editing has been reported in organelles of a variety of organisms where it appears to have evolved independently multiple times and involve various mechanisms (29, 37, 44, 45).

However, in the majority of reported cases the editing is limited from one to three nucleotides, usually at either the 3' or the 5' end of the acceptor stem. Previously the most extensive tRNA editing (up to five nucleotides) was found in mitochondrial tRNAs of the centipede *Lithobius forficatus* (5). The tRNA editing found in onychophoran mitochondria and reported in this study is much more extensive and involves posttranscriptional addition of up to 34 nucleotides, as found in *Oroperipatus* sp. $tRNA^{Thr}_{UGU}$ and $tRNA^{Asn}_{GUU}$. The pattern of tRNA editing in both onychophorans is similar, but more extreme in *Oroperipatus* sp., where the entire TΨC arm can be added posttranscriptionally. *Oroperipatus* sp. appears to have evolved an additional novel mechanism that rebuilds this arm *de novo* with a similar sequence of nucleotides.

Evolutionary conservation of tRNA editing

In addition to its unprecedented extent, onychophoran tRNA editing is also unusual in its phylogenetic persistence. In general, tRNA editing is regarded as a mechanism that allows animal mitochondria "to stay fit without sex" (46) in the face of accumulation of deleterious mutations driven by Muller's Ratchet (47). However, the evolution of an editing mechanism, while providing a short-term solution to the fitness decline, relaxes selection on tRNA genes that leads to their further degeneration and makes tRNA editing a requirement for the survival of organisms (5). It might be inferred that the energy and time investment required for RNA editing makes an organism less fit in the long run and eventually leads to its demise. Supporting this type of reasoning, tRNA editing has been primarily observed in individual species rather than clades of animals. The apparent conservation of tRNA editing reported in this study provides an interesting exception to this observation. Onychophora is an ancient

group of animals, possibly related to marine Lobopoda, known from the Early Cambrian (48, 49). The two onychophorans used for this study belong to two recognized extant families, Peripatidae and Peripatopsidae that have diverged 175-140 million years ago (50). We infer that tRNA editing was already present in the common ancestor of these two groups and retained thereafter. Such a long persistence of tRNA editing may be due either to some unknown benefits or, more likely, to the relaxed selection pressure on this niche-specific group of animals.

A novel editing mechanism is required for tRNA editing observed in Onychophora

Previous cases of tRNA 3'- and 5'-editing can be explained by either a template-independent mechanism, where specific nucleotides (usually As or, more rarely, Cs) are added to the 3' end of the molecule or by a template-dependent mechanism, where one part of the tRNA structure is used, either directly or indirectly, as a template for another (44). The editing observed in onychophoran tRNAs appears to be template-dependent as it adds specific sequences to individual tRNAs to match the 5' sequence of their acceptor stems and, in some cases, the missing parts of the TΨC arm. Yet, there is no internal template to rebuild the entire TΨC arm, which is not encoded in several tRNA genes of *Oroperipatus* sp. but is present in the corresponding tRNAs. Although, external templates, such as guide RNAs (gRNAs) are known to be used in mRNA editing in unicellular eukaryotes, particularly in trypanosomes (11), two observations mitigate against the involvement of such molecules in onychophoran tRNA editing. First, editing using gRNAs is usually limited to insertions and deletions of U residues, while all four nucleotides are present in the *de novo* generated TΨC arm in onychophoran tRNAs. Second, and more critically, there is no template downstream

of the missing TΨC arm for the gRNA 5' anchor sequence to anneal to. Furthermore, the observation that the sequence of the rebuilt TΨC arm is either identical or similar among different tRNA species, yet variable for each tRNA argues against the possibility that tRNA molecules are combined from separate, individually transcribed parts, as found in the archaeal parasite *Nanoarchaeum equitans* (51). Thus, a different, potentially template-independent, mechanism has likely evolved in *Oroperipatus* sp. tRNAs to generate a simple sequence (usually GGA_xT_yCC) for the missing TΨC arms.

As a final point we would like to note that although the tRNA editing described in onychophoran mtDNA is undoubtedly extreme and bizarre, it might not be unique among animals. Recently, the presence of severely truncated tRNA genes has been reported in the mitochondrial genomes of arachnids (52), and of gall midges (53). It would be very interesting to investigate whether tRNA genes are similarly edited in these animals and the molecular machinery involved in this process.

Materials and Methods

Specimen collection and preservation

The specimen of *Peripatoides sympatrica* was collected in at the Hakarimata Walkway, near Huntly town in the Waikato region, North Island, New Zealand. The specimen of *Oroperipatus* sp. was collected at the Belize Foundation for Research and Environmental Education in the Toledo district of Belize, bordering the Bladen Nature Reserve. Both specimens were stored in 80% ethanol at -20ºC.

Nucleic acid extraction and tRNA circularization

Total RNA was prepared from each specimen using TRIZOL® Reagent (Invitrogen). The DNA was prepared from the same individuals using the 2x CTAB buffer and phenol-chloroform extraction (54). To circularize tRNA molecules, total RNA was ligated using T4 RNA ligase (Fermentas) as previously described (55).

Sequencing of mitochondrial DNA

Regions of *rns* and *cox3* (*P. sympatrica*) and *cox1* and *nad5* (*Oroperipatus* sp.) were amplified using animal-specific primers for these genes (56) and used to design species-specific primers. Complete mtDNA from both species was amplified in two overlapping fragments using the TaKaRa LA-PCR kit and sheared into overlapping fragments as described in (56). For *Peripatoides sympatrica,* these fragments were barcoded and used for the GS FLX Titanium library preparation (454 Life Sciences) along with other samples. Pyrosequencing was carried out on a Genome Sequencer FLX Instrument (454 Life Sciences) at the University of Indiana Center for Genomics and Bioinformatics. For *Oroperipatus* sp. sheared fragments were processed using Invitrogen TOPO® Shotgun Subcloning Kit following the manufacturer's protocol. Clones were sequenced on an Applied Biosystems 3730xl DNA Analyzer at Iowa State University DNA Facility. All sequences were assembled using the STADEN package v. 1.6.0. Gaps and uncertainties in the assembly were filled/resolved by primer walking using conventional Sanger sequencing (57). The assembled genomes were annotated as previously described (58).

cDNA synthesis, PCR amplification, and cloning of tRNA

Primers used for reverse transcription and subsequent PCR amplification were designed based on complete mitochondrial sequences (Supplementary Table 1, Supplementary Material). The nine 5' nucleotides in some primers (in lower case) were added to create a restriction site for HindIII or BamHI plus three terminal nucleotides. Reverse transcription was performed using SuperScript III Reverse Transcriptase (Invitrogen) under conditions described by the manufacturer. PCR amplification of cDNA products was performed using recombinant *Taq* DNA polymerase (Invitrogen). PCR products were cloned using TOPO TA Cloning Kit for Sequencing (Invitrogen). At least 10 clones for each tRNA have been sequenced at Iowa State University DNA facility.

Acknowledgements

We would like to thank Judy and Dan Dourson, Jacob Marlin and other members of the Belize Foundation for Research and Environmental Education (www.bfreebz.org) for their hospitality and help in finding specimens, and Georg Mayer of the University of Leipzig for his help in identifying this specimen. This research project was supported by funds from Iowa State University.

References

1. Podsiadlowski L, Braband A, Mayer G (2008) The complete mitochondrial genome of the onychophoran Epiperipatus biolleyi reveals a unique transfer RNA set and provides further support for the ecdysozoa hypothesis. *Mol Biol Evol* 25:42–51.

2. Lavrov DV (2001) Arthropod phylogeny based on gene arrangement and other characters from mitochondrial DNA. 196.

3. Hou YM, Schimmel P (1988) A simple structural feature is a major determinant of the identity of a transfer RNA. *Nature* 333:140–145.

4. McClain WH (1995) in *tRNA: Structure, Biosynthesis, and Function*, eds Söll D, RajBhandary U (American Society for Microbiology, Washington, DC), pp 335–347.

5. Lavrov DV, Brown WM, Boore JL (2000) A novel type of RNA editing occurs in the mitochondrial tRNAs of the centipede *Lithobius forficatus*. *Proc Natl Acad Sci U S A* 97:13738–13742.

6. Price DH, Gray MW (1998) in *Modification and Editing of RNA*, eds Grosjean H, Benne R (AMS Press, Washington, D.C.), pp 289–305.

7. Powell LM, Wallis SC, Pease RJ, Edwards YH, Knott TJ, Scott J (1987) A novel form of tissue-specific RNA processing produces apolipoprotein-B48 in intestine. *Cell* 50:831–840.

8. Chen SH, Habib G, Yang CY, Gu ZW, Lee BR, Weng SA, Silberman SR, Cai SJ, Deslypere JP, Rosseneu M (1987) Apolipoprotein B-48 is the product of a messenger RNA with an organ-specific in-frame stop codon. *Science* 238:363–366.

9. Bass BL, Weintraub H (1988) An unwinding activity that covalently modifies its double-stranded RNA substrate. *Cell* 55:1089–1098.

10. Sommer B, Kohler M, Sprengel R, Seeburg PH (1991) RNA editing in brain controls a determinant of ion flow in glutamate-gated channels. *Cell* 67:11–19.

11. Benne R, Van den Burg J, Brakenhoff JP, Sloof P, Van Boom JH, Tromp MC (1986) Major transcript of the frameshifted coxII gene from trypanosome mitochondria contains four nucleotides that are not encoded in the DNA. *Cell* 46:819–826.

12. Feagin JE, Abraham JM, Stuart K (1988) Extensive editing of the cytochrome c oxidase III transcript in Trypanosoma brucei. *Cell* 53:413–422.

13. Mahendran R, Spottswood M, Miller DL (1991) RNA editing by cytidine insertion in mitochondria of Physarum polycephalum. *Nature* 349:434–438.

14. Mahendran R, Spottswood M, Ghate A, Ling ML, Jeng K, Miller DL (1994) Editing of the mitochondrial small subunit rRNA in Physarum polycephalum. *EMBO J* 13:232–240.

15. Antes T, Costandy H, Mahendran R, Spottswood M, Miller D (1998) Insertional editing of mitochondrial tRNAs of Physarum polycephalum and Didymium nigripes. *Mol Cell Biol* 18:7521–7527.

16. Covello PS, Gray MW (1989) RNA editing in plant mitochondria. *Nature* 341:662–666.

17. Gualberto JM, Lamattina L, Bonnard G, Weil JH, Grienenberger JM (1989) RNA editing in wheat mitochondria results in the conservation of protein sequences. *Nature* 341:660–662.

18. Hiesel R, Wissinger B, Schuster W, Brennicke A (1989) RNA editing in plant mitochondria. *Science* 246:1632–1634.

19. Bock R (2004) Studying RNA editing in transgenic chloroplasts of higher plants. *Methods Mol Biol* 265:345–356.

20. Thomas SM, Lamb RA, Paterson RG (1988) Two mRNAs that differ by two nontemplated nucleotides encode the amino coterminal proteins P and V of the paramyxovirus SV5. *Cell* 54:891–902.

21. Volchkov VE, Volchkova VA, Muhlberger E, Kolesnikova LV, Weik M, Dolnik O, Klenk HD (2001) Recovery of infectious Ebola virus from complementary DNA: RNA editing of the GP gene and viral cytotoxicity. *Science* 291:1965–1969.

22. Doria M, Neri F, Gallo A, Farace MG, Michienzi A (2009) Editing of HIV-1 RNA by the double-stranded RNA deaminase ADAR1 stimulates viral infection. *Nucleic Acids Res* 37:5848–5858.

23. Randau L, Stanley BJ, Kohlway A, Mechta S, Xiong Y, Soll D (2009) A cytidine deaminase edits C to U in transfer RNAs in Archaea. *Science* 324:657–659.

24. Yang W, Chendrimada TP, Wang Q, Higuchi M, Seeburg PH, Shiekhattar R, Nishikura K (2006) Modulation of microRNA processing and expression through RNA editing by ADAR deaminases. *Nat Struct Mol Biol* 13:13–21.

25. Borchert GM, Gilmore BL, Spengler RM, XIng Y, Lanier W, Bhattacharya D, Davidson BL (2009) Adenosine deamination in human transcripts generates novel microRNA binding sites. *Hum Mol Genet* 18:4801–4807.

26. Kim DD, Kim TT, Walsh T, Kobayashi Y, Matise TC, Buyske S, Gabriel A (2004) Widespread RNA editing of embedded alu elements in the human transcriptome. *Genome Res* 14:1719–1725.

27. Paz-Yaacob N, Levanon EY, Nevo E, Kinar Y, Harmelin A, Jacob-Hirsch J, Amariglio N, Eisenberg E, Rechavi G (2010) Adenosine-to-inosine RNA editing shapes transcriptome diversity in primates. *Proc Natl Acad Sci USA* 107:12174–12179.

28. Brennicke A, Marchfelder A, Binder S (1999) RNA editing. *FEMS Microbiol Rev* 23:297–316.

29. Price DH, Gray MW (1999) A novel nucleotide incorporation activity implicated in the editing of mitochondrial transfer RNAs in Acanthamoeba castellanii. *RNA* 5:302–317.

30. Laforest MJ, Roewer I, Lang BF (1997) Mitochondrial tRNAs in the lower fungus Spizellomyces punctatus: tRNA editing and UAG 'stop' codons recognized as leucine. *Nucl Acids Res Nucleic Acids Research* 25:626–632.

31. Forget L, Ustinova J, Wang Z, Huss VA, Lang BF (2002) Hyaloraphidium curvatum: a linear mitochondrial genome, tRNA editing, and an evolutionary link to lower fungi. *Mol Biol Evol* 19:310–319.

32. Yokobori S, Pääbo S (1995) Transfer RNA editing in land snail mitochondria. *Proc Natl Acad Sci USA Proceedings of the National Academy of Sciences of the United States of America* 92:10432–10435.

33. Tomita K, Ueda T, Watanabe K (1996) RNA editing in the acceptor stem of squid mitochondrial tRNA(Tyr). *Nucl Acids Res Nucleic Acids Research* 24:4987–4991.

34. Yokobori SI, Pääbo S (1995) tRNA editing in metazoans. *Nature Nature* 377:490.

35. Leigh J, Lang F (2004) Mitochondrial 3' tRNA editing in the jakobid Seculomonas ecuadoriensis: a novel mechanism and implications for tRNA processing. *RNA* 10:615–621.

36. Janke A, Pääbo S (1993) Editing of a tRNA anticodon in marsupial mitochondria changes its codon recognition. *Nucleic Acids Res* 21:1523–1525.

37. Gott JM, Somerlot BH, Gray MW (2010) Two forms of RNA editing are required for tRNA maturation in Physarum mitochondria. *RNA* 16:482–488.

38. Trewick S (1998) Sympatric cryptic species in New Zealand Onychophora. *Biological Journal of the Linnean Society* 63:307–329.

39. Rota-Stabelli O, Kayal E, Gleeson D, Daub J, Boore JL, Telford MJ, Pisani D, Blaxter M, Lavrov DV (2010) Ecdysozoan mitogenomics: evidence for a common origin of the legged invertebrates, the panarthropoda. *Genome Biol Evol* 2:425–440.

40. Braband A, Cameron SL, Podsiadlowski L, Daniels SR, Mayer G (2010) The mitochondrial genome of the onychophoran Opisthopatus cinctipes (Peripatopsidae) reflects the ancestral mitochondrial gene arrangement of Panarthropoda and Ecdysozoa. *Mol Phylogenet Evol*

41. Braband A, Podsiadlowski L, Cameron SL, Daniels S, Mayer G (2010) Extensive duplication events account for multiple control regions and pseudo-genes in the mitochondrial genome of the velvet worm Metaperipatus inae (Onychophora, Peripatopsidae). *Mol Phylogenet Evol*

42. Chen JY, Joyce PB, Wolfe CL, Steffen MC, Martin NC (1992) Cytoplasmic and mitochondrial tRNA nucleotidyltransferase activities are derived from the same gene in the yeast Saccharomyces cerevisiae. *J Biol Chem* 267:14879–14883.

43. Lonergan KM, Gray MW (1993) Editing of transfer RNAs in Acanthamoeba castellanii mitochondria. *Science* 259:812–816.

44. Schürer H, Schiffer S, Marchfelder A, Mörl M (2001) This is the end: processing, editing and repair at the tRNA 3'-terminus. *Biol Chem* 382:1147–1156.

45. Borner GV, Morl M, Janke A, Paabo S (1996) RNA editing changes the identity of a mitochondrial tRNA in marsupials. *EMBO J* 15:5949–5957.

46. Börner GV, Yokobori S, Mörl M, Dörner M, Pääbo S (1997) RNA editing in metazoan mitochondria: staying fit without sex. *FEBS Lett* 409:320–324.

47. Muller HJ (1964) The relation of recombination to mutational advance. *Mutat Res* 106:2–9.

48. Bergström J, Hou XG (2001) Cambrian Onychophora or xenusians. *Zoologischer Anzeiger-A Journal of Comparative Zoology* 240:237–245.

49. Monge-Nájera J, Xianguang H (2002) Experimental taphonomy of velvet worms (Onychophora) and implications for the Cambrian" explosion, disparity and decimation" model. *Revista de biología tropical* 50:1133–1138.

50. Monge-Najera J (1995) Phylogeny, biogeography and reproductive trends in the Onychophora. *Zoological Journal of the Linnean Society* 114:21–60.

51. Randau L, Munch R, Hohn MJ, Jahn D, Soll D (2005) Nanoarchaeum equitans creates functional tRNAs from separate genes for their 5'- and 3'-halves. *Nature* 433:537–541.

52. Masta SE, Boore JL (2008) Parallel evolution of truncated transfer RNA genes in arachnid mitochondrial genomes. *Mol Biol Evol* 25:949–959.

53. Beckenbach AT, Joy JB (2009) Evolution of the mitochondrial genomes of gall midges (Diptera: Cecidomyiidae): rearrangement and severe truncation of tRNA genes. *Genome Biol Evol* 2009:278–287.

54. Saghai-Maroof MA, Soliman KM, Jorgensen RA, Allard RW (1984) Ribosomal DNA spacer-length polymorphisms in barley: mendelian inheritance, chromosomal location, and population dynamics. *Proc Natl Acad Sci USA* 81:8014–8018.

55. Price DH, Gray MW (1999) Confirmation of predicted edits and demonstration of unpredicted edits in Acanthamoeba castellanii mitochondrial tRNAs. *Curr Genet* 35:23–29.

56. Burger G, Lavrov DV, Forget L, Lang BF (2007) Sequencing complete mitochondrial and plastid genomes. *Nat Protoc* 2:603–614.

57. Staden R (1996) The Staden sequence analysis package. *Molecular biotechnology* 5:233–241.

58. Lavrov DV, Brown WM, Boore JL (2004) Phylogenetic position of the Pentastomida and (pan)crustacean relationships. *Proc R Soc Lond B Biol Sci* 271:537–544.

Figures and Table

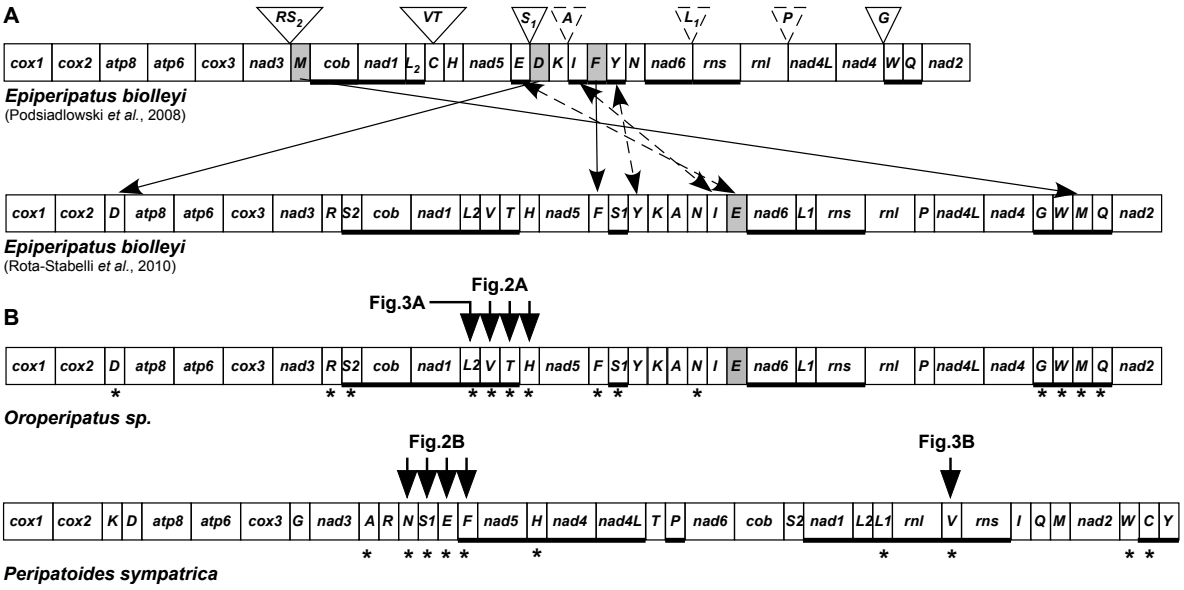


Fig 1. Mitochondrial gene orders in Onychophora. (*A*) Comparison of mitochondrial gene arrangements in *Epiperipatus biolleyi* inferred by Podsiadlowski et al. (1) and in our earlier study (39). (*B*) Inferred mitochondrial gene order in *Oroperipatus* sp. and *Peripatoides sympatrica* investigated for this study. Underlined genes are transcribed from right to left; other genes are transcribed in the opposite direction. Arrows indicate incongruence in tRNA gene annotations. Triangles indicate genes that were reported missing by Podsiadlowski et al. (1) but found by Rota-Stabelli et al. (39). Solid triangles and solid arrow lines indicate inferences supported by the present study. Asterisks under tRNA genes denote tRNAs for which cDNA sequences were amplified and sequenced.

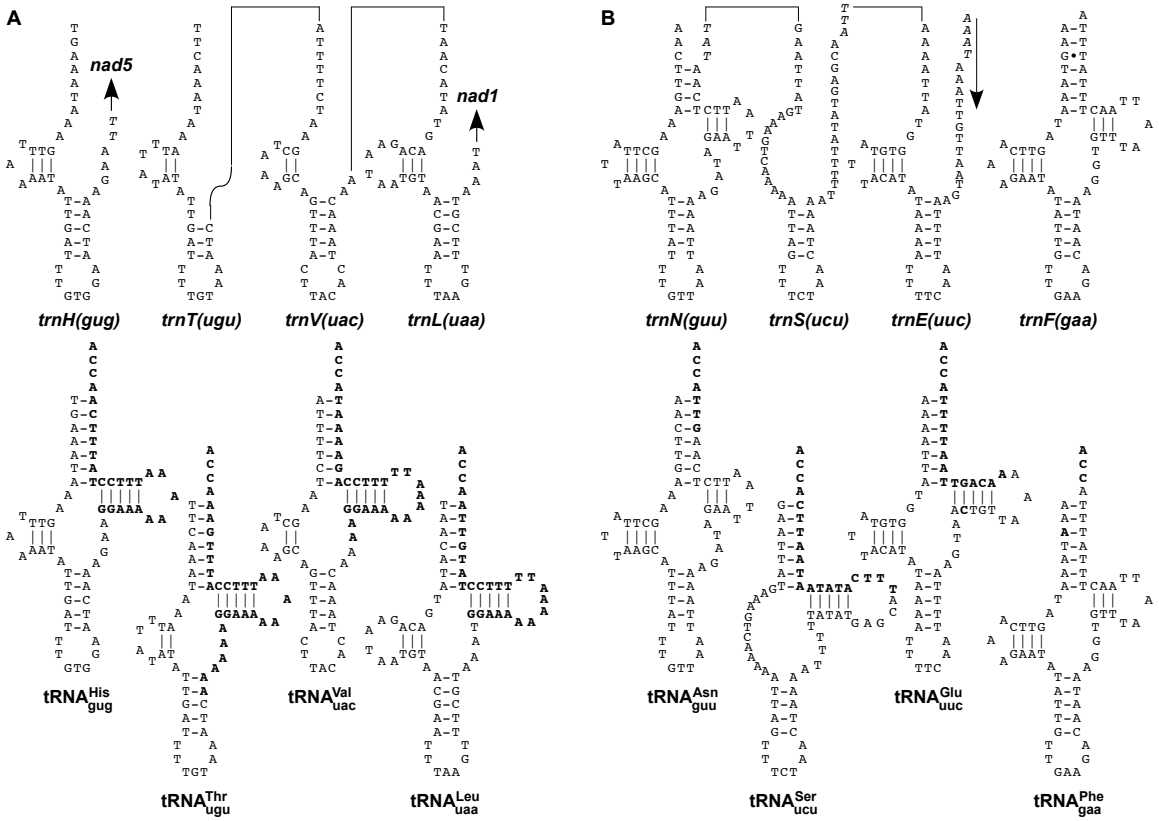


Fig 2. Secondary structures of *Oroperipatus* sp. (A) and *Peripatoides sympatrica* (B) tRNAs as inferred from the genomic sequence (Upper part) and as modified at the RNA level (Lower part). Edited nucleotides are in bold.

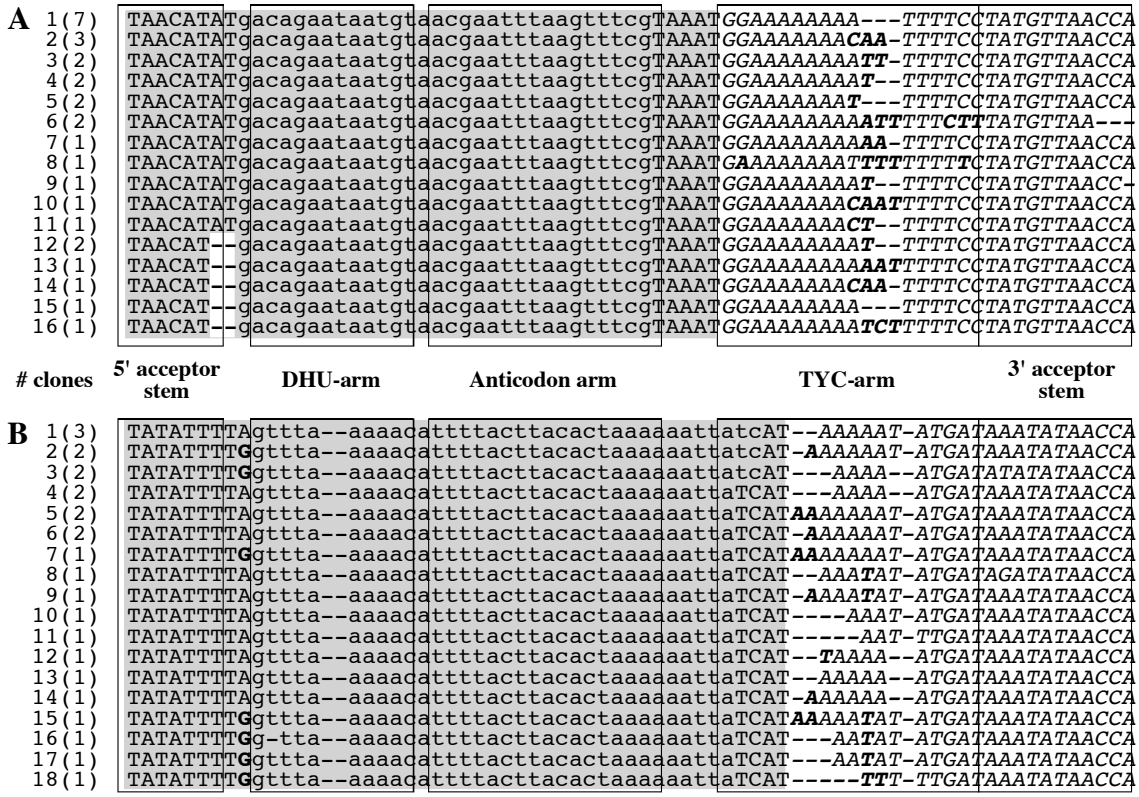
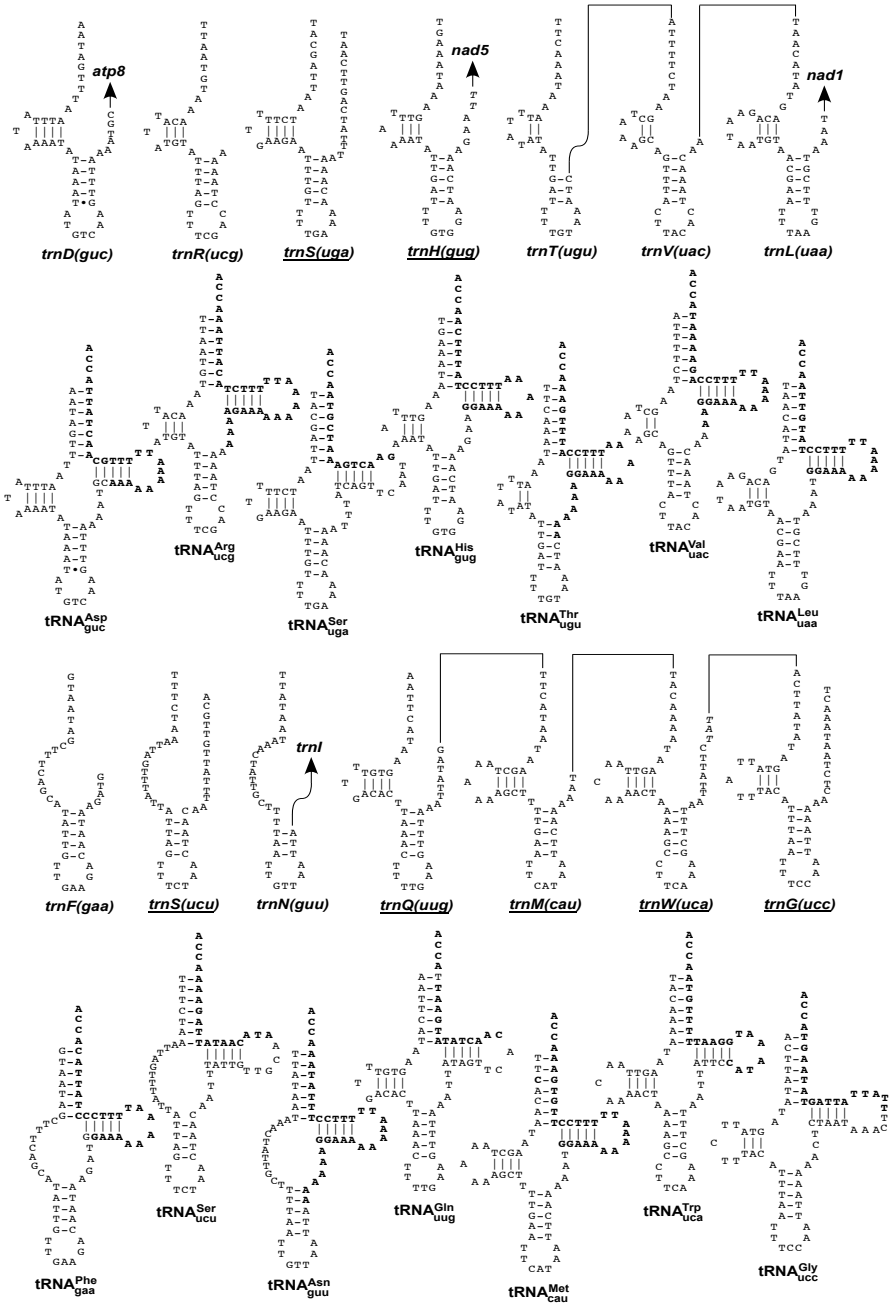
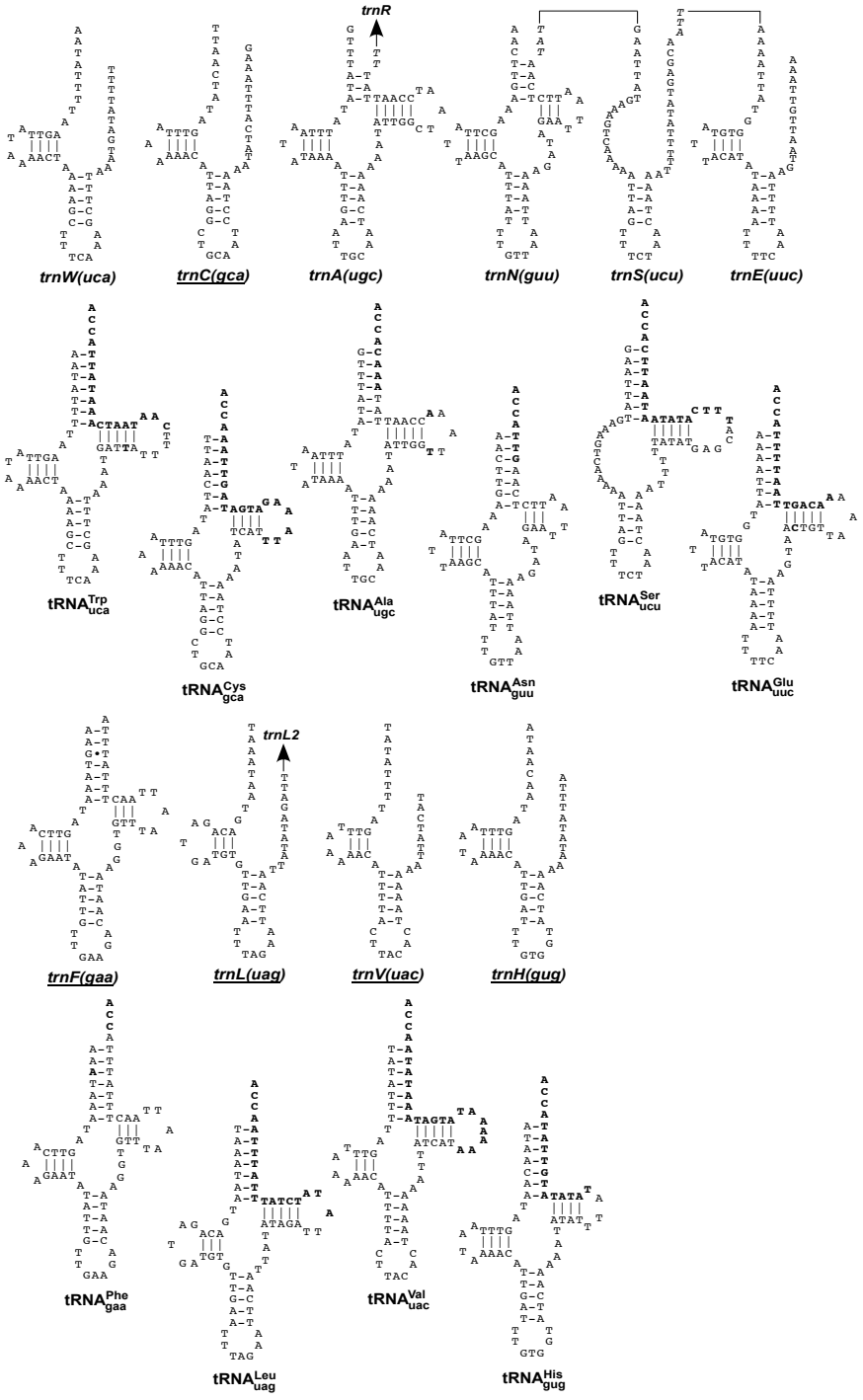


Fig 3. cDNA sequence variation in edited tRNAs. (*A*) *Oroperipatus* sp. mt-$tRNA^{Leu}_{UAA}$. (*B*) *Peripatoides sympatrica* mt-$tRNA^{Val}_{UAC}$. Sequence encoded in the genome is presented on grey background. Sequence added post-transcriptionally is shown on white background and is italicized. Region used to design primers is shown in lower case. Variations among the cDNA sequences are shown in bold.

Supplementary Material



Supplementary fig. 1. Secondary structures of 14 *Oroperipatus* sp. tRNAs. (Upper part) inferred from mtDNA sequence. (Lower part) inferred from cDNA sequences. Edited nucleotides are in bold. tRNA genes encoded by the β strand are underlined.



Supplementary fig. 2. Secondary structures of 10 *Peripatoides sympatrica* tRNAs. (Upper part) inferred from mtDNA sequence. (Lower part) inferred from cDNA sequences. Edited nucleotides are in bold. tRNA genes encoded by the β strand are underlined.

Primers used in reverse transcription and PCR amplification of circularized tRNAs in *Oroperipatus* sp.

tRNA	5’ (RT) Primer	3’ Primer
Q	5’ -cacaagcttGTTTAAGTGTCAAACACTTATG- 3’	5’ -ataggatccGACACTTAAACTTTTGAAG- 3’
W	5’ -cacaagcttAGGCTTTTAGTTTTGTTAAC- 3’	5’ -ataggatccAACTAAAAGCCTTCAAAGCT- 3’
N	5’ -cacaagcttAAAAGCAATAGTTTATTATA- 3’	5’ -ataggatccAACTATTGCTTTTAATTGTTA- 3’
S1	5’ -cacaagcttACTAATAATAAACTAATTTAG- 3’	5’ -ataggatccAGTTTATTATTAGTTTCTAAC- 3’
F	5’ -cacaagcttCTTCAACAATATGCTGAAAGC- 3’	5’ -ataggatccGCATATTGTTGAAGACAATAAG- 3’
S2	5’ -cacaagcttAACAAATTCTTCAAAAGATTA- 3’	5’ -ataggatccTGAAGAATTTGTTTTGAAAAC- 3’
R	5’ -cacaagcttAACTAAATACATAATGTTTAC- 3’	5’ -ataggatccAATAGTATTTAGTTTCGACC- 3’
D	5’ -cacaagcttATTTATATTTTATAAATTAAAC- 3’	5’ -ataggatccAATATAAATATGTCAAGTTTA- 3’
L2	5’ -cacaagcttATTCGTTACATTATTCTGTC- 3’	5’ -ataggatccGTAACGAATTTAAGTTTCG- 3’
T	5’ -cacaagcttTCAATATATAAATTTATTTG- 3’	5’ -ataggatccATATATTGATTTTGTAAATC- 3’
H	5’ -cacaagcttCCACAAATCAATCTTTTAAAC- 3’	5’ -ataggatccTATTGATTTGTGGAATCAAAG- 3’
V	5’ -cacaagcttAGTAAACTGCTTTAGCTTAG- 3’	5’ -ataggatccGCAGTTTACTTACACTAAAC- 3’
G	5’ -cacaagcttGGAAATTAAATGTAAATAATAC- 3’	5’ -ataggatccCATTTAATTTCCAATTAAAAAC- 3’
M	5’ -cacaagcttGAATTCAAAAGCTTTTTTAGC- 3’	5’ -ataggatccGCTTTTGAATTCATAATTC- 3’

Primers used in reverse transcription and PCR amplification of circularized tRNAs in *Peripatoides sympatrica*

tRNA	5’ (RT) Primer	3’ Primer
E	5’ -TTTTATATGTAATACACCATA- 3’	5’ -CATAAAATTTTCAATTTTAAG- 3’
W	5’ -GAAAGCTTTAGTTTTATAAC- 3’	5’ -AAGCTTTCAAAGCTTTAAATG- 3’
A	5’ -GCAATTCAAATTTTATATTA- 3’	5’ -TGAATTGCAATCAAAAAATA- 3’
F	5’ -CAACAATATATTCTTTGAACTA- 3’	5’ -TATTGTTGAAGACAATAAGGTG- 3’
N	5’ -ATAAATGCTTAATAAGCTTTC- 3’	5’ -TATTTGTTAATTAAAAGATAG- 3’
C	5’ -CCTAATGTTTATTAAACTATAGC- 3’	5’ -AGGCTGCAATCCTAAAATATC- 3’
L1	5’ -TTCAACACATCATCTGTCA- 3’	5’ -GTGTTGAATTTAGAATTCAATTA- 3’
H	5’ -CAATGTTTTATTAAACTATTG- 3’	5’ -AAAACATTGATTTGTGGTATC- 3’
S1	5’ -ataggatccAGTTAGAAACTAATTTTTGAC- 3’	5’ -cacaagcttTCTAACTAAAATTTTTATATG- 3’
V	5’ -cacaagcttGTAAGTAAAATGTTTTTTAAC- 3’	5’ -ataggatccAAAACATTTTACTTACACTAA- 3’ 5’ -ataggatccTACTTACACTAAAAAAATATC- 3’

Supplementary table 1. All primer pairs that were used for reverse transcription and subsequent PCR amplification in *Oroperipatus* sp and *Peripatoides sympatrica* tRNAs. The nine 5' nucleotides in some primers (in lower case) were added to create a restriction site for HindIII (5' AAGCTT) or BamHI (5' GGATCC) plus three terminal nucleotides.

CHAPTER 3: THE COMPLETE MITOCHONDRIAL DNA SEQUENCES OF THE ONYCHOPHORANS *OROPERIPATUS* SP (PERIPATIDAE) AND *PERIPATOIDES SYMPATRICA* (PERIPATOPSIDAE)

A paper to be submitted to the *Mitochondrion*

Romulo Segovia[1], Walker Pett[2], and Dennis V. Lavrov[3]

Abstract

We determined the complete mitochondrial DNA sequences of the onychophorans *Oroperipatus* sp. (Peripatidae) from Belize and *Peripatoides sympatrica* (Peripatopsidae) from New Zealand, and found that they contain 36 and 37 genes respectively. *Oroperipatus* sp. mtDNA lacks *trnC(gca)* and its gene order is similar to that of *Epiperipatus biolleyi*. Both Onychophoran mitochondrial genomes are characterized by the presence of extreme reduced tRNA genes similar to those found in arachnids and gall midges, including the loss of the variable loop, TΨC arms, and the 3' side of the aminoacyl stems. Remarkably, in *Oroperipatus* sp. *trnT(ugu)* and *trnN(guu)* the editing starts at position 42 in a standard tRNA structure, which makes them the most reduced and yet functional mt-tRNA genes in animals to date. In addition, we found two stem loop structures in *Oroperipatus* sp. mtDNA. One

---

1 Graduate Student and primary author, Department of Ecology, Evolution, and Organismal Biology, Iowa State University.

2 Graduate Student, Department of Ecology, Evolution, and Organismal Biology, Iowa State University.

3 Professor and author for correspondence, Department of Ecology, Evolution, and Organismal Biology, Iowa State University, dlavrov@iastate.edu

stem loop is encoded in each strand. These structures are conserved among the "New World" onychophorans since *E. biolleyi* and *Metaperipatus inae* also display similar features. Because of similarities to the human stem loop structure associated with the origin of L-strand replication (association also confirmed in some invertebrates), we suggest that an analogue replication mechanism may exist in *Oroperipatus* sp. mtDNA. *P. sympatrica* mtDNA gene order is identical to the Ancestral Arthropod Gene Order (AAGO) with an exception of *trnQ(uug)* inversion. This indicates that *P. sympatrica* gene arrangement represents the ancestral Onychophoran gene order.

Introduction

Animal mitochondrial DNA contains a limited number of essential genes required for the electron transport chain and a minimal translation system. The typical animal mitochondrial genome is a circular molecule of approximately 16 kbp in size and is composed of 37 genes: 2 for the small and large subunit ribosomal RNA (*rns* and *rnl*), 22 for transfer RNAs (tRNAs), and 13 for protein subunits of complexes I, III, IV, and V involved in oxidative phosphorylation (NADH dehydrogenase subunits 1-6, *nad1-6*; cytochrome b, *cob*; cytochrome c oxidase subunits I-III, *cox1-3*; and ATP synthase subunits 6 and 8, *atp6* and *atp8*). In bilaterian animals, the mtDNA normally contains a single noncoding region containing hairpin-like structures that were probed to be the mtDNA origin of replication in a few animals (Hixson et al., 1986; Zhang et al., 1995).

Onychophora, also known as velvet worms or claw bearers, is a group of ancient animals whose appearance dates back to the early Cambrian (Bergström and Hou, 2001; Monge-Nájera and Xianguang, 2002). Based on molecular information, the relationship of

Onychophora to the Arthropoda and Tardigrada in the monophyletic group Panarthropoda within a clade called the Ecdysozoa that includes Cycloneuralia is currently widely accepted (Aguinaldo et al., 1997; Podsiadlowski et al., 2008; Telford et al., 2008) (Rota-Stabelli et al., 2010). Over 150 species of onychophorans have been described to date, which are distributed in two families worldwide: Peripatopsidae and Peripatidae. Members of the Peripatopsidae family are found in Chile, South Africa, Australia (including Tasmania), New Guinea, and New Zealand, whereas members of the Peripatidae family are found in Central and tropical South America, Equatorial West Africa, and South East Asia.

Despite their ancient origin, important phylogenetic position, and interesting biology, onychophorans remain to be relatively understudied group of animals. In particular, only four complete mtDNA from representatives of four different genera have been sequenced to date. Two genera correspond to species from the "New World": *Epiperipatus biolleyi* (Peripatidae) from Central America and *Metaperipatus inae* (Peripatopsidae) from Chile. The remaining two genera correspond to *Opisthopatus cinctipes* (Peripatopsidae) from South Africa and *Peripatoides* sp. (Peripatopsidae) from New Zealand species. The mitochondrial genomes from the sampled onychophorans revealed a diversity of architectures, including numerous gene order rearrangements and strand asymmetry variations (Rota-Stabelli et al., 2010). Furthermore, there were contradictory reports on the gene content (Podsiadlowski et al., 2008)(Rota-Stabelli et al., 2010), which is due to a highly unusual structure of tRNA genes. In addition, *O. cinctipes* and *M. inae* complete mitochondrial genomes were made available in two studies by Braband (Braband et al., 2010a; Braband et al., 2010b). *O. cinctipes* was found to partially conserve the ancestral mitochondrial gene arrangement of Panarthropoda

and Ecdysozoa (Seven tRNA genes are missing from the Ancestral Arthropod Gene Order pattern represented by Limulus Polyphemus mt-DNA (Lavrov et al., 2000b). *Peripatoides* sp. on the other hand seemed to retain the AAGO pattern (Rota-Stabelli et al., 2010). *M. inae* mtDNA was presented as a molecule whose gene organization reflects dramatic rearrangements compared to the AAGO, even though all protein-coding and ribosomal RNA genes are encoded on the same strands, which gave ground to suggest that this genome evolved by partial genome duplication. (Braband et al., 2010b)

Here we present the complete mtDNA sequence of two distant onychophorans, *Oroperipatus* sp. (Peripatidae) a representative sample from Belize, Central America, and *Peripatoides sympatrica* (Peripatopsidae) from New Zealand. Besides their description and analysis in gene content, we are comparing their gene orders to all other onychophoran gene orders available to date. We are also presenting sequences that produce stem loop structures in *Oroperipatus* sp., and their similarities to other organisms stem loops. In addition, we show the ancestral gene order in Onychophora represented by the *P. sympatrica* gene order. The sequencing of the *Oroperipatus* sp. and *P. sympatrica* complete genomes confirm the high diversity of gene arrangements in Onychophora.

Materials and Methods

DNA extraction, cloning and sequencing

The specimen of *Oroperipatus* sp. was collected at the Belize Foundation for Research and Environmental Education (BFREE) station in the Toledo district of Belize. The specimen of *Peripatoides sympatrica* was collected at the Hakarimata Walkway bordering

Huntly town, North Island, New Zealand. Both specimens were stored in 80% ethanol at -20C. Total DNA was extracted from both specimens using 2x CTAB buffer and phenol-chloroform method (Saghai-Maroof et al., 1984). Primers designed to match conserved regions of animal mtDNA (Burger et al., 2007) were used to amplify small fragments of *rns* and *cox3* (*P. sympatrica*) and *cox1* and *nad5* (*Oroperipatus sp.)*, and used to design specific primers for long-range PCR amplification of the entire molecule in two fragments using Takara LA-PCR kit under conditions recommended by the manufacturer. For *Oroperipatus* sp., these fragments were sheared and barcoded for the GS FLX Titanium library preparation (454 Life Sciences) along with other samples. Pyrosequencing was carried out on a Genome Sequencer FLX Instrument (454 Life Sciences) at the University of Indiana Center for Genomics and Bioinformatics. For *P. sympatrica*, random clone libraries were constructed from the purified PCR products using the TOPO Shotgun Subcloning Kit from Invitrogen. Clones were sequenced at the Iowa State University office of Biotechnology DNA facility.

Annotation of genes and further analysis

Assembling of reads was carried out using the STADEN package v. 1.6.0 (Staden, 1996). Problematic regions were reamplified directly from PCR templates by primer walking using conventional Sanger sequencing. All genes were identified by similarity searches in local databases using the FASTA program (Pearson, 1994). In addition fourteen severe-reduced *Oroperipatus sp.* tRNA genes (*trnD, R, S, H, T, V, L(uaa), F, S(ucu), N, Q, M, W,* and *G*) and ten *P. sympatrica* tRNA genes (*trnW, C, A, N, S(ucu), E, F, L(uag), V*, and *H*) were more accurately later defined based on their respective cDNA sequences obtained when studying transfer RNA editing. All tRNA secondary structures were manually folded. The

codon usage table was generated using the CUSP program available in the EMBOSS package (Rice et al., 2000).

Results and Discussion

Genome organization and nucleotide composition

The mitochondrial genomes of *Oroperipatus* sp. and *Peripatoides sympatrica* are circular molecules of 14801 bp and ~14.2 kb in size containing 36 and 37 genes respectively. Both genomes contain 13 protein-coding genes and two for the small and large subunits of rRNA, whereas the complete set of 22 tRNA genes is found in *P. sympatrica,* in *Oroperipatus* sp. is found 21 tRNA genes (fig. 1) just as *E. biolleyi* where *trnC* was the single missing gene. *Oroperipatus* sp. mtDNA is characterized by a large noncoding region (l-ncr) (1579 nt) located between *trnI(gau)* and *nad6* genes, and a small noncoding region (s-ncr) (56 nt) between *trnK(uuu)* and *trnA(ugc)*. *P. sympatrica* mtDNA, on the other hand, contains a single noncoding region between *trnQ(uug)* and *trnM(cau)* (we could not amplify this region completely due to its high content in AT runs). Both genomes are characterized by gene overlaps and few intergenic nucleotides among those with the same transcriptional polarity (Table 1), but above all for the presence of a severe reduced set of tRNA genes, many of them encoded in clusters.

Among the "New World onychophorans" we found that *Oroperipatus* sp. mtDNA gene order is very similar to that of *E. biolleyi* (Rota-Stabelli et al., 2010) (fig. 2), with the sole differences of the translocation of *trnE(uuc)* inside the l-ncr, as well as the s-ncr absence in *E. biolleyi*. Among members of the two families, a large amount of rearrangements is

observed between *Oroperipatus* sp. and *M. inae* gene orders (fig. 2). Translocations of cox2-atp8-atp6, cob, nad1, and rnl-nad4L genes are observed between them. Among members of the Peripatopsidae family, a large amount of rearrangements is also observed between *P. sympatrica* and *M. inae* gene orders (fig. 2). As *P. sympatrica* and *Peripatoides* sp. are species from the same genus that display the same gene organization. 85% of the protein nucleotide identity is shared between them (Data not showed). *M. inae* mtDNA seems to be the more derived since its gene order displays fewer correspondences to other members of the same family.

On the other hand, despite *O. cinctipes* (Braband et al., 2010a) and *P. sympatrica* geographical separation, they still conserve the Ancestral Arthropod Gene Order (AAGO) represented by the gene order of the horseshoe crab *L. polyphemus* (Lavrov et al., 2000a) (fig. 2). However *O. cinctipes* differs from the AAGO by seven tRNA genes, and translocations of *trnS1* and *trnW(uca)* (The authors did not found *trnD, R, N, L2, Q,* and *C*). Conversely, we found that *P. sympatrica* conserves the identical AAGO with the single exception of an inversion of *trnQ(uug)*. Based on this information, we infer that the *P. sympatrica* mitochondrial gene organization and members of the Peripatidae genus from New Zealand represents the ancestral gene order in Onychophora (*Peripatoides* sp. displays the same gene order as well). Likewise, only *atp6-atp8* and *cob-trnS2* gene boundaries are conserved among all onychophorans. All Peripatopsid members share the following gene boundaries: *nad6-cob-trnS2, trnV-rns, trnH-nad4, trnM-nad2,* and *trnY-cox1*.

In O*roperipatus* sp., protein genes accounts for 75.4% of the entire genome (11157 bp), rRNA genes for 11.9% (1765 bp), tRNA genes for 6.3% (928 bp) and noncoding regions

for 7.6% (1132 bp). Whereas in *P. sympatrica,* protein genes accounts for 78% (11085 bp), rRNA genes for 12.7% (1808 bp), and tRNA genes for 7.9% (~1133 bp). The AT content of the *Oroperipatus* sp. and *P. sympatrica* mtDNAs are 76.3% and 79.5% respectively, similar to those onychophora whose complete mt genomes were sequenced: *E. biolleyi* (74.1%), *Peripatoides* sp. (80.5%), *M. inae* (78.3%), and *O. cinctipes* (77.5%). The strand profiles in both onychophorans presented in this paper are as follows: There is a small negative AT-skew (-0.09) and slightly stronger positive GC-skew (0.11) between the two DNA strands of *P. sympatrica*, whereas stronger negative and positive AT (-0.13) and GC (0.19) skews in *Oroperipatus* sp. respectively. Interestingly, all protein-coding genes in both onychophorans display negative AT-skews (table 1), meanwhile positive and negative GC-skews are observed in all protein-coding genes encoded in the Alfa and Beta strands respectively of *Oroperipatus* sp. Finally, all protein-coding genes of *P. sympatrica* display positive GC skews with the single exception of *cob* (-0.07).

Protein genes

Oroperipatus sp. protein coding genes are more similar to *E. biolleyi* in size (table 1) and in sequence similarity compared to other onychophorans (table 2). Seven genes in *Oroperipatus* sp. (*cox2, atp6, cox3, nad3, nad4L, nad4,* and *nad2*) are the same size, three (*nad6, cob,* and *cox1*) are slightly smaller (3, 6, and 9 nt respectively) and two (*atp8* and *nad5*) are slightly larger (6 and 9 nt respectively) than *E. biolleyi*. Sequence comparisons among *Oroperipatus* sp. and other onychophorans (table 2) reflect a broader degree of variation and reveal the same pattern of gene conservation observed in arthropods (Crease, 1999) showing *cox1* as the most conserved gene (86.9% similar to *E. biolleyi*) and *nad6,*

atp8, and *nad2* as the least conserved genes in the same order. In general an overall protein sequence identity of 77.2, 68.5, 67.5, and 67.7% is observed between *Oroperipatus* sp. and *E. biolleyi, P. sympatrica, M. inae,* and *O. cinctipes* respectively (table 2) reflecting more conservation among species of the same family and less conservation among species of the two families. Again *M. inae* appears to hold the less conserved set of protein coding genes within the Peripatopsidae family and between the two families as well. 68.5, 73.5, and 74% identities are observed between *P. sympatrica* and *E. biolleyi, M. inae,* and *O. cinctipes* respectively.

Most coding sequences in *Oroperipatus* sp. and *P. sympatrica* use ATG as a start codon (table 1). Alternative initiator codons normally found in invertebrate mitochondria are also used: ATA is used by *Oroperipatus* sp. *cox1* and *atp8, and P. sympatrica atp8*. ATT is used by *Oroperipatus* sp. *nad1* only. Interestingly, the unusual start codon TTA is used by *P. sympatrica cox1*. This nonstandard TTA codon was also found being used by the *L. polyphemus cox1* (AAGO) (Staton et al., 1997; Lavrov et al., 2000a). *P. sympatrica* does not use two stop codons: TAG and CGC which recognize arginine. On the other hand, codons ending with T or A are preferred compared to those ending with G or C (86.6 and 93.7% in *Oroperipatus* sp. and *P. sympatrica* respectively). This indicates that the codon usage of onychophoran mtDNA is highly biased, especially that of *P. sympatrica*.

Truncated transfer RNA genes

We determined the presence of 21 mt-tRNA genes in *Oroperipatus* sp. (fig. 3), which is typical for bilaterian animals with the exception of *trnC,* just as in *E. biolleyi,* and the complete set of 22 mt tRNA genes in *P. sympatrica* (fig. 4). The majority of tRNA genes in

Oroperipatus sp. are severely reduced. They encode incomplete tRNA products that can lack sequences at the 3' side of the acceptor stem, the TΨC arm, the anticodon stem, and even the last two nucleotides of the acceptor stem. From 21 tRNA genes, the exact nucleotide composition and boundaries of 14 genes were confirmed at the RNA level when studying RNA editing for another article (fig. 3). Confirmation that the remaining 7 tRNA genes are real genes is supported by the same localization in *E. biolleyi*, with the sole exception of *trnE(uuc),* which in *Oroperipatus* sp. is encoded inside the large noncoding region. Whereas in *E. biolleyi* this gene is found in the major tRNA cluster between *trnK(uuu)* and *trnI(gau)*. Therefore, we cannot state that *Oroperipatus* sp. *trnE(uuc)* is not a pseudogene. The tRNA genes that are more severely reduced are *trnT(ugu)* and *trnN(guu)* since only 33 nucleotides are encoded in both genes (positions 42 and 43 in a standard tRNA structure also appear to be missing). *Oroperipatus* sp. *trnT(ugu)* and *trnN(guu)* represent the most reduced and yet functional reported mt-tRNA genes in animals to date.

The same pattern of tRNA gene truncation is seen in *P. sympatrica*, although to a lesser degree. Many of their tRNA genes still conserve the complete TΨC arm (*trnA(ugc), trnS(uga), trnL(uaa), trnI(gau),* and *trnQ(uug)*). Interestingly *trnF(gaa)* encodes the entire tRNA structure. From the complete set of 22 tRNA genes in *P. sympatrica*, 10 were confirmed at the RNA level by amplification of their edited cDNA products (fig. 4).

The possession of a set of severely reduced tRNA genes is not unique to onychophorans, since it has also been recently reported similar genes in the mitochondrial genomes of arachnids (Masta and Boore, 2008), and of gall midges (Beckenbach and Joy, 2009). In general, eleven *Oroperipatus* sp. and fourteen *P. sympatrica* tRNA genes are

encoded by the α strand (the strand encoding most of the protein coding genes), and ten by the β strand (fig. 1). On average the AT content of tRNA genes is 80.8% in *Oroperipatus* sp., and 81.2% in *P. sympatrica*, which is slightly higher than the AT composition of the genome.

Noncoding regions and stem-loop structures

There are two well-defined noncoding regions (ncr) in *Oroperipatus* sp. mtDNA. The large ncr (l-ncr) located between *trnI(gau)* and *nad6* genes is 1076 nt in size, 82.4% AT rich, and contains the *trnE(uuc)* and sequences for one large stem loop structure. The small ncr (s-ncr) located between *trnK(uuu)* and *trnA(ugc)* is 56 nt long and 78.2% AT rich (fig. 1). *P. sympatrica* contains a single ncr located between *trnQ(uug)* and *trnM(cau)*. We found two sequences in *Oroperipatus* sp. encoded in opposite strands with the potential to form nearly identical stem-loop structures (fig. 5). The first stem-loop structure (SL1) is encoded in the alfa strand in a tRNA gene cluster between *trnY(gua)* and *trnK(uuu)*. The second stem-loop structure (SL2) is located in the beta strand in the largest noncoding region. The stems of both structures are composed of 9 base pairs whereas the loops are comprised of 11 and 9 thymine runs each. We expect that the stem loop structure corresponding to *P. sympatrica* is located in a region between *trnQ(uug)* and *trnM(cau)*, however we could not obtain any sequences for this region due to its high content in AT runs.

When analyzing stem loops from other velvet worms, we observed a high degree of conservation among the "New World" onychophorans (fig. 5). *E. biolleyi* mtDNA contains two analogue stem loop structures located in the same position, and 92.6% identical in sequence. Even though *M. inae* apparently contains three stem loop structures (Braband et al., 2010b), two of them that are encoded in different strands are also similar (44.4% of

nucleotide identity). Conversely, *O. cinctipes* (Braband et al., 2010a) displays only one stem-loop structure, which differs substantially from the "New World" onychophorans, resembling the replication origins of related structures found in the insects *L. migratoria, S. gregaria,* and *C. parallelus* (Zhang et al., 1995; Saito et al., 2005).

Despite occupying a different position, sequences configuring stem loop structures were proved to be the origin of replication of the minor coding strand in different species. This was proved mostly in humans (Hixson et al., 1986) and in some invertebrates: Drosophila (Clary and Wolstenholme, 1987) and in the locust *L. migratoria* (Saito et al., 2005). Due to typical hairpin-like structures we infer that the stem loops SL1 and SL2 in *Oroperipatus* sp. represent the origin of mtDNA replication. Furthermore they display the GAT sequence consensus at the 3' end. This G(A)nT motif is also present in insects, mammals and diverse organisms as the mitochondrial L-strand replication origin (Zhang et al., 1995), which indicates its universal conservation and functional importance related to replication origins in onychophora. The GAT motif is present in *E. biolleyi* flanking both stem loop structures.

Interestingly, we found that the human stem loop structure associated with the origin of L-strand replication (Hixson et al., 1986) (Wong and Clayton, 1985), resembles the *Oroperipatus* sp. stem loop structures. Therefore if they are functional analogues, a novel replication mechanism may exist, which utilizes both of these structures for the separate initiation of replication on each strand of *Oroperipatus* sp. mtDNA. The thymine runs in both stem loops may serve for the recognition and synthesis of RNA primers by a mitochondrial DNA primase, a requirement for L-strand synthesis. The transition to DNA synthesis may

occur in specific sequences flanking the base of the stem. It is also interesting to mention that the sequence TCTAAA flanking the 5' of the stem is present in both human and *Oroperipatus* sp. mtDNA. This sequence in humans is related to the position where RNA/DNA transitions take place. Perhaps this position plays a similar role in Onychophora. Interestingly two stem-loop structures have also been observed in millipedes (Lavrov et al., 2002).

References

Aguinaldo, A.M.A., Turbeville, J.M., Linford, L.S., Rivera, M.C., Garey, J.R., Raff, R.A. and Lake, J.A.: Evidence for a clade of nematodes, arthropods and other moulting animals. Nature (London) 387 (1997) 489-493.

Beckenbach, A.T. and Joy, J.B.: Evolution of the mitochondrial genomes of gall midges (Diptera: Cecidomyiidae): rearrangement and severe truncation of tRNA genes. Genome Biol Evol 2009 (2009) 278-287.

Bergström, J. and Hou, X.G.: Cambrian Onychophora or xenusians. Zoologischer Anzeiger-A Journal of Comparative Zoology 240 (2001) 237-245.

Braband, A., Cameron, S.L., Podsiadlowski, L., Daniels, S.R. and Mayer, G.: The mitochondrial genome of the onychophoran Opisthopatus cinctipes (Peripatopsidae) reflects the ancestral mitochondrial gene arrangement of Panarthropoda and Ecdysozoa. Mol Phylogenet Evol (2010a)

Braband, A., Podsiadlowski, L., Cameron, S.L., Daniels, S. and Mayer, G.: Extensive duplication events account for multiple control regions and pseudo-genes in the mitochondrial genome of the velvet worm Metaperipatus inae (Onychophora, Peripatopsidae). Mol Phylogenet Evol (2010b)

Burger, G., Lavrov, D.V., Forget, L. and Lang, B.F.: Sequencing complete mitochondrial and plastid genomes. Nat. Protoc. 2 (2007) 603-614.

Clary, D.O. and Wolstenholme, D.R.: Drosophila mitochondrial DNA: conserved sequences in the A + T-rich region and supporting evidence for a secondary structure model of the small ribosomal RNA. J. Mol. Evol. Journal of Molecular Evolution 25 (1987) 116-125.

Crease, T.J.: The complete sequence of the mitochondrial genome of Daphnia pulex (Cladocera: Crustacea). Gene Gene (Amsterdam) 233 (1999) 89-99.

Hixson, J.E., Wong, T.W. and Clayton, D.A.: Both the conserved stem-loop and divergent 5'-flanking sequences are required for initiation at the human mitochondrial origin of light-strand DNA-replication. J. Biol. Chem. Journal of Biological Chemistry 261 (1986) 2384-2390.

Lavrov, D.V., Boore, J.L. and Brown, W.M.: The complete mitochondrial DNA sequence of the horseshoe crab *Limulus polyphemus*. Mol. Biol. Evol. 17 (2000a) 813-824.

Lavrov, D.V., Boore, J.L. and Brown, W.M.: Complete mtDNA sequences of two millipedes suggest a new model for mitochondrial gene rearrangements: duplication and nonrandom loss. Mol. Biol. Evol. 19 (2002) 163-169.

Lavrov, D.V., Brown, W.M. and Boore, J.L.: A novel type of RNA editing occurs in the mitochondrial tRNAs of the centipede *Lithobius forficatus*. Proc Natl Acad Sci U S A 97 (2000b) 13738-13742.

Masta, S.E. and Boore, J.L.: Parallel evolution of truncated transfer RNA genes in arachnid mitochondrial genomes. Mol Biol Evol 25 (2008) 949-959.

Monge-Nájera, J. and Xianguang, H.: Experimental taphonomy of velvet worms (Onychophora) and implications for the Cambrian" explosion, disparity and decimation" model. Revista de biología tropical 50 (2002) 1133-1138.

Pearson, W.R.: Using the FASTA program to search protein and DNA sequence databases. Methods Mol Biol 25 (1994) 365-389.

Podsiadlowski, L., Braband, A. and Mayer, G.: The complete mitochondrial genome of the onychophoran Epiperipatus biolleyi reveals a unique transfer RNA set and provides further support for the ecdysozoa hypothesis. Mol Biol Evol 25 (2008) 42-51.

Rice, P., Longden, I. and Bleasby, A.: EMBOSS: the European Molecular Biology Open Software Suite. Trends Genet 16 (2000) 276-277.

Rota-Stabelli, O., Kayal, E., Gleeson, D., Daub, J., Boore, J.L., Telford, M.J., Pisani, D., Blaxter, M. and Lavrov, D.V.: Ecdysozoan mitogenomics: evidence for a common origin of the legged invertebrates, the panarthropoda. Genome Biol Evol 2 (2010) 425-440.

Saghai-Maroof, M.A., Soliman, K.M., Jorgensen, R.A. and Allard, R.W.: Ribosomal DNA spacer-length polymorphisms in barley: mendelian inheritance, chromosomal location, and population dynamics. Proc. Natl. Acad. Sci. USA 81 (1984) 8014-8018.

Saito, S., Tamura, K. and Aotsuka, T.: Replication origin of mitochondrial DNA in insects. Genetics 171 (2005) 1695-1705.

Staden, R.: The Staden sequence analysis package. Molecular biotechnology 5 (1996) 233-241.

Staton, J.L., Daehler, L.L. and Brown, W.M.: Mitochondrial gene arrangement of the horseshoe crab Limulus polyphemus L.: conservation of major features among arthropod classes. Mol Biol Evol 14 (1997) 867-874.

Telford, M.J., Bourlat, S.J., Economou, A., Papillon, D. and Rota-Stabelli, O.: The evolution of the Ecdysozoa. Philos Trans R Soc Lond B Biol Sci 363 (2008) 1529-1537.

Wong, T.W. and Clayton, D.A.: In vitro replication of human mitochondrial DNA: accurate initiation at the origin of light-strand synthesis. Cell Cell 42 (1985) 951-958.

Zhang, D.X., Szymura, J.M. and Hewitt, G.M.: Evolution and structural conservation of the control region of insect mitochondrial DNA. J Mol Evol 40 (1995) 382-391.

Figures and Tables

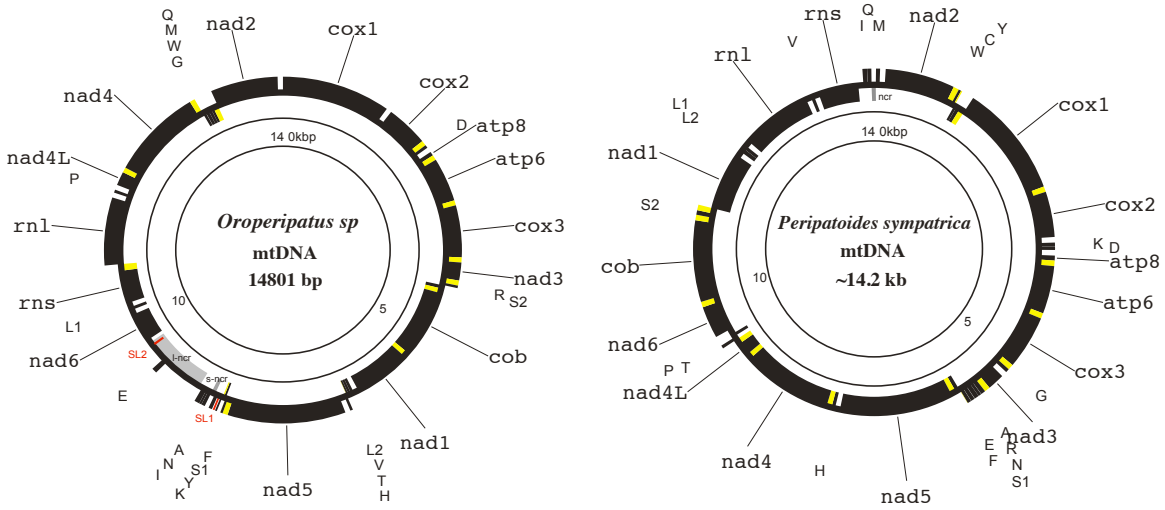


Fig 1. Gene maps of *Oroperipatus* sp. and *Peripatoides sympatrica* mtDNAs. Protein and rRNA genes are abbreviated as follows: *atp6* an *atp8* (genes for subunits 6 an 8 of the F0 ATPase), *cox1-cox3* (genes for cytochrome c oxidase subunits 1-3), *cob* (gene for apocytochrome b), *nad1-nad6* and *nad4L* (genes for NADH dehydrogenase subunits 1-6 and 4L), and *rns* and *rnl* (genes for small and large subunit rRNAs). The tRNAs genes are identified by the one-letter code for the corresponding amino acid. Two leucine and two serine tRNA genes are differentiated by their anticodon sequence with *trnL(uag)* marked as L1, *trnL(uaa)* as L2, *trnS(ucu)* as S1, and *trnS(uga)* as S2. Dark lines on the outside represent genes that are transcribed clockwise and inside lines counterclockwise. Regions in yellow indicate overlapped genes, SL1-SL2 (red) stem loops structures, and l-ncr and s-ncr (gray) denote large and small noncoding regions respectively.

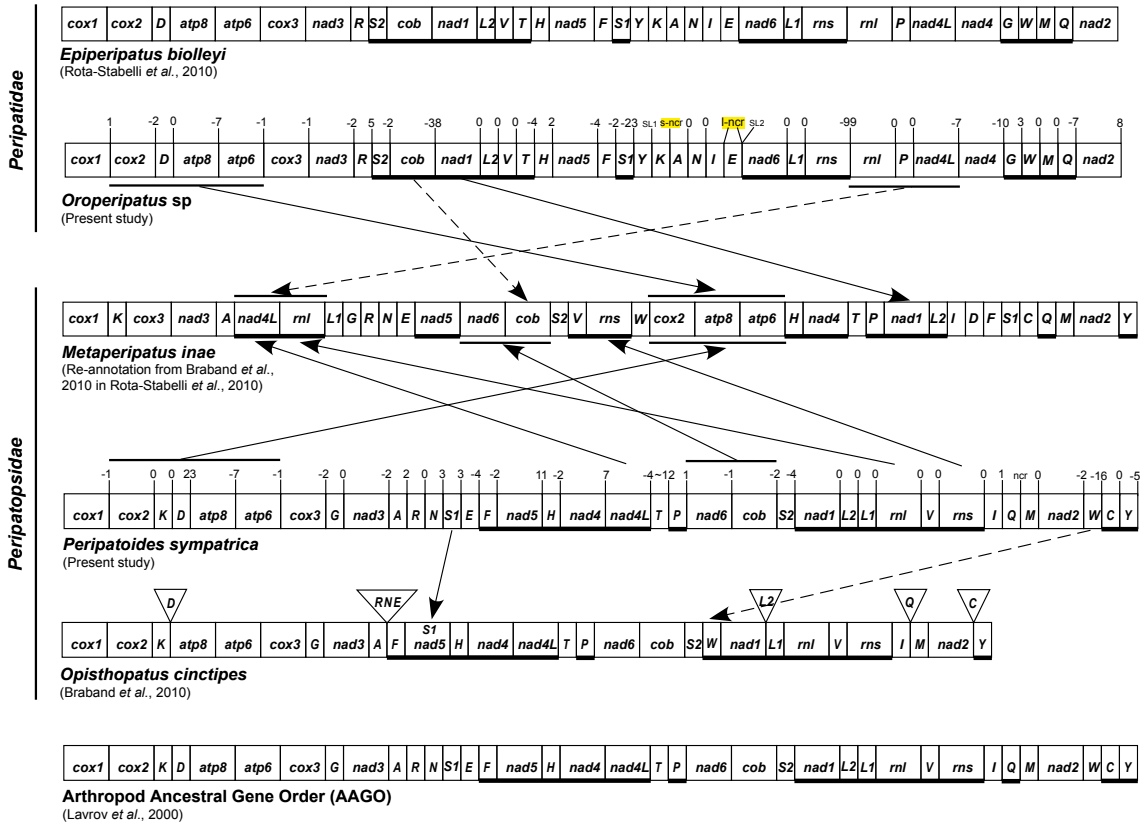


Fig 2. Mitochondrial gene orders in Onychophora. Comparison of mitochondrial gene arrangements of all onychophoran species available to date and the AAGO (exemplified by *Limulus Polyphemus*). tRNA genes are labeled by the one-letter code for their corresponding amino acids. Genes are transcribed from left to right unless underlined. Arrows indicate inferred genome rearrangements and dashed arrows inversions. Multiple tRNA gene rearrangements have been omitted for clarity except between *P. sympatrica* and *O. cinctipes*. Positive numbers at *Oroperipatus* sp. and *P. sympatrica* gene boundaries indicate the number of intergenic nucleotides; negative numbers indicate the number of overlapping nucleotides; sl stands for stem loop structure, s-ncr for small noncoding region, and l-ncr for large noncoding region. Triangles at *O. cinctipes* indicate tRNA genes that were missed from the AAGO.

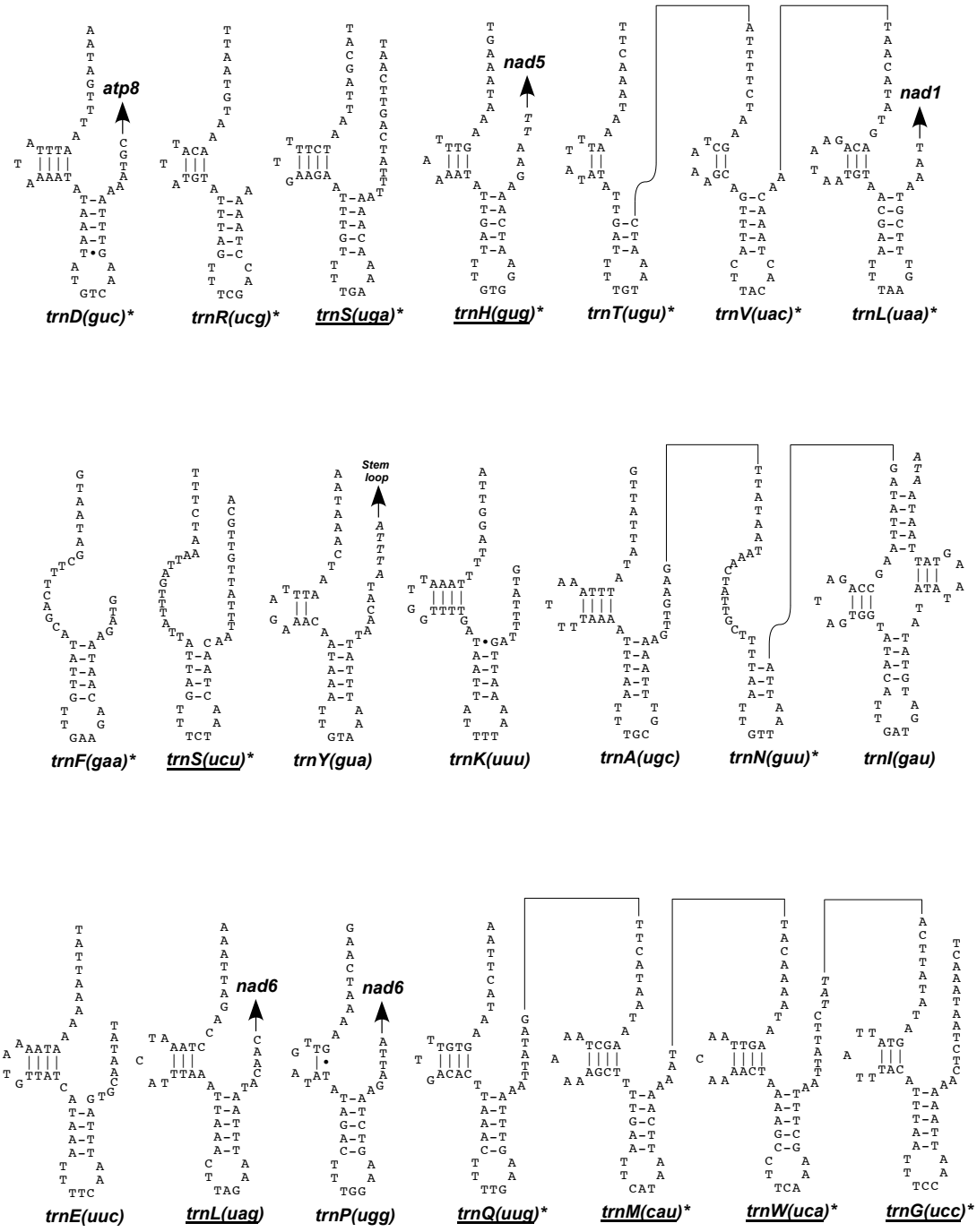


Fig 3. Secondary structures of the 21 inferred mitochondrial tRNA genes of *Oroperipatus* sp. Those encoded by the β strand are underlined. Asterisks indicate those tRNA genes that were predicted from cDNA sequences.

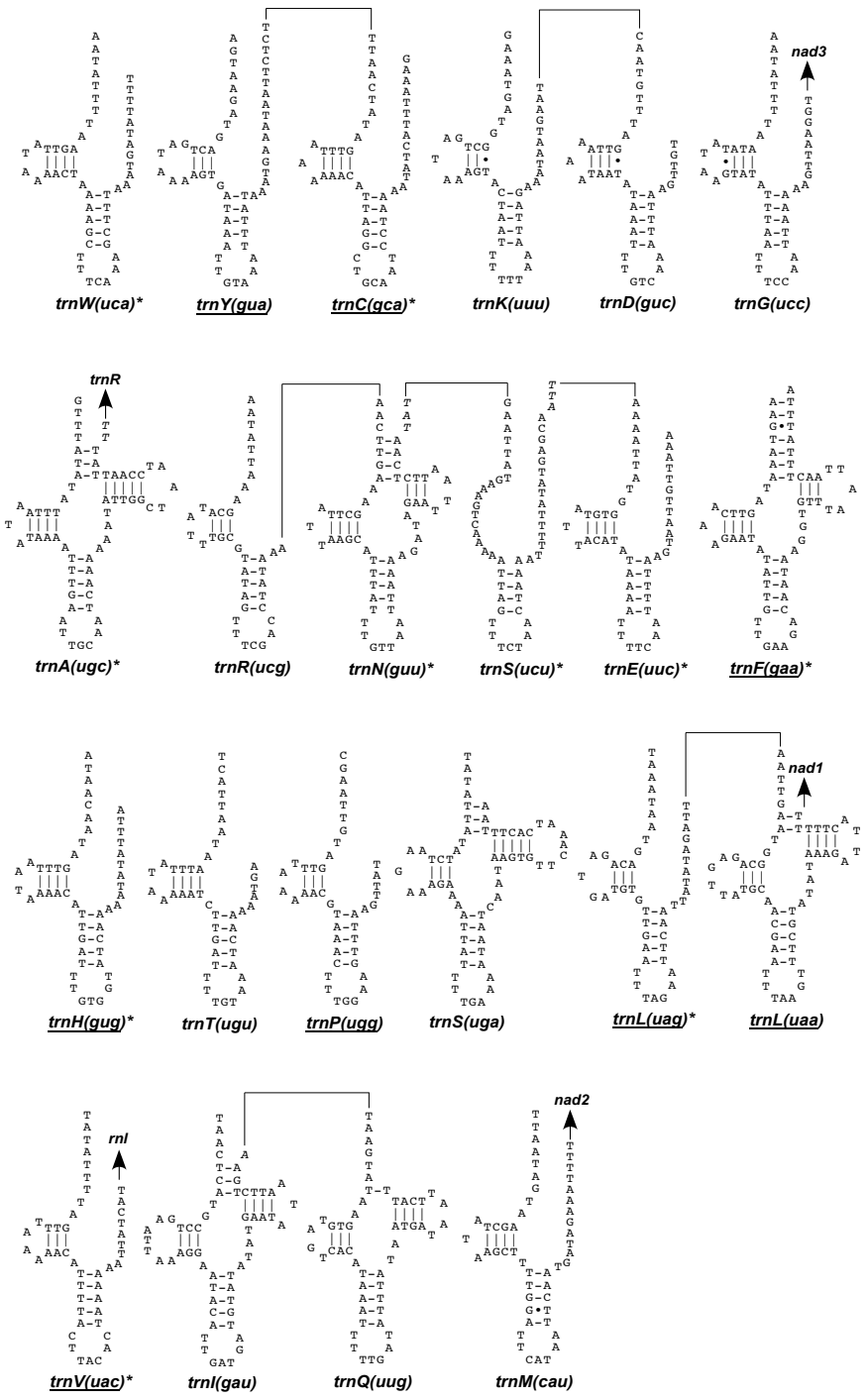


Fig 4. Secondary structures of the 22 inferred mitochondrial tRNA genes of *Peripatoides sympatrica*. Those encoded by the β strand are underlined. Asterisks indicate those tRNA genes that were predicted from cDNA sequences.

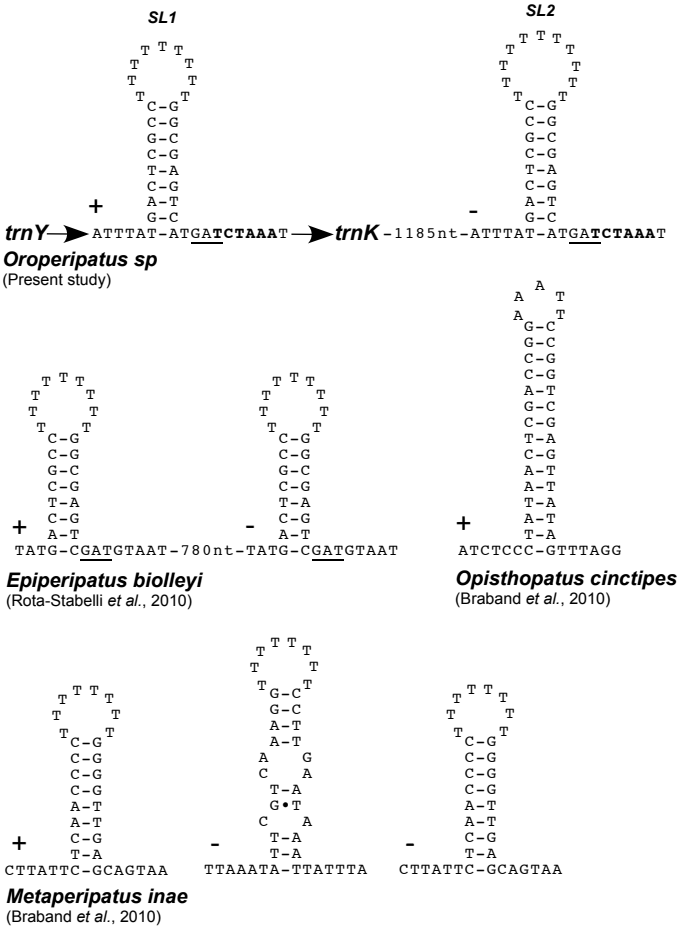


Fig 5. Comparison of stem loop structures of onychophoran species studied to date. Structures were found encoded at the α strand (+) and β strand (-). In *Oroperipatus* sp. SL1 refers to the stem loop structure found between *trnY(gua)* and *trnK(uuu)*. SL2 refers to the stem loop structure found in the large noncoding region. Adjacent tRNA genes are show by arrows indicating their transcriptional polarities. In *Oroperipatus* sp. and *E. biolleyi* the underlined sequences in the flanking regions are the conserved motif G(A)nT present in diverse organisms including humans at the mitochondrial L-strand origin of replication structures. In *Oroperipatus* sp. the flanking sequence TCTAAA in bold is also present in human mtDNA. Note similar stem loop structures in all "New World" onychophorans.

Table 1
Organization of the Mitochondrial Genome of *Oroperipatus* sp (Os) and *Peripatoides sympatrica* (Ps)

Gene		Strand		Size (nt)		GC-Skew		Start Codon		Stop Codon		Intergenic nt	
Os	Ps	Os	Ps	Os	Ps	Os	Ps	Os	Ps	Os	Ps	Os	Ps
Cox1	*Cox1*	+	+	1524	1536	0.26	0.10	ATA	TTA	TAA	TAA	1	-1
Cox2	*Cox2*	+	+	678	679	0.37	0.08	ATG	ATG	T–>trnD	T–>trnK	-2	0
trnD	*trnK*	+	+	44	49							0	0
Atp8	*trnD*	+	+	165	~44	0.52		ATA		TAG		-7	23
Atp6	*Atp8*	+	+	678	147	0.31	0.18	ATG	ATA	TAA	TAA	-1	-7
Cox3	*Atp6*	+	+	786	678	0.27	0.08	ATG	ATG	TAA	TAA	-1	-1
Nad3	*Cox3*	+	+	352	784	0.40	0.09	ATG	ATG	T–>trnR	T–>trnG	-2	-2
trnR	*trnG*	+	+	37	49							5	0
trnS2	*Nad3*	-	+	53	352		0.17		ATG		T–>trnA	-2	-2
Cob	*trnA*	-	+	1132	60	–0.23		ATG		T–>trnS2		-38	2
Nad1	*trnR*	-	+	966	39	–0.19		ATT		TAA		0	0
trnL2	*trnN*	-	+	43	57							0	3
trnV	*trnS1*	-	+	37	50							0	3
trnT	*trnE*	-	+	33	51							-4	-4
trnH	*trnF*	+	-	40	61							2	-2
Nad5	*Nad5*	+	-	1719	1689	0.24	0.09	ATG	ATG	TAA	T–>trnF	-4	11
trnF	*trnH*	+	-	38	50							-2	-2
trnS1	*Nad4*	-	-	49	1342		0.04		ATG		T–>trnH	-23	7
trnY	*Nad4L*	+	-	48	288		0.14		ATG		TAA	0	-4
SL1	*trnT*	+	+	46	~45							0	~12
trnK	*trnP*	+	-	40	~43								1
trnA	*Nad6*	+	+	48	510		0.04		ATG		TAA	0	-1
trnN	*Cob*	+	+	33	1132		–0.07		ATG		T–>trnS2	0	-2
trnI	*trnS2*	+	+	58	63								-4
trnE	*Nad1*	+	-	38	933		0.18		ATG		TAA		0
SL2	*trnL2*	-	-	47	57								0
Nad6	*trnL1*	-	-	504	49	-0.38		ATG		TAA		0	0
trnL1	*rnl*	-	-	45	1116		0.25					0	0
rns	*trnV*	-	-	660	46	-0.07						-99	0
rnl	*rns*	+	-	1103	692	0.41	0.14					0	0
trnP	*trnI*	+	+	44	58							0	1
Nad4L	*trnQ*	+	+	297	52	0.44		ATG		TAA		-7	
Nad4	*trnM*	+	+	1356	51	0.32		ATG		TAG		-10	0
trnG	*Nad2*	-	+	52	1015		0.09		ATG		T–>trnW	3	-2
trnW	*trnW*	-	+	48	51							0	-16
trnM	*trnC*	-	-	44	52							0	0
trnQ	*trnY*	-	-	46	56							-7	-5
Nad2		+		1002		0.57		ATG		n.a.		8	

Table 2
Comparison of the mitochondrial genes of the Onychophorans *Oroperipatus* sp (Os), *Peripatoides sympatrica* (Ps), *Epiperipatus biolleyi* (Eb), *Metaperipatus inae* (Mi), and *Opisthopatus cinctipes* (Oc)

Genes	N° of encoded nucleotides					Percentage of sequence identity (nt)						
	Os	Ps	Eb	Mi	Oc	Os/Ps	Os/Eb	Os/Mi	Os/Oc	Ps/Eb	Ps/Mi	Ps/Oc
Cox1	1524	1536	1533	1533	1533	81.2	86.9	79.2	79.6	80.6	82.4	83.7
Cox2	678	679	678	681	681	72.3	83.3	72.3	71.8	71.8	75.5	77.7
Atp8	165	147	159	156	156	62.4	65.9	65.9	64.2	65.6	68.0	64.9
Atp6	678	678	678	678	675	66.8	73.0	65.7	65.5	71.0	76.5	76.3
Cox3	786	784	786	789	792	72.9	79.6	72.4	73.1	73.5	76.6	76.3
Nad3	352	352	354	378	312	69.3	78.0	68.6	66.8	67.5	74.2	72.3
Cob	1132	1132	1140	1126	1137	71.0	75.6	69.6	69.2	70.3	75.7	77.0
Nad1	966	933	930	933	936	69.0	81.5	68.1	69.9	69.5	74.5	75.2
Nad5	1719	1689	1710	1736	1684	67.3	79.4	66.2	66.9	67.7	71.0	70.6
Nad6	504	510	507	507	483	60.6	69.0	55.0	58.6	60.9	69.2	70.7
rns	660	692	691	586	669	62.5	82.5	64.7	67.4	65.0	65.7	67.0
rnl	1103	1116	1166	1289	973	61.9	74.2	64.6	61.6	60.2	70.5	73.2
Nad4L	297	288	297	279	279	68.1	78.0	65.9	65.3	63.8	71.8	72.5
Nad4	1356	1342	1356	1335	1338	67.7	77.8	66.1	66.1	67.4	71.8	73.7
Nad2	1002	1015	1002	1002	1002	62.4	75.7	62.5	63.4	60.7	68.5	71.1

Table 3
Codon usage among 13 protein genes in *Oroperipatus* sp. mitochondrial genome

Aa	Codon	N°	Aa	Codon	N°	Aa	Codon	N°	Aa	Codon	N°
Phe	TTT	335	Ser	TCT	86	Tyr	TAT	136	Cys	TGT	29
	TTC	29		TCC	6		TAC	18		TGC	4
Leu	TTA	373		TCA	102	Stop	TAA	9	Trp	TGA	75
	TTG	72		TCG	10	Stop	TAG	2		TGG	24
Leu	CTT	45	Pro	CCT	65	His	CAT	75	Arg	CGT	20
	CTC	5		CCC	1		CAC	3		CGC	4
	CTA	33		CCA	49	Gln	CAA	43		CGA	22
	CTG	1		CCG	6		CAG	14		CGG	6
Ile	ATT	363	Thr	ACT	65	Asn	AAT	130	Ser	AGT	37
	ATC	24		ACC	3		AAC	15		AGC	5
Met	ATA	260		ACA	56	Lys	AAA	97		AGA	111
	ATG	61		ACG	6		AAG	23		AGG	36
Val	GTT	123	Ala	GCT	69	Asp	GAT	67	Gly	GGT	53
	GTC	3		GCC	6		GAC	4		GGC	1
	GTA	91		GCA	42	Glu	GAA	60		GGA	101
	GTG	21		GCG	12		GAG	28		GGG	46

Table 4
Codon usage among 13 protein genes in *Peripatoides sympatrica* mitochondrial genome

Aa	Codon	N°	Aa	Codon	N°	Aa	Codon	N°	Aa	Codon	N°
Phe	TTT	341	Ser	TCT	92	Tyr	TAT	158	Cys	TGT	43
	TTC	20		TCC	7		TAC	8		TGC	2
Leu	TTA	449		TCA	98	Stop	TAA	9	Trp	TGA	94
	TTG	30		TCG	3	Stop	TAG	0		TGG	11
Leu	CTT	48	Pro	CCT	59	His	CAT	72	Arg	CGT	12
	CTC	2		CCC	1		CAC	5		CGC	0
	CTA	19		CCA	57	Gln	CAA	65		CGA	40
	CTG	2		CCG	1		CAG	1		CGG	2
Ile	ATT	404	Thr	ACT	62	Asn	AAT	183	Ser	AGT	38
	ATC	24		ACC	4		AAC	9		AGC	1
Met	ATA	302		ACA	62	Lys	AAA	112		AGA	120
	ATG	24		ACG	3		AAG	10		AGG	5
Val	GTT	59	Ala	GCT	79	Asp	GAT	60	Gly	GGT	37
	GTC	2		GCC	4		GAC	5		GGC	10
	GTA	54		GCA	44	Glu	GAA	85		GGA	109
	GTG	8		GCG	4		GAG	7		GGG	18

CHAPTER 4: GENERAL CONCLUSION

After obtaining cDNA sequences from 14 and 10 tRNAs from *Oroperipatus* sp. and *Peripatoides sympatrica* mtDNAs bearing the missing parts and displaying the complete cloverleaf standard secondary structures, it was firmly probed that tRNA editing is the responsible mechanism for the restoration of severe reduced tRNA genes in onychophora that otherwise would be nonfunctional molecules. In this process, up to 34 nucleotides are added posttranscriptionally to the tRNA sequences encoded in the genome, rebuilding the amino-acyl acceptor stem, the TΨC arm, and in some cases even the variable arm and up to two nucleotides in the anticodon stem. Furthermore, in *Oroperipatus* sp. when the entire TΨC arm is added post-transcriptionally, its sequence is identical or nearly identical both among different clones of the same tRNA and among different tRNAs. Importantly, it was never discovered before the presence of such extreme tRNA editing. As the two onychophorans are representatives from extant families that have diverged 175-140 million years ago, we deduce that tRNA editing was already present in the common onychophoran ancestor and retained thereafter.

The mitochondrial genomes of *Oroperipatus* sp. and *P. sympatrica* display two different gene arrangement patterns, consequently few gene boundaries sharing. Whereas *P. sympatrica* gene order conserve the Ancestral Arthropod Gene Order (AAGO), hence representing the ancestral onychophora gene order, *Oroperipatus* sp. gene order shows multiple gene arrangements to the AAGO and similar organization to that of *E. biolleyi*. They are also characterized by presenting strand asymmetry composition revealed by their AT and GC skews and a high degree of tRNA gene reduction as a consequence of accumulation of

deleterious mutations driven by Muller's Ratchet. In addition, the two identical stem loop structures identified in both strands of the large and small noncoding regions of *Oroperipatus* sp. similar to other "New World" onychophorans, resemble the human stem loop structure associated with the origin of L-strand replication, suggesting that an analogue replication mechanism may that utilizes both stem loops structures for the separate initiation of each strand of the onychophoran mtDNA.

Since the presence of severely truncated tRNA genes has been reported in the mitochondrial genomes of arachnids and of gall midges. It would be very interesting to determine whether tRNA genes are similarly edited in these animals, and most interesting yet it would be to investigate the molecular machinery involved in the tRNA editing process and enzymes implicated in this. It is probable that the identical sequences used for the restoration of the TΨC arms in many tRNAs are encoded somewhere in the nuclear genome. If that were the case, the identification of these sequences would represent revealing information to better understand the process and their origin. The sequence of additional complete mitochondrial genomes from species of onychophorans not represented yet would be very valuable in inferring the phylogeny of this phylum and molecular evolution of the tRNA editing process as well.

ACKNOWLEDGEMENTS

First and foremost I thank my major professor Dr. Dennis Lavrov for his guidance, patience and opportunity to learn from him throughout the course of my master's study and research. I thank my committee members Drs. Lyric Bartholomay and Bing Yang. I appreciate their comments and contributions to this work. I am indebted to all lab members, but especially to Karri Haen and Ehsan Kayal for their always disposition to help, assistance and contributions to my project. I am also grateful to the Fulbright and Barsa Scholarship Programs for the financial support and possibility to fulfill a dream. Special thanks to Anna Holz for her constant and unconditional support. Thank you to all the others that have helped me in small but important ways.