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Extensive duplication events account for multiple control regions and pseudo-genes in the mitochondrial genome of the velvet worm *Metaperipatus inae* (Onychophora, Peripatopsidae)

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ABSTRACT

The phylogeny of Onychophora (velvet worms) is unresolved and even the monophyly of the two major onychophoran subgroups, Peripatidae and Peripatopsidae, is uncertain. Previous studies of complete mitochondrial genomes from two onychophoran species revealed two strikingly different gene arrangement patterns from highly conserved in a representative of Peripatopsidae to highly derived in a species of Peripatidae, suggesting that these data might be informative for clarifying the onychophoran phylogeny. In order to assess the diversity of mitochondrial genomes among onychophorans, we analyzed the complete mitochondrial genome of *Metaperipatus inae*, a second representative of Peripatopsidae from Chile. Compared to the proposed ancestral gene order in Onychophora, the mitochondrial genome of *M. inae* shows dramatic rearrangements, although all protein-coding and ribosomal RNA genes are encoded on the same strands as in the ancestral peripatopsid genome. The retained strand affiliation of all protein-coding and ribosomal RNA genes and the occurrence of three control regions and several pseudo-genes suggest that the derived mitochondrial gene arrangement pattern in *M. inae* evolved by partial genome duplications, followed by a subsequent loss of redundant genes. Our findings, thus, confirm the diversity of the mitochondrial gene arrangement patterns among onychophorans and support their utility for clarifying the phylogeography of Onychophora, in particular of the Peripatopsidae species from South Africa and Chile.

1. Introduction

Onychophora or velvet worms are a group of moulting animals (Ecdysozoa), which are closely related to arthropods and show an ancient pattern of distribution dating back to the existence of Gondwana (Brinck, 1957; Monge-Nájera, 1995; Reid, 1996; Allwood et al., 2009; Braband et al., 2010). Among the two major onychophoran subgroups, the Peripatidae are distributed circumequatorially in Central America, West Africa and South-East Asia, whereas the Peripatopsidae are confined to the southern hemisphere and occur in Chile, South Africa and Australasia (Fig. 1A; Brinck, 1957; Monge-Nájera, 1995; Reid, 1996). Despite their ancient pattern of distribution, the biogeography of Onychophora remains ambiguous since their phylogeny is unresolved (Fig. 1B–D; Monge-Nájera, 1995; Reid, 1996; Allwood et al., 2009; Daniels et al., 2009; Mayer and Tait, 2009).

Recent studies of mitochondrial sequences from various onychophoran species revealed that mitochondrial data, including gene arrangement patterns, might help clarify the onychophoran phylogeny and biogeography (Gleeson et al., 1998; Trewick, 2000; Podsiadlowski et al., 2008; Allwood et al., 2009; Daniels et al., 2009; Braband et al., 2010). However, only two complete mitochondrial genomes are known from Onychophora, which show two distinct gene arrangement patterns. The mitochondrial gene order in *Opisthopatus cinctipes* (Peripatopsidae) resembles the ancestral pattern of the Panarthropoda (Onychophora + Tardigrada + Arthropoda) or even the Ecdysozoa (Braband et al., 2010) whereas *Epiperipatus biolleyi* (Peripatidae) shows a highly derived gene arrangement with numerous translocated genes (Podsiadlowski et al., 2008). Hence, the study of mitochondrial genomes from additional onychophoran species might reveal a high diversity of gene arrangement patterns, which may be useful for clarifying the onychophoran phylogeny.

In this paper, we analyze the complete mitochondrial genome of the onychophoran *Metaperipatus inae* (Peripatopsidae) and pro-

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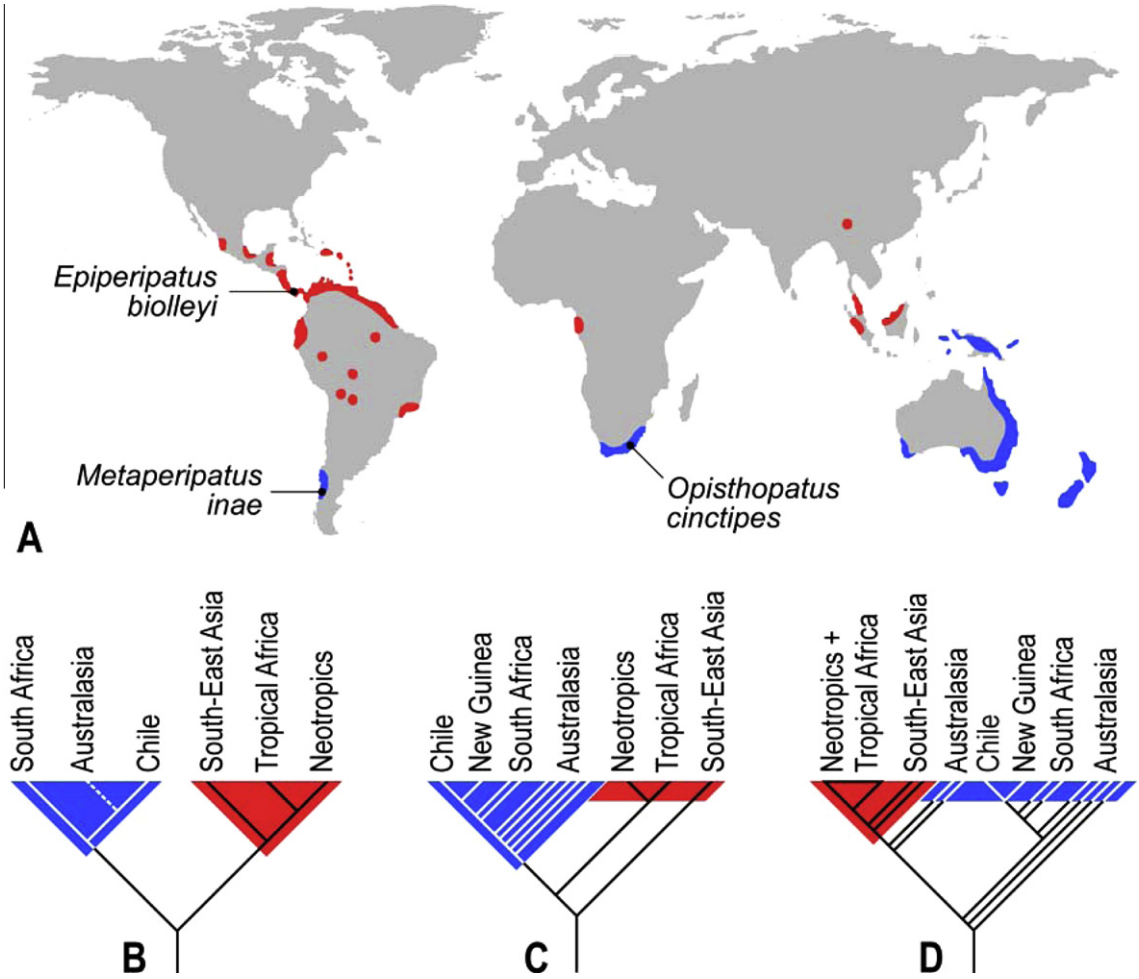

Fig. 1. Distribution and phylogeny of Onychophora. Peripatidae are highlighted in red, Peripatopsidae in blue. Onychophoran subgroups are designated by their geographical distribution in the cladograms. (A) Simplified map illustrating the distribution of the Peripatidae and Peripatopsidae (modified after Reid, 1996). The localities of species, the complete mitochondrial genomes of which have been analyzed, are also shown: *Epiperipatus biolleyi* from Costa Rica (Podsiadlowski et al., 2008), *Opisthopatus cinctipes* from South Africa (Braband et al., 2010), and *Metaperipatus inae* from Chile (this study). (B–D) Controversial hypotheses for the phylogenetic relationships within Onychophora (modified from Mayer and Tait, 2009). (B) Both Peripatopsidae and Peripatidae are monophyletic (Monge-Nájera, 1995). (C) Peripatidae are not monophyletic (Reid, 1996). (D) Peripatopsidae are not monophyletic (Reid, 1996). (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

pose a scenario explaining the derived gene order in this species with multiple control regions and pseudo-genes. Our findings confirm the high diversity of mitochondrial gene arrangement patterns among the onychophoran species and provide a baseline for future studies of the phylogeny and biogeography of Onychophora.

2. Materials and methods

2.1. Animals, DNA extraction, amplification, and sequencing

Specimens of *Metaperipatus inae* Mayer, 2007 were collected in Chile as described previously (Mayer, 2007). DNA was extracted with the DNeasy Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol with a slight modification: a few grains of sea sand were added to a tube containing tissue, lysis buffer and proteinase K to promote tissue digestion, and the tissue was then grinded vigorously with a pistil. Initial selected amplification was carried out by PCR for the third domain of the ribosomal RNA gene *srRNA* and for the protein-coding gene *COI* by using universal primers (Supplementary Table 1; Folmer et al., 1994; Braband et al., 2006). The PCR primers were extended at the 5′ end with complement sequences of standard primers $T7_{term}$ (5′-AAAG CTAGTTATTGCTCAGCGG-3′) and $T7_{prom}$ (5′-AAATAATACGACTCACT ATAGGG-3′) to facilitate standard cycle sequencing reactions (Cho et al., 1995). PCR conditions for the *srRNA* fragment followed a standard three-step protocol with 40 cycles of (1) denaturing for 30 s at 94 °C, (2) annealing for 45 s at 45 °C, and (3) extension for 45 s at 72 °C. PCR conditions for the *COI* fragment followed the protocol of Folmer et al. (1994). Amplicons were sequenced by dideoxy-chain termination using Cy5-labelled $T7_{term}$ and $T7_{prom}$ standard primers (4 pmol each) on ALF sequencers (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. Initial *srRNA* and *COI* sequences were used to design two pairs of specific primers (Supplementary Table 1) for long range PCR, which resulted in two long overlapping amplicons (~8.3 and ~7.5 kb) spanning the entire mitochondrial genome. Long range PCRs were carried out using the LongeRange PCR Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. To a volume of 50 µl containing ~500 ng total DNA, Q-solution (PCR additive provided by the manufacturer) was added and 0.4 µM of each primer were used under the following conditions: 15 s at 93 °C (denaturing), 30 s at 44.2 and 48.5 °C, respectively (anneal-

ing) (Supplementary Table 1), and 15 min at 68 °C (extension) for 40 cycles. The optimal annealing conditions (30 s at 44.2 and 48.5 °C) were determined by using a gradient thermocycler (Eppendorf AG, Hamburg, Germany). The obtained amplicons were shotgun-cloned and the clones sequenced in commission at the Max Planck Institute for Molecular Genetics (Berlin, Germany).

2.2. Sequence annotation

In the present study, we apply the nomenclature of the mitochondrial gene names provided by Boore (1999). Protein-coding and ribosomal RNA genes were identified by BLAST searches using the NCBI database. Gene boundaries were determined by alignments with sequences from the onychophoran species *Epiperipatus biolleyi* and *Opisthopatus cinctipes* (Podsiadlowski et al., 2008; Braband et al., 2010) and various arthropod species. Positions of tRNA genes, tRNA structures, and tRNA scan scores were determined by using the tRNA scan-SE Search Server (Lowe and Eddy, 1997) and the ARWEN software (Laslett and Canbäck, 2008). Despite an additional inspection by eye for putative tRNA-like structures and anticodons, none of the remaining tRNAs were detected. Sequence data were deposited in the NCBI database (NC_010961).

3. Results

3.1. Mitochondrial genome composition

The complete mitochondrial genome of *Metaperipatus inae* consists of 15,347 bp and contains the complete set of two ribosomal RNA genes and 13 protein-coding genes, which are encoded on both strands (Fig. 2; Supplementary Table 2). Among the transfer RNA (tRNA) genes, six are missing: *C*, *F*, *H*, *L(CUN)*, *R*, and *Y*. In addition to the 16 remaining tRNA genes, we detected five pseudo-tRNA genes: p-*D*, p1-*L(UUR)*, p2-*L(UUR)*, p3-*L(UUR)*, and p-*N* (Fig. 2; Supplementary Table 2). Most pseudo-genes, except for p3-*L(UUR)*, are adjacent to or lie close to the three putative control regions CR1, CR2, and CR3 (Fig. 2). The occurrence of three putative pseudo-genes encoding the tRNA L(UUR) on the one hand and the lack of the essential tRNA gene *L(CUN)* in the mitochondrial genome of *M. inae* on the other hand suggest that one of the three pseudo-tRNAs L(UUR) might be modified to L(CUN) by post-transcriptional RNA editing (Lavrov et al., 2000). However, this suggestion needs to be verified by future experimental work.

3.2. Putative control regions

According to our sequence data, there are three non-coding regions in the mitochondrial genome of *M. inae* separated by one to three tRNA genes (Figs. 2 and 3; Supplementary Table 2). We designate these regions as the putative control regions (CR1–CR3) since they show the hairpin-like structures and high A + T content typical of mitochondrial control regions. The A + T content of the three control regions is 81% (CR1), 82% (CR2), and 79% (CR3), respectively, which is slightly higher than in the remaining mitochondrial genome (78%). A sequence alignment of the three control regions reveals a high overall identity in base composition between CR1 and CR3 (51.6%), with an even higher identity in the core region (60.4%) (Supplementary Fig. S1). Furthermore, the hairpin-like

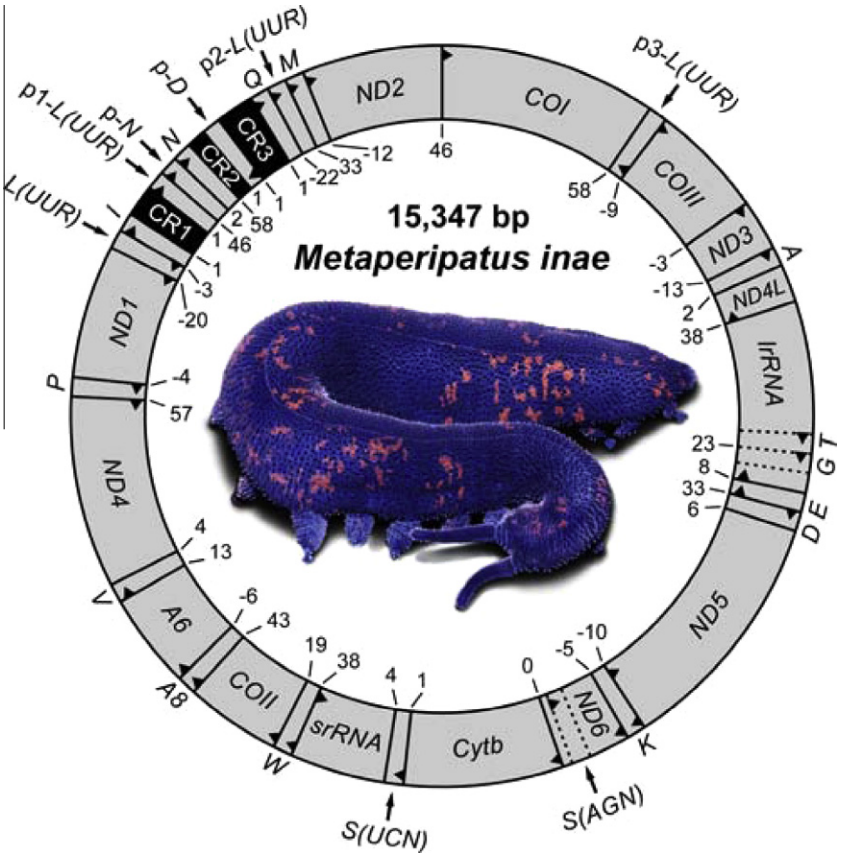

Fig. 2. Ring diagram of the mitochondrial genome of the onychophoran *Metaperipatus inae* (Peripatopsidae). The relative size is approximated for all regions and genes except for tRNA genes, which are not to scale. The orientation of genes either on the heavy strand (clockwise) or light strand (counterclockwise) is indicated by arrowheads. Numbers at gene borders refer to the number of nucleotides in the non-coding (positive values) and overlapping gene regions (negative values). CR1, CR2, and CR3 are the three putative control regions. Pseudo-tRNA genes are designated by the following names: p-*D*, p1-*L(UUR)*, p2-*L(UUR)*, p3-*L(UUR)*, and p-*N*. Dotted lines indicate the location of tRNA genes, which are encoded within the *lrRNA* and *ND6* sequences.

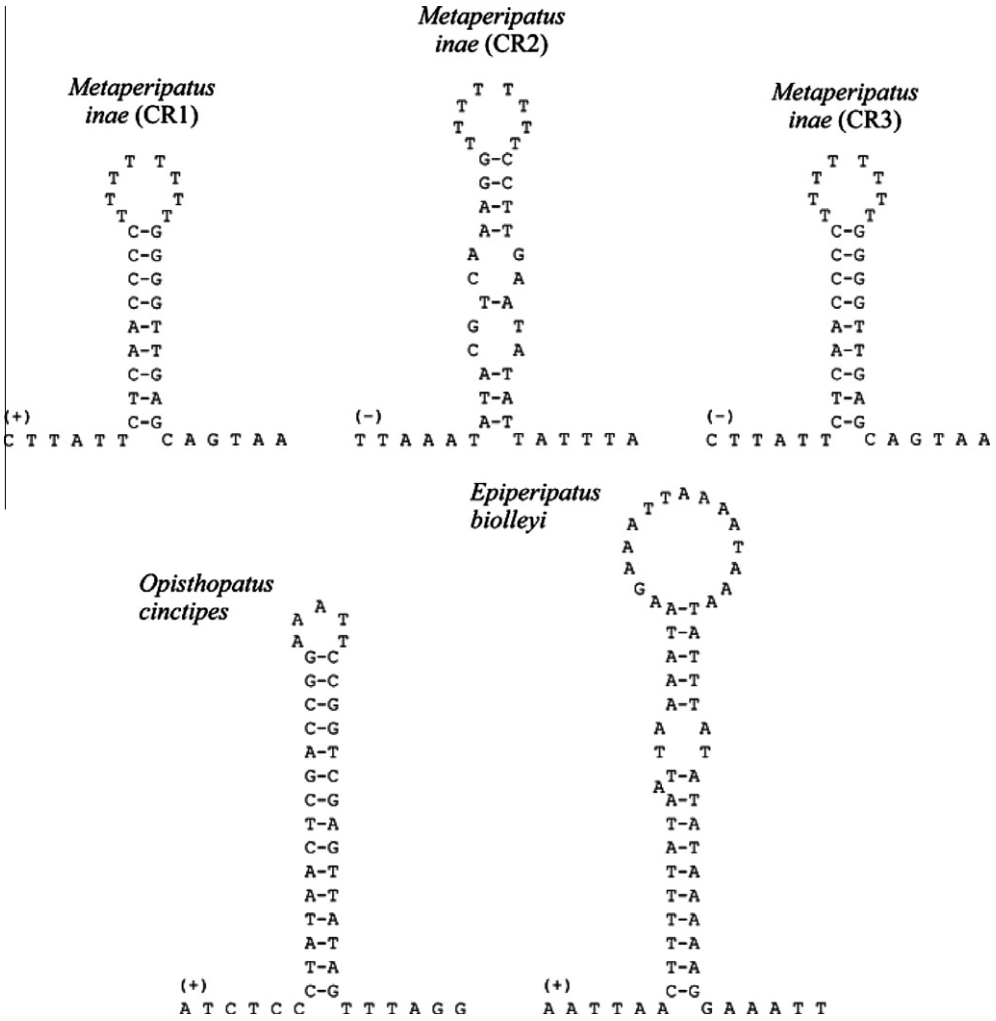

Fig. 3. Comparison of hairpin-like structures from putative control regions of three onychophoran species studied thus far. CR1, CR2, and CR3 refer to the three control regions in *Metaperipatus inae*. The hairpin-like structures were determined for the heavy strand (+) or the light strand (−), respectively. The hairpin-like structure of *Opisthopatus cinctipes* is according to Braband et al. (2010). Sequence data from *Epiperipatus biolleyi* were obtained from the NCBI database (NC_009082; Podsiadlowski et al., 2008). Note the same loop structure in all three control regions of *M. inae* and the identical hairpin-like structure in CR1 and CR3, despite their position on opposite strands.

structures of CR1 and CR3 are absolutely identical, although their sequences are complementary and, thus, encoded on opposite strands (Fig. 3). The stems of both control regions are composed of nine nucleotide pairs whereas the loops comprise eight T nucleotides. In contrast, CR2 shows a different stem sequence, with 12 nucleotide pairs including four mismatches. However, the loop of CR2 is composed of eight T nucleotides and is therefore identical to the CR1 and CR3 loops (Fig. 3). A sequence analysis of the putative control regions from two other onychophoran species, *O. cinctipes* and *E. biolleyi*, reveals hairpin-like structures with a different nucleotide composition. In particular, the loop structures from these two species differ from those in *M. inae* in both the nucleotide number and composition (Fig. 3).

3.3. Pseudo-tRNA genes

Among the five pseudo-tRNA genes, three encode the tRNAs transferring leucine: p1-*L(UUR)*, p2-*L(UUR)*, and p3-*L(UUR)* (Fig. 4A). Two of them, p1-*L(UUR)* and p2-*L(UUR)*, are located on the heavy strand whereas p3-*L(UUR)* lies on the light strand, as does the putatively functional tRNA gene *L(UUR)* (Figs. 2 and 5A). A sequence alignment of all *L(UUR)* genes reveals a high similarity in base composition, in particular in a region adjacent to the anticodon (Supplementary Fig. S2). However, the secondary tRNA structures of the three pseudo-*L(UUR)* genes might be aberrant and unlikely to fold into functional tRNAs (Fig. 4A). The tRNA p1-L(UUA) shows an asymmetrical anticodon loop consisting of 11 instead of seven nucleotides whereas the tRNAs p2-L(UUA) and p3-L(UUA) have lost their TψC stems (Fig. 4A), suggesting that these three pseudo-tRNAs are unstable and the corresponding genes are degenerating copies.

The same applies to the tRNA transferring asparagine (encoded by p-*N*), which shows both an increased number of nucleotides in the anticodon loop (13 instead of 7) and a missing TψC stem (Fig. 4B). In the mitochondrial genome, p-*N* is flanked by *N* and p1-*L(UUR)*, all of which are adjacent to CR1 on the heavy strand (Figs. 2 and 5A). An alignment of the p-*N* and *N* sequences reveals several identical regions, despite the aberrant secondary structure of p-N(AAU) (Supplementary Fig. S2).

The fifth pseudo-tRNA gene, p-*D* (encoding the tRNA for aspartic acid) is located on the light strand between the control regions CR2 and CR3 (Figs. 2 and 5A). A sequence alignment of p-*D* with the putative *D* sequence reveals several albeit short identical regions (Supplementary Fig. S2). The tRNAs transcribed by both genes might be unstable since the TψC stem is absent from their secondary structures and their anticodon loops contain more than seven

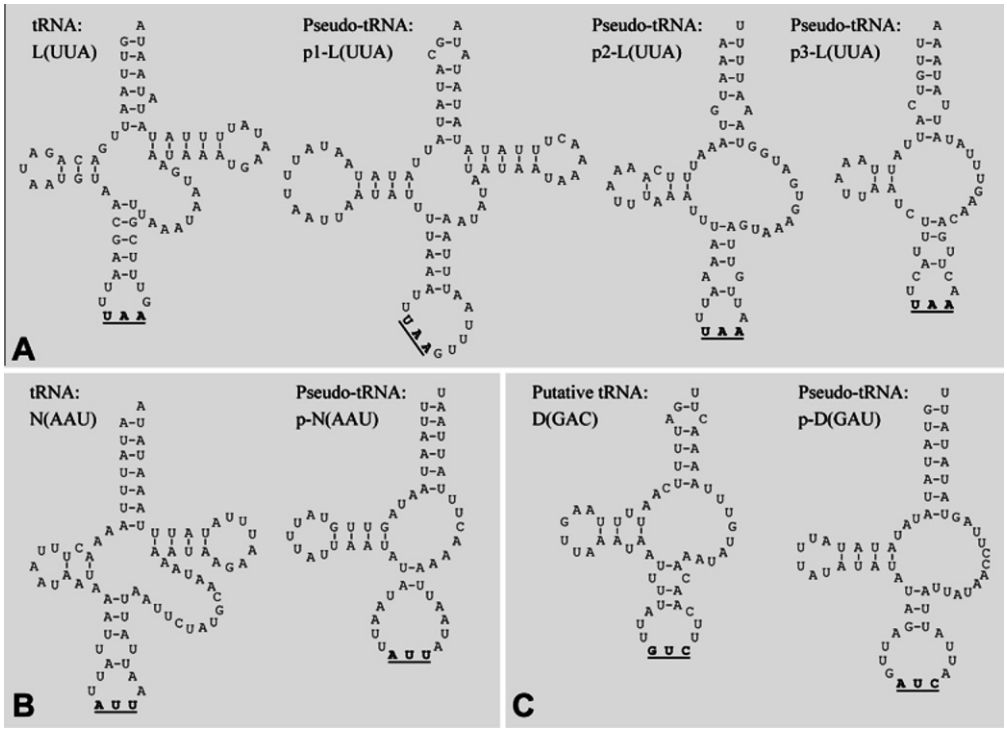

Fig. 4. Putative secondary structures of tRNAs and corresponding pseudo-tRNAs from the onychophoran *Metaperipatus inae*. (A) Comparison of putatively functional L(UUA) with three pseudo-tRNAs: p1-L(UUA) shows an aberrant anticodon loop whereas p2-L(UUA) and p3-L(UUA) have lost their TψC stems. (B) Putatively functional N(AAU) and aberrant p-N(AAU). The latter shows an increased number of nucleotides in the anticodon loop and a missing TψC stem. (C). The putative tRNA D(GAC) and the pseudo-tRNA p-D(GAU). Both secondary structures show an increased number of nucleotides in the anticodon loop and a missing TψC stem.

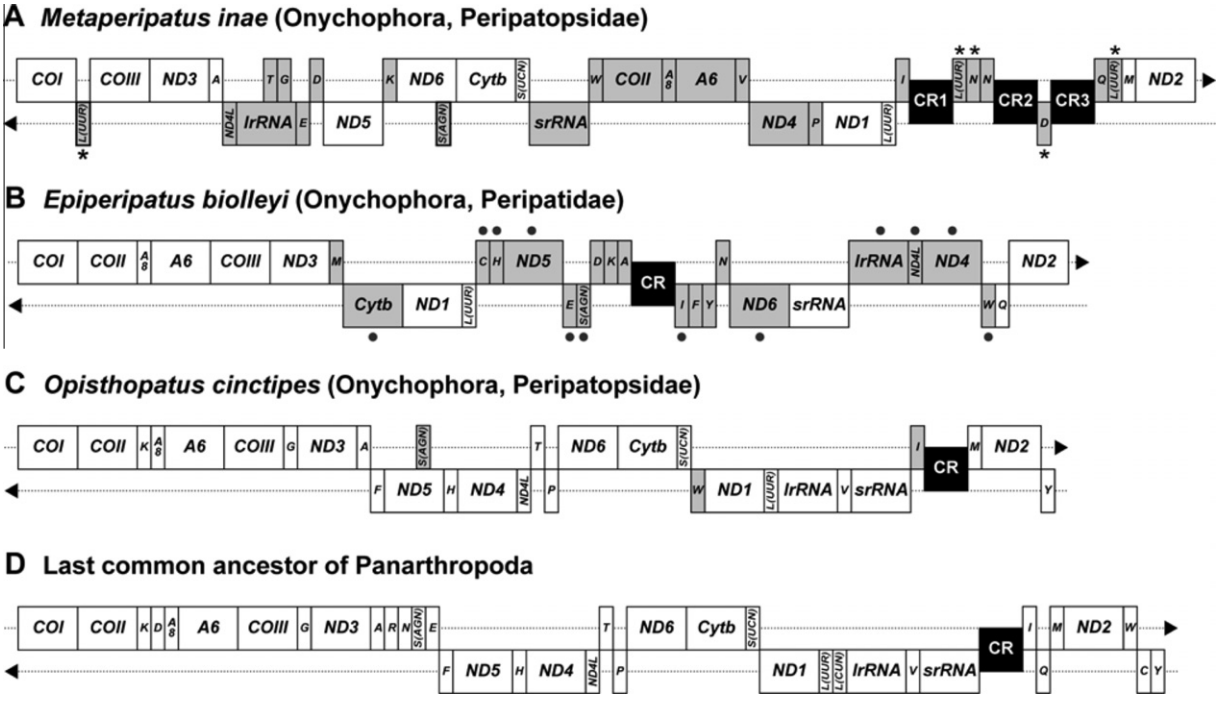

Fig. 5. Comparison of linearized mitochondrial genomes from three representatives of Onychophora (A–C) with the ancestral gene arrangement pattern in Panarthropoda (D) (B combined and modified after Podsiadlowski et al., 2008; Braband et al., 2010; C and D modified from Braband et al., 2010). Genes that deviate in position from the ancestral pattern in Panarthropoda are illustrated in grey. The control regions (black boxes) have not been assigned to a strand. Asterisks indicate the pseudo-tRNA genes in *Metaperipatus inae*. Filled black circles in *Epiperipatus biolleyi* designate genes that have been transposed to the opposite strand, thus suggesting numerous translocation events instead of genome duplication (Podsiadlowski et al., 2008). Note the highly conserved gene arrangement pattern in *Opisthopatus cinctipes* (see Braband et al., 2010).

nucleotides (Fig. 4C). However, due to the higher tRNA scan score of 12.01 (nematode model) for D(GAC), as compared to only 6.82 for p-D(GAU), we regard D(GAC) as a putatively functional tRNA and p-D(GAU) as an aberrant pseudo-tRNA copy.

4. Discussion

4.1. Evolution of multiple control regions

Analyses of the mitochondrial genomes from *E. biolleyi* (Peripatidae) and *O. cinctipes* (Peripatopsidae) revealed a single control region in each of these two distantly related onychophoran species (Fig. 5B and C; Podsiadlowski et al., 2008; Braband et al., 2010). This suggests that only one control region was present in the last common ancestor of Onychophora, which also holds true for the ground pattern of Panarthropoda (Fig. 5D). In contrast, there are three putative control regions in the mitochondrial genome of *M. inae*, which display identical hairpin loops and are situated close to each other on the mitochondrial DNA (Figs. 3 and 5A). Although the hairpin-like structures in the three control regions of *M. inae* are similar, they differ fundamentally from the corresponding structures in the control regions of *E. biolleyi* and *O. cinctipes* in the number and composition of nucleotides in the loop region (Fig. 3). The existence of three control regions with similar hairpin-like structures in *M. inae* suggests that CR1, CR2, and CR3 share a single origin and evolved by at least two duplication events.

Notably, the entire hairpin-like structure of the control regions CR1 and CR3 is identical, suggesting that these two control regions arose by a recent duplication event. In contrast, the less similar hairpin-like structure of CR2 indicates a more ancient duplication, if we assume a constant mutation rate in all three control regions. According to the identical albeit complementary loop sequences, the hairpin-like structures of CR2 and CR3 lie on the opposite strand compared to CR1. This position might be due to an inversion event, which occurred after duplication of the control regions.

We suggest that CR1 is located on the heavy strand since its heavy strand sequence shows typical structural elements, which have been reported from insects (review Zhang and Hewitt, 1997): an $R(T)_n$ motif at the beginning of the CR1 sequence adjacent to the tRNA gene *I* is followed by a $TA(A)_n$ stretch, which is adjacent to the hairpin loop (Supplementary Fig. S1). In contrast to insects, however, we found an $R(T)_n$ motif instead of $R(T)_nR$ and no apparent G + A-rich block downstream of the hairpin in *M. inae*. The position of both tRNA genes flanking CR1 on the heavy strand also supports our suggestion that the origin of replication in this control region lies on the heavy strand.

In addition to the 100% hairpin sequence identity of CR3 and CR1, CR3 shows the same base compositional elements (e.g., $R(T)_n$ followed by a $TA(A)_n$ motif) at the 5′ end and we therefore assume that it is located on the light strand (Supplementary Fig. S1). The situation is less clear for CR2 since only the $TA(A)_n$ motif is present, which is an integral part of the hairpin-like structure. In addition, the adjacent tRNA genes *N*, p-*D*, and *Q* are located on either the heavy or the light strands (Fig. 5A), thus impeding a clear decision. However, due to the complementary sequences of their hairpin-like structures, the putative origins of replication of CR2 and CR3 are most likely encoded on the opposite strand to CR1, i.e., on the light strand (Supplementary Fig. S1).

4.2. Evolution of mitochondrial gene arrangement

In *M. inae*, the relative order of most protein-coding genes and the two ribosomal RNA genes is strikingly different from the reconstructed ground pattern of Onychophora and Panarthropoda (Fig. 5A and D; Braband et al., 2010). However, since most genes are encoded on the same strand as in the ancestral mitochondrial genome, the derived gene order in *M. inae* is unlikely to have resulted from numerous translocation and inversion events, which is the case in *E. biolleyi* (Fig. 5B; Podsiadlowski et al., 2008). We instead suggest that the mosaic gene arrangement pattern in *M. inae* evolved by partial genome duplications, followed by deletions of the redundant genes (Fig. 6A–G). This mechanism has been proposed to elucidate the mitochondrial genome rearrangements in other animals (e.g., Moritz et al., 1987; Boore, 2000; Bensch and Härlid, 2000; Lavrov et al., 2002; Zhang et al., 2008).

According to our findings, the most parsimonious scenario explaining the gene arrangement pattern in *M. inae* suggests four duplication events. We assume the ancestral gene order of Onychophora as the starting point, in which initially the control region was duplicated (Fig. 6A). This step was followed by a duplication spanning almost the entire mitochondrial genome and a subsequent loss of redundant genes (Fig. 6B and C). A second partial genome duplication, followed by a random deletion of genes, gave rise to the gene arrangement pattern found in *M. inae*, but with only two control regions instead of three (Fig. 6D and E). Finally, the two control regions were duplicated and inverted, after which one of them was reduced (Fig. 6F). This final step has led to the mitochondrial gene arrangement currently found in *M. inae* (Fig. 6G).

This scenario seems most likely since any other assumption of duplication events would result either in a less parsimonious scenario with additional duplication steps required or in an inappropriate position of the three control regions. Moreover, our scenario reflects sequence correspondences of the three control regions (CR1 more similar to CR3) as it suggests an ancient plus a second more recent duplication of the control regions (Fig. 6A and F). The proposed inversion event in our scenario (Fig. 6F) is consistent with the complementary sequence of the hairpin loop in CR1 as compared to CR2 and CR3. The assumption of partial genome duplications in our scenario agrees with the typical pattern frequently found in animal mitochondrial genomes (e.g., Boore, 2000) since all duplicated regions either include or border one of the control regions. This specific pattern might be due to replication errors within the control regions, which may lead to duplication of the adjacent genomic regions (Moritz et al., 1987; Boore, 2000; Lavrov et al., 2002).

Our duplication scenario is also supported by the occurrence of five pseudo-genes in *M. inae*, which are missing from the mitochondrial genomes of two other onychophoran species showing no indication of genome duplication (Fig. 5B and C; Podsiadlowski et al., 2008; Braband et al., 2010). Mitochondrial pseudo-genes are commonly regarded as degenerating copies of functional genes, which have arisen by genome duplications (Moritz et al., 1987; Lavrov et al., 2002; Zhang et al., 2008). The occurrence of three pseudo-*L(UUR)* genes in *M. inae*, in addition to a functional *L(UUR)* gene, suggests that there were at least two genome duplication events, thus supporting our scenario.

4.3. Conclusions and perspectives

The three onychophoran species studied thus far (Fig. 1A) display three different gene arrangement patterns ranging from highly conserved in *O. cinctipes* to highly derived in *E. biolleyi* and *M. inae* (this study; Podsiadlowski et al., 2008; Braband et al., 2010). Our findings revealed that the derived gene arrangement pattern in *M. inae* might have evolved by an entirely different mechanism from that in *E. biolleyi* as it involved several duplication events. This hypothesis receives support from the retained strand affiliation of all protein-coding and ribosomal RNA genes and the occurrence of multiple control regions and pseudo-genes in *M. inae*. We therefore suggest that the derived and dissimilar gene orders in *E. biolleyi* and *M. inae* have evolved convergently.

The apparently high diversity of mitochondrial gene arrangement patterns among the onychophoran species contrasts with their conserved morphology, thus providing an additional dataset for clarifying the phylogeny and biogeography of the Onychophora

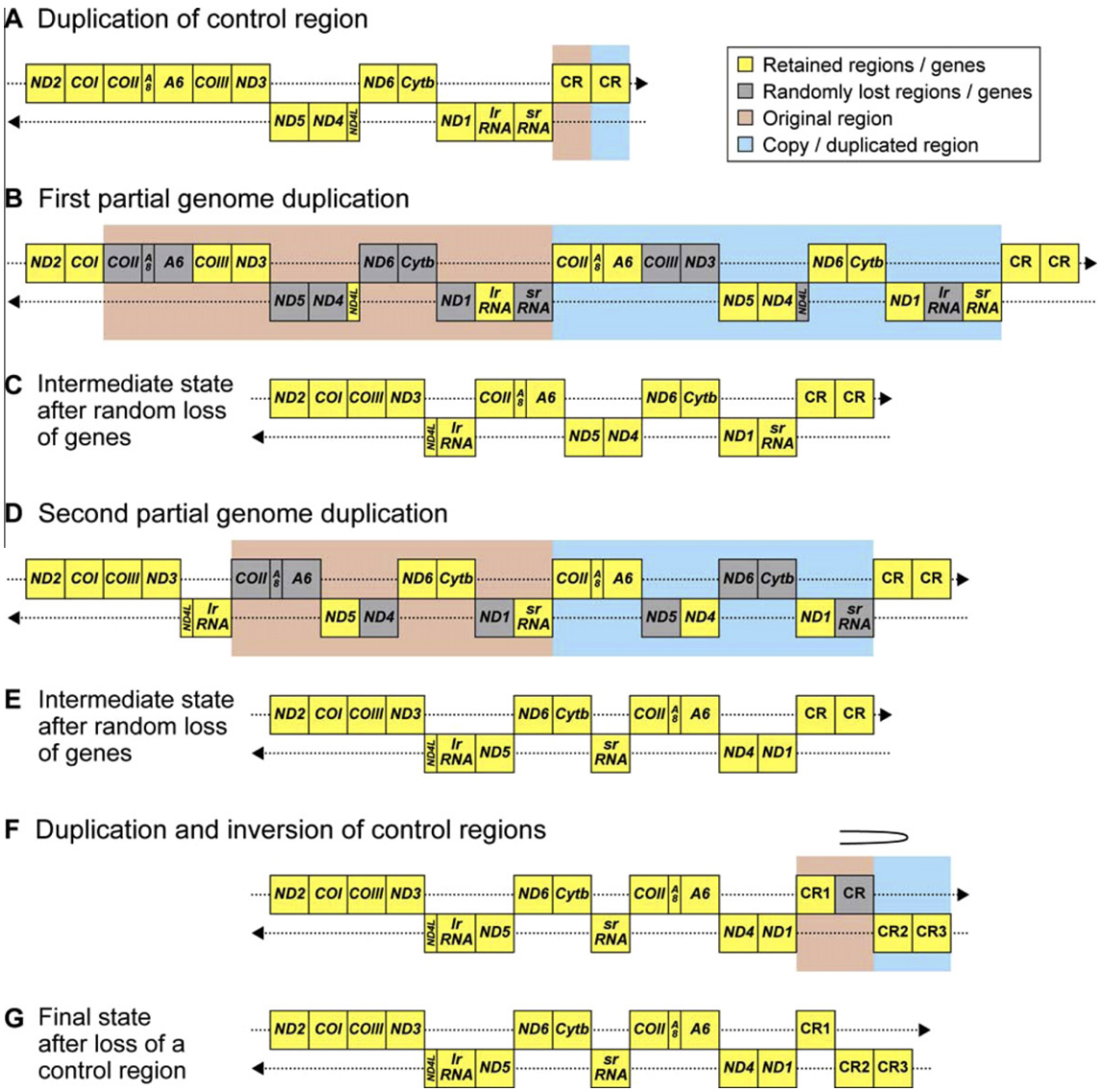

Fig. 6. Most parsimonious evolutionary scenario explaining the mitochondrial gene order in *Metaperipatus inae* (A–G), starting with the putative ancestral gene arrangement pattern of Onychophora (Braband et al., 2010). The derived gene order in *M. inae* evolved by four duplication events, followed by a random loss of redundant genes and an inversion of the control regions (convoluted line in F). Transfer RNA genes were disregarded in this scenario since they are usually considered as movable or transposable elements and their rearrangement patterns are therefore difficult to reconstruct (e.g., Moritz et al., 1987; Saccone et al., 2002; Podsiadlowski et al., 2008).

(cf. Fig. 1A–D). Recent phylogenetic analyses revealed that the divergence of several subgroups of Peripatopsidae might have preceded the breakup of Gondwana (Reid, 1996; Allwood et al., 2009). In particular, the results of molecular phylogenetic analyses suggest that the two South African genera, *Opisthopatus* and *Peripatopsis*, are not sister taxa since *Peripatopsis* sister groups with *Metaperipatus* from Chile (Allwood et al., 2009). The study of the mitochondrial genome of *Peripatopsis* might thus provide additional support for this sister group relationship, provided that a similar gene arrangement pattern occurs in *Peripatopsis* and *M. inae*. Alternatively, analyzing the mitochondrial genome of *Peripatopsis* might reveal a gene arrangement intermediate between the ancestral pattern (as exemplified by *O. cinctipes*) and the highly derived arrangement found in *M. inae*. A study of the mitochondrial genomes in the *Peripatopsis* species will therefore provide a more detailed picture on the evolution of the mitochondrial genomes within the Peripatopsidae and help clarify the phylogeny within the Onychophora.

Author contributions

A.B. conceived and performed the molecular experiments and data analyses (PCR, DNA sequencing, sequence analyses, and annotations). G.M. collected, identified and dissected the specimens, and helped analyze the data. G.M. and A.B. wrote the manuscript. All authors participated in the discussion of the results and the preparation of the final manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2010.05.012.

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