Molecular Phylogenetics and Evolution 57 (2010) 285–292

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

The mitochondrial genome of the onychophoran *Opisthopatus cinctipes* (Peripatopsidae) reflects the ancestral mitochondrial gene arrangement of Panarthropoda and Ecdysozoa

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ARTICLE INFO

Article history:
Received 20 January 2010
Revised 8 May 2010
Accepted 13 May 2010
Available online 20 May 2010

Keywords:
Arthropods
Gene order
Onychophora
Phylogeny
Velvet worms

ABSTRACT

The ancestral genome composition in Onychophora (velvet worms) is unknown since only a single species of Peripatidae has been studied thus far, which shows a highly derived gene order with numerous translocated genes. Due to this lack of information from Onychophora, it is difficult to infer the ancestral mitochondrial gene arrangement patterns for Panarthropoda and Ecdysozoa. Hence, we analyzed the complete mitochondrial genome of the onychophoran *Opisthopatus cinctipes*, a representative of Peripatopsidae. Our data show that *O. cinctipes* possesses a highly conserved gene order, similar to that found in various arthropods. By comparing our results to those from different outgroups, we reconstruct the ancestral gene arrangement in Panarthropoda and Ecdysozoa. Our phylogenetic analysis of protein-coding gene sequences from 60 protostome species (including outgroups) provides some support for the sister group relationship of Onychophora and Arthropoda, which was not recovered by using a single species of Peripatidae, *Epiperipatus biolleyi*, in a previous study. A comparison of the strand-specific bias between onychophorans, arthropods, and a priapulid suggests that the peripatid *E. biolleyi* is less suitable for phylogenetic analyses of Ecdysozoa using mitochondrial genomic data than the peripatopsid *O. cinctipes*.

1. Introduction

Studies of animal mitochondrial genomes have become a useful tool for inferring the evolutionary relationships of animals for two reasons. First, the mitochondrial sequence data can be directly used to infer phylogenetic relationships and, second, offer the possibility to trace back the evolution of gene rearrangements, thus, providing an additional source of information for phylogenetic reconstructions (e.g., Boore et al., 1995, 1998; Blanchette et al., 1999; Dowton et al., 2002; Bleidorn et al., 2007).

As close relatives to arthropods (Nielsen, 2001; Kusche et al., 2002; Mallatt and Giribet, 2006; Roeding et al., 2007; Dunn et al., 2008), Onychophora or velvet worms are an important group for understanding the evolution of Panarthropoda and Ecdysozoa (Fig. 1). So far, however, complete mitochondrial genomic data are available only from one species of Peripatidae, *Epiperipatus biolleyi*, which has a highly derived gene order and numerous gene rearrangements (Podsiadlowski et al., 2008). Moreover, the use of mitochondrial sequence data from this species for a phylogenetic analysis resulted in an unresolved polytomy within the Ecdysozoa. This might be due to a strand bias in this species, which highly deviates from that of arthropods (Rota-Stabelli and Telford, 2008).

In the present study, we investigate the complete mitochondrial genome of the onychophoran *Opisthopatus cinctipes* (Peripatopsidae). We compare the mitochondrial gene arrangement patterns between arthropods, onychophorans, and other ecdysozoans to clarify the major transformation events and reconstruct the ancestral mitochondrial gene order in Onychophora, Panarthropoda, and Ecdysozoa. Furthermore, we perform an amino acid-based phylogenetic analysis of Panarthropoda and Ecdysozoa to clarify the phylogenetic position of Onychophora. In addition to *O. cinctipes*, we include data from another representative of Peripatopsidae, *Metaperipatus inae*, to increase taxon sampling. A detailed analysis of the mitochondrial genome of *M. inae* is presented in another paper from this issue (Braband et al., 2010) as it is beyond the scope of the present work to describe the complex pattern of gene rearrangements and genome composition in this species.

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doi:10.1016/j.ympev.2010.05.011

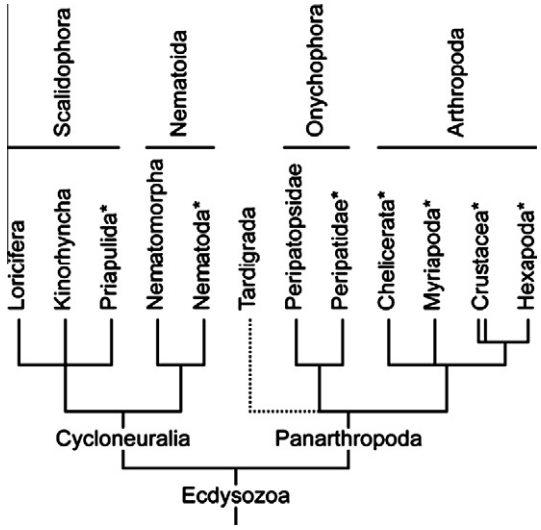

Fig. 1. Phylogenetic relationships of major animal groups within Ecdysozoa (combined from various sources: Schmidt-Rhaesa et al., 1998; Nielsen, 2001; Dunn et al., 2008; Edgecombe, 2009; Hejnol et al., 2009). The phylogeny of Scalidophora is unresolved. The position of Tardigrada is uncertain (dotted line) as they might be the sister group to arthropods, to onychophorans, to onychophorans plus arthropods, or to one of the cycloneuralian taxa (review Mayer and Whitington, 2009a). Myriapoda might be either the sister group to Chelicerata or to Crustacea + Hexapoda (e.g., Rota-Stabelli and Telford, 2008; Edgecombe, 2009; Mayer and Whitington, 2009b). The double line for Crustacea indicates that this grouping might be non-monophyletic (Richter, 2002; Edgecombe, 2009). Asterisks designate the ecdysozoan subgroups, for which at least one complete mitochondrial genome sequence is available.

2. Materials and methods

2.1. Animals, DNA extraction, amplification, and sequencing

Specimens of *Opisthopatus cinctipes* Purcell, 1899 were collected in the Cathedral Peak Nature Reserve (Drakensberg Mountain Range, KwaZulu-Natal, South Africa, 28°57′590″S, 29°13′659″E). DNA was extracted with the DNeasy Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Initial PCRs amplified small portions of the *COI* and *srRNA* genes using the primer pairs LCOI-1490 and HCOI-2198 from Folmer et al. (1994) and 12Sai and 12Smb from Kocher et al. (1989), respectively, and the AmpliTaq Gold DNA Polymerase (Applied Biosystems, Carlsbad, USA). These initial PCR products were sequenced using the BigDye v3.0 Cycle Sequencing Kit (Applied Biosystems) and used to design specific primers for long PCR amplifications. The remainder of the mitochondrial genome was amplified by long range PCR using the Elongase Enzyme Mix (Invitrogen, Carlsbad, USA) in three pieces: *COI–ND4* (ONY1/OCP7), *ND4–srRNA* (N4-J-8944/ONY5), and *srRNA–COI* (ONY2/ONY8) (Supplementary Table 1). Long range PCR amplicons were sequenced using several primers and primer walking strategy as in previous studies of mitochondrial genomes (Supplementary Table 1; Cameron et al., 2006; Fenn et al., 2008).

2.2. Gene identification

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 *E. biolleyi* (Podsiadlowski et al., 2008) and various arthropod species. Positions of 15 tRNA genes and secondary tRNA structures were identified by using the tRNAscan-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_HM008997). Since we discovered several inconsistencies in the original annotation of several tRNA genes in the onychophoran *E. biolleyi*, we re-annotated the mitochondrial sequences from this species using the ARWEN server and found two additional candidate sequences for the tRNA genes *S(AGN)* (position 13,694–13,747 on the light strand) and *A* (position 13,933–13,994 on the heavy strand) (see also fig. 5 in Braband et al., 2010).

2.3. Determination of GC-skew values

The GC-skew values were determined for all codon positions and for each separate gene according to Perna and Kocher's (1995) method: GC-skew = (G – C)/(G + C). In addition, GC-skew values were calculated for entire mitochondrial genomes, including both coding and non-coding regions (Supplementary Table 2). The sequence data from additional species were obtained from the GenBank (Supplementary Table 3).

2.4. Phylogenetic analysis

The species included in the phylogenetic analysis cover all four major arthropod groups (Chelicerata, Myriapoda, Crustacea, and Hexapoda), three species of Onychophora, one representative of Priapulida, and fifteen species of Lophotrochozoa (including representatives of Annelida, Brachiopoda, Ectoprocta, Entoprocta, and Mollusca) as outgroups (Supplementary Table 3). Nematoda were excluded from the analysis because they exhibit a highly dissimilar strand-specific bias and a highly deviating G + C content compared to those in arthropods. These deviations may interfere with the stationarity of the model and cause the so-called "random outgroup effect" in phylogenetic analyses of mitochondrial datasets (Rota-Stabelli and Telford, 2008).

A concatenated amino acid sequence alignment of nearly all protein-coding genes (the *A8* sequence was excluded due to its small size) from 60 protostome species was performed with Clustal W (Thompson et al., 1994), as implemented in BioEdit 7.0.9 (Hall, 1999) under default settings. The obtained alignment showed 3901 amino acid positions.

Maximum Likelihood analyses were performed with Treefinder (Jobb et al., 2004). We calculated the appropriate amino acid model by invoking the options "propose model" and "mtProt" with the concatenated alignment to set limits to mitochondrial data-specific

Table 1
Occurrence (✓) of the mitochondrial tRNA genes in three onychophoran species studied thus far: *Opisthopatus cinctipes*, *Metaperipatus inae*, and *Epiperipatus biolleyi*. Note that the tRNA genes *L(CUN)* and *R* are missing in all three species.

tRNA genes	*A*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*K*	*L1*	*L2*	*M*	*N*	*P*	*Q*	*R*	*S1*	*S2*	*T*	*V*	*W*	*Y*
O. cinctipes	✓				✓		✓	✓	✓	✓		✓		✓			✓	✓	✓	✓	✓	✓
M. inae	✓		✓	✓		✓		✓	✓	✓		✓	✓	✓	✓		✓	✓	✓	✓	✓	
E. biolleyi	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓		✓	✓		✓						✓	✓

Abbreviations: *L1 = L(UUR)*, *L2 = L(CUN)*, *S1 = S(AGN)*, and *S2 = S(UCN)*.

models. The proposed model was mtART + G. Furthermore, 100 bootstrap replicates were performed to calculate the best tree. An additional dataset was obtained by applying the software Gblocks, version 0.91b (Castresana, 2000), with default parameters except for "allowed gap positions = with half". This dataset was also analyzed with Treefinder by proposing the same model (mtART + G). However, this analysis resulted in a less resolved tree than that using the total alignment. Bayesian analysis using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) was also performed, but resulted in an equally poor resolution (not shown).

3. Results

3.1. Mitochondrial genome content

The mitochondrial genome of *O. cinctipes* consists of 13,599 bp and contains the complete set of two ribosomal RNA genes and 13 protein-coding genes (Fig. 2). Of the 22 tRNA genes present in most animal mitochondrial genomes, only 15 occur in *O. cinctipes* whereas *C*, *D*, *E*, *L(CUN)*, *N*, *Q* and *R* are missing (Fig. 2, Table 1, Supplementary Table 4).

3.2. Gene arrangement

The relative order of all the protein-coding genes and the control region in *O. cinctipes* corresponds exactly to that in the chelicerate *Limulus polyphemus* (see Staton et al., 1997), and thus resembles the putative arthropod ground pattern (Fig. 3C and E). Furthermore, the position of most tRNA genes is strikingly similar between these two panarthropod species. Among the 15 tRNA genes that have been retained in *O. cinctipes*, only the positions of *S(AGN)*, *I* and *W* are different from those found in *L. polyphemus*. The tRNA gene *I* is situated on the other side of the control region adjacent to *srRNA* whereas *W* is located next to *ND1* on the light strand instead of being adjacent to *ND2* on the heavy strand. The tRNA gene *S(AGN)* is located within the protein-coding gene *ND5*, but is encoded on the heavy strand (Figs. 2 and 3E).

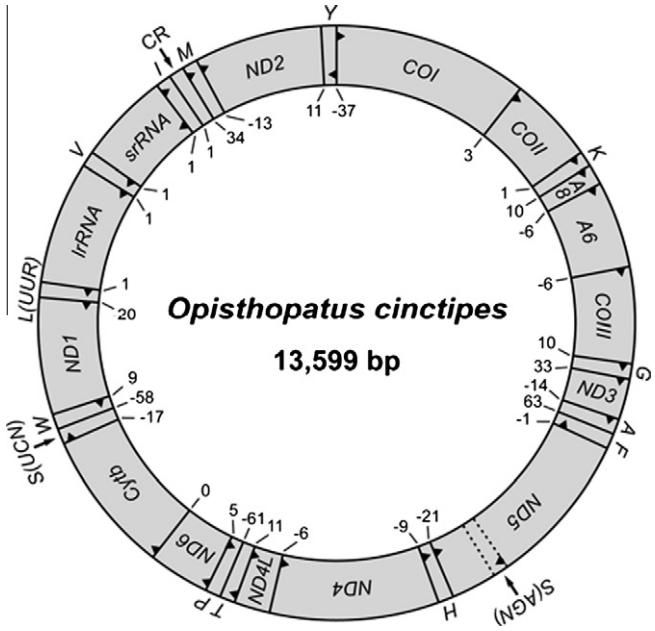

Fig. 2. Map of the mitochondrial genome of the onychophoran *Opisthopatus cinctipes* (Peripatopsidae). The relative gene size is illustrated, except for the tRNA genes and the control region (CR). Numbers at gene borders indicate nucleotide number in the non-coding (positive values) and overlapping regions (negative values) between two adjacent genes. Arrowheads show the orientation of genes either on the heavy strand (clockwise) or light strand (counterclockwise). Dotted lines indicate the location of the tRNA gene *S(AGN)*, which is encoded within the *ND5* sequence, but on the opposite strand.

3.3. Base composition

Analysis of nucleotide composition in *O. cinctipes* reveals a moderate bias between both strands, with a GC-skew value for the entire mitochondrial genome near zero (Supplementary Table 2). Furthermore, the GC-skew values for most genes encoded on the heavy (+) strand are positive, except for *A8*, *ND6*, and *Cytb*. The GC-skew values for most genes on the light (−) strand are also positive, apart from *ND4L* (Supplementary Table 2).

3.4. Structure of the putative control region

According to our sequence data, there is one non-coding region in *O. cinctipes* (Figs. 2 and 3E). We designate this region as a putative control region (CR) since it shows a hairpin-like structure (Supplementary Fig. S1), which resembles the stem-loop structures reported from the mitochondrial control regions of various arthropods and from tardigrades (e.g., Kilpert and Podsiadlowski, 2006; Fahrein et al., 2007; Ryu et al., 2007; Pie et al., 2008). The hairpin-like structure in the control region of *O. cinctipes* has a loop composed of 5 unpaired nucleotides and a G + C rich stem with 15 nucleotide pairs (Supplementary Fig. S1). The A + T content in the control region (70.3%) is lower than in the remaining mitochondrial genome (77.6%), but its position in *O. cinctipes* between the genes *srRNA* and *ND2* is conserved and corresponds with that in *L. polyphemus* (Fig. 3C and E).

3.5. Phylogenetic analysis

We performed a phylogenetic analysis with concatenated amino acid sequences of 12 genes (all protein-coding genes except for *A8*) from 41 representative arthropod species, three onychophorans, one priapulid, and 15 species of Lophotrochozoa as outgroups (Supplementary Table 3). The resulting Maximum Likelihood tree supports the monophyly of the Panarthropoda (data from complete mitochondrial genomes of tardigrades were unavailable for our analysis), with a monophyletic Onychophora sister grouping with a monophyletic Arthropoda (Fig. 4). Within the Onychophora, *O. cinctipes* and *M. inae* are sister species within the monophyletic Peripatopsidae to the exclusion of a single Peripatidae species, which is *E. biolleyi*. The position of the Priapulida is unresolved, but they are placed outside the Onychophora + Arthropoda clade. The Annelida, Mollusca, Entoprocta, Ectoprocta, and Brachiopoda are united in a monophyletic Lophotrochozoa, with a high bootstrap support value for each group (Fig. 4). The internal phylogeny of the Arthropoda is unresolved and none of the four major arthropod groups, including the Chelicerata, Myriapoda, Crustacea, and Hexapoda, are monophyletic (Fig. 5).

4. Discussion

4.1. Evidence for a sister group relationship of Onychophora and Arthropoda

Our phylogenetic analysis of protein-coding mitochondrial genes supports the monophyly of both Onychophora and Arthropoda and provides some evidence for their sister group relationship. Despite a low bootstrap value (<50%) for this grouping, our findings agree with results of phylogenetic analyses based on various other datasets (Kusche et al., 2002; Mallatt and Giribet, 2006; Roeding et al., 2007; Dunn et al., 2008; Hejnol et al., 2009) and contradict

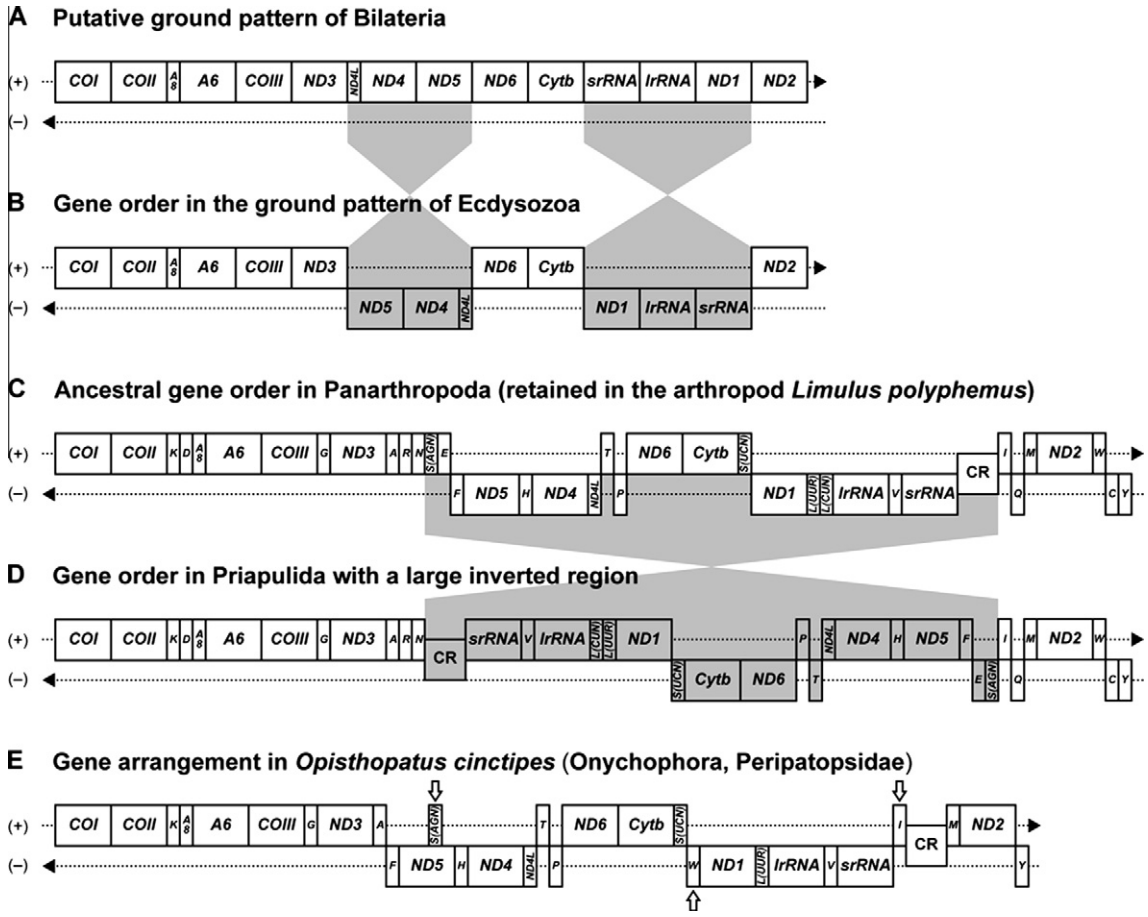

Fig. 3. Evolution of the mitochondrial gene arrangement patterns in Ecdysozoa. Major inversion events are demonstrated in grey. Heavy and light strands are designated by (+) and (−), respectively. The control regions (CR) are not assigned to any strand. (A) Putative arrangement of protein-coding and ribosomal RNA genes in the ground pattern of Bilateria (modified from Mwinyi et al., 2009). The position of tRNA genes is not illustrated. Note the position of all genes on one strand. (B) Inferred ancestral arrangement of protein-coding and ribosomal RNA genes in Ecdysozoa. Two inversion events (grey) most likely occurred in the Ecdysozoan stem-lineage. (C) Ancestral gene arrangement in Panarthropoda, which has been retained in the chelicerate *Limulus polyphemus* (modified from Staton et al., 1997). (D) Gene arrangement in the priapulid *Priapulus caudatus* (modified from Webster et al., 2006) showing a large inverted genome region (grey). (E) Mitochondrial gene order in the onychophoran *Opisthopatus cinctipes* analyzed in this study. Open arrows indicate three translocated tRNA genes. Note the striking similarity of gene order between *O. cinctipes*, *L. polyphemus*, and *P. caudatus*, taking into consideration the inversion of a large genomic portion in the latter.

previous proposals of a sister group relationship between Onychophora and Chelicerata (Ballard et al., 1992; Strausfeld et al., 2006). Our analysis, however, does not resolve the monophyly and relationship of the four major arthropod groups. This unresolved phylogeny might be due to the strand-specific bias (usually measured as GC-skew), which differs considerably between arthropods and outgroups used. Previous analyses of mitochondrial datasets have demonstrated that the strand-specific nucleotide biases can lead to misleading phylogenetic signals even when mitochondrial genomes are analyzed as translated amino acid sequences (e.g., Hassanin et al., 2005; Jones et al., 2007; Rota-Stabelli and Telford, 2008).

Indeed, a comparison of GC-skew values reveals positive skews for most genes in onychophorans encoded on the heavy strand whereas the corresponding values are mostly negative in arthropods and priapulids (Supplementary Table 2; Hassanin et al., 2005; Hassanin, 2006). Despite the striking similarity of the mitochondrial gene order in the onychophoran *O. cinctipes* and the chelicerate *L. polyphemus*, the GC-skew values for most protein-coding genes and the entire mitochondrial genome differ considerably between these two panarthropod species (Fig. 5A). In contrast, the GC-skew values in *O. cinctipes* and the priapulid *Priapulus caudatus* resemble each other, despite the inversion of a large portion of the *P. caudatus* mitochondrial genome (Webster et al., 2006, 2007). The divergent GC-skews might explain why the inclusion of Priapulida and Onychophora in the analysis does not help resolve the internal phylogeny of Arthropoda or recover the monophyly of the four major arthropod groups. Conversely, similar GC-skew values in *P. caudatus* and *O. cinctipes* might account for a well-resolved phylogeny and monophyly of Onychophora in our analysis, despite the low taxon sampling (Fig. 5A).

Differences in GC-skew values between the onychophoran species might be the reason why a previous analysis using only one species, *E. biolleyi*, was unable to resolve the relationships within the Ecdysozoa, finding an unresolved polytomy between the Priapulida, Onychophora, and Arthropoda (Podsiadlowski et al., 2008). In light of the present study, this previous result might be due to a limited taxon sampling and deviating GC-skew values in *E. biolleyi* from those in *O. cinctipes*, *M. inae*, and *P. caudatus* (Fig. 5A and B; Supplementary Table 2). In summary, the similarity of GC-skew values between the priapulid species and the two representatives of Peripatopsidae suggest that these species are potentially suitable for future phylogenetic analyses of Ecdysozoa using additional mitochondrial genomic data, in particular from Kinorhyncha, Loricifera, Nematomorpha, and Tardigrada.

4.2. Ancestral mitochondrial gene order in Onychophora

By comparing the mitochondrial gene order from three onychophoran species, *O. cinctipes* (Peripatopsidae; this study), *M. inae*

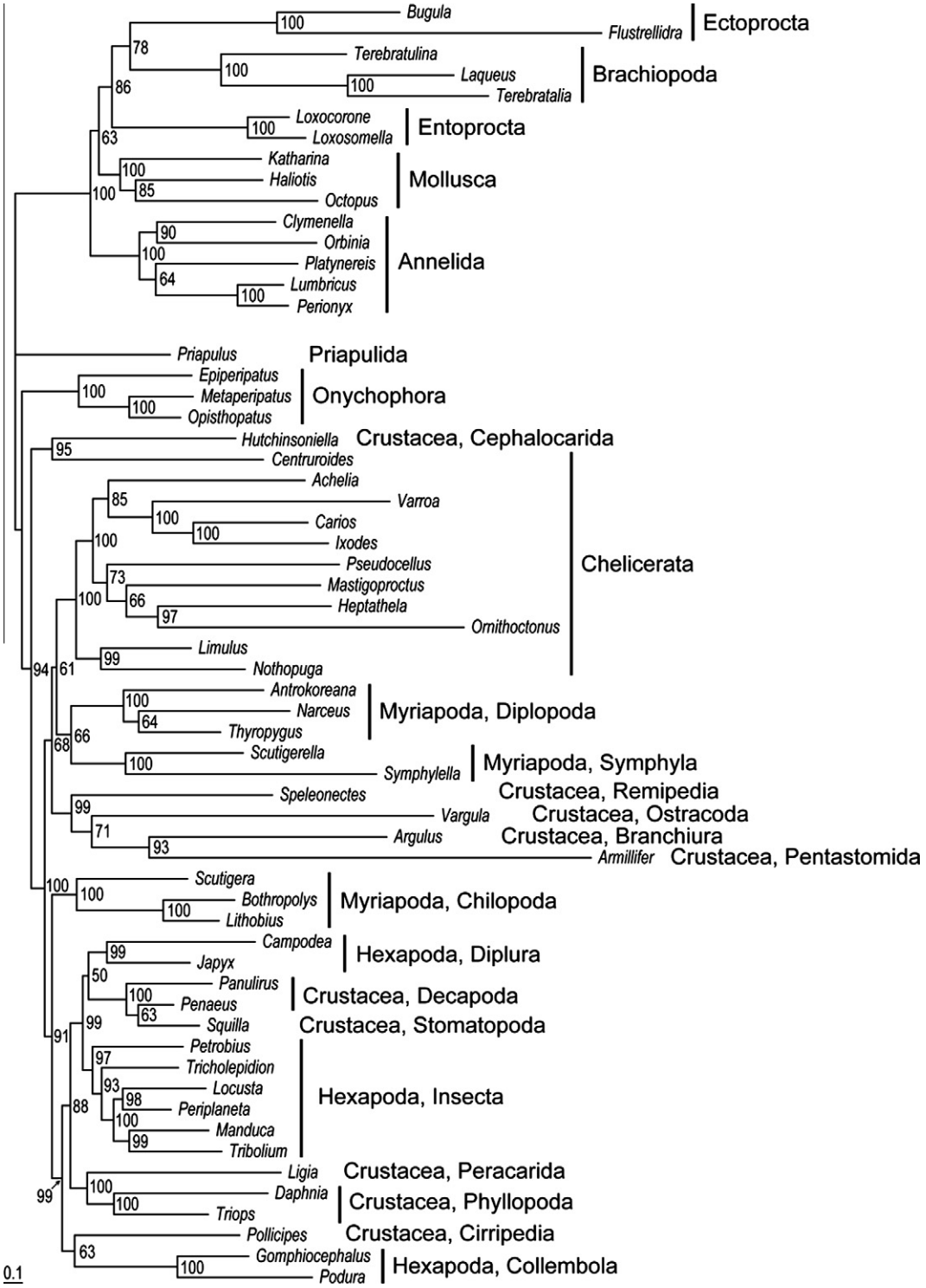

Fig. 4. Maximum likelihood analysis of 12 concatenated mitochondrial protein-coding genes from various species of Ecdysozoa and several species of Lophotrochozoa as outgroups. Bootstrap support values of ⩾50 are indicated at each node. Scale bar represents genetic distance (substitutions per site).

(Peripatopsidae; Braband et al., 2010), and *E. biolleyi* (Peripatidae; Podsiadlowski et al., 2008), we find three different gene arrangement patterns. The mitochondrial gene orders in *M. inae* and *E. biolleyi* differ considerably from those reported from various arthropods and the priapulid *P. caudatus* (e.g., Clary and Wolstenholme, 1985; Valverde et al., 1994; Staton et al., 1997; Webster et al., 2006, 2007). The highly derived gene order reported from *E. biolleyi* might be due to numerous gene translocation events whereas that in *M. inae* can be explained by extensive genome duplications, followed by a random loss of genes (Podsiadlowski et al., 2008; Braband et al., 2010).

In contrast, the mitochondrial gene order in *O. cinctipes* resembles that in the chelicerate *L. polyphemus*, despite three translocated and seven missing tRNA genes in *O. cinctipes* (Fig. 3C and E). The missing tRNA genes include both twofold and fourfold degenerate isotypes, as in *M. inae* (Braband et al., 2010). The differ-

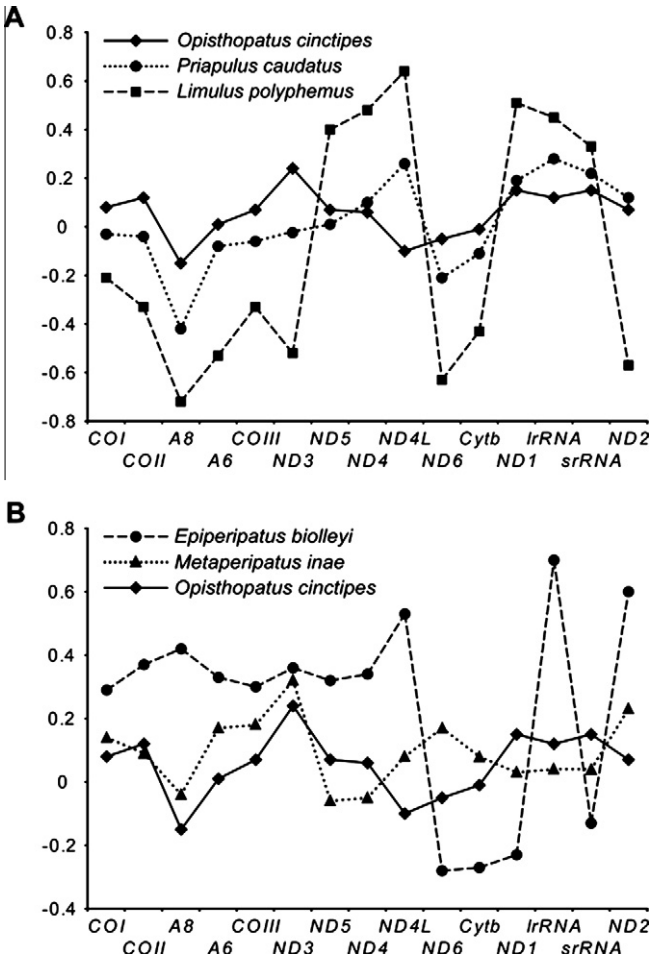

Fig. 5. Comparison of GC-skew values of mitochondrial genes (A) between the priapulid *Priapulus caudatus*, the onychophoran *Opisthopatus cinctipes* and the arthropod *Limulus polyphemus*, and (B) between three onychophoran species *Epiperipatus biolleyi*, *Metaperipatus inae* and *O. cinctipes*. Note the similar and more balanced GC-skew values among different genes in *P. caudatus* and *O. cinctipes*, as compared to *L. polyphemus*. Among the three onychophoran species, the GC-skew values in *E. biolleyi* (Peripatidae) differ from those in *M. inae* and *O. cinctipes* (both Peripatopsidae species), which are similar to each other.

ent patterns of gene loss in each onychophoran species (Table 1) suggest that the missing tRNA genes have been lost convergently in these species. The ancestral set of the mitochondrial tRNA genes in Onychophora, thus, might have comprised at least 20 tRNA genes since only *L(CUN)* and *R* are absent from all three distantly related onychophoran species studied thus far (Table 1).

In *O. cinctipes*, 12 out of 15 remaining tRNA genes show an identical position to the corresponding genes in *L. polyphemus* and *P. caudatus*, if the large inversion in the priapulid genome is taken into account (Fig. 3C–E). This suggests that the position of at least these 12 tRNA genes is conserved in *O. cinctipes* and was the same in the last common ancestor of Onychophora. Such conservatism is unusual since the mitochondrial tRNA genes are commonly known as movable or transposable elements and many of them have been translocated in various animals, including the onychophorans *E. biolleyi* and *M. inae* (Moritz et al., 1987; Saccone et al., 1999, 2002; Podsiadlowski et al., 2008; Braband et al., 2010). Currently, it is impossible to infer the ancestral location of the remaining tRNA genes in Onychophora since they are either missing or translocated to another position in the three onychophoran species studied.

In summary, a comparison of our data from *O. cinctipes* with those from *L. polyphemus* as an outgroup reveals an identical arrangement of all 13 protein-coding genes, two ribosomal genes, 12 transfer RNA genes, and the control region in these two species (Fig. 3C and E). Since this specific correspondence is unlikely to have evolved by chance, we suggest that this arrangement is an ancestral feature of onychophorans and was also present in the last common ancestor of Onychophora and Arthropoda.

4.3. Ancestral mitochondrial gene order in Panarthropoda

A mitochondrial gene order strikingly similar to that found in *O. cinctipes* and *L. polyphemus* occurs in partial mitochondrial genome fragments (~3.8 kb) available from the tardigrades *Batillipes pennaki* and *Pseudobiotus* sp. (Ryu et al., 2007). In particular, the ribosomal RNA genes *lrRNA* and *srRNA*, and the protein-coding genes *ND2* and *COI* show the same arrangement and strand identity as in the last common ancestor of both Onychophora and Arthropoda. Furthermore, most analyzed tRNA genes lie in the corresponding positions in tardigrades, except for *I*, which is missing in *Pseudobiotus* sp. and translocated in *B. pennaki* (see Ryu et al., 2007).

A comparison of the mitochondrial gene order in *L. polyphemus* and the priapulid *P. caudatus* (as an outgroup taxon to Panarthropoda) reveals an identical gene arrangement, taking into account the inversion of a large genomic portion including 18 genes and the control region in *P. caudatus* (Fig. 3C–E). Notably, even the relative positions of all 22 tRNA genes are the same in the two species, if the inversion in *P. caudatus* is considered. The absence of a large inverted genomic portion from the mitochondrial genome of tardigrades, onychophorans and arthropods suggests that this large inversion was not present in the last common ancestor of these three panarthropod groups but might have evolved in the cycloneuralian lineage (cf. Fig. 1). Thus, a gene arrangement without inverted region, as retained in *L. polyphemus*, belongs to the ground pattern of Panarthropoda (Fig. 3C).

4.4. Ancestral mitochondrial gene order in Ecdysozoa

To clarify whether the inversion of a large genomic portion found in *P. caudatus* is an ancestral character of Ecdysozoa, or a derived feature of Cycloneuralia or a cycloneuralian subgroup, additional ecdysozoan species have to be analyzed. So far, only the data from nematodes are available for comparison (Fig. 1). It has been demonstrated that the mitochondrial genome composition within nematodes is highly variable, but the arrangement in the early-branching representative, *Trichinella spiralis*, does not have an inverted region similar to that found in the priapulid mitochondrial genome (Lavrov and Brown, 2001). In *T. spiralis*, 10 out of 18 genes from the inverted region of *P. caudatus* show the same non-inverted order as in arthropods whereas the remaining eight genes from this region are translocated to other positions different from both the arthropod and priapulid genomes. The partially conserved gene arrangement in *T. spiralis* implies that the inversion of a large genomic portion does not belong to the ground pattern of Cycloneuralia.

In conclusion, the similar mitochondrial gene arrangement patterns in an onychophoran, various arthropods, and a priapulid suggest that the gene order, which is found in *L. polyphemus*, was present in the last common ancestor of Arthropoda, Onychophora, and Priapulida, i.e., in the ground pattern of Ecdysozoa (Fig. 3B). Furthermore, a comparison with the putative ancestral mitochondrial gene order in Bilateria (Mwinyi et al., 2009) reveals two inversion events of genome regions containing *ND4L–ND4–ND5* and *srRNA–lrRNA–ND1* in the stem-lineage of Ecdysozoa (Fig. 3A and B). Studies of additional ecdysozoan subgroups, in particular Kinorhyncha, Loricifera, Nematomorpha, and Tardigrada are necessary to further clarify the transformation events and the evolution of the mitochondrial genomes in the Ecdysozoa.

Acknowledgments

This work was supported by grants from the German Research Foundation (DFG) to A. Braband (Scho 442/8-1), L. Podsiadlowski (Ba 1520/10-1), and G. Mayer (Ma 4147/2-1, and Ma 4147/3-1). A. Braband and L. Podsiadlowski participated in the high priority project 1174 "Deep Metazoan Phylogeny" of the DFG. G. Mayer is an Emmy Noether Group Leader supported by the DFG. The work of S.L. Cameron was supported by grants from the U.S. National Science Foundation (DEB0120718, and DEB0444972).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2010.05.011.

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