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

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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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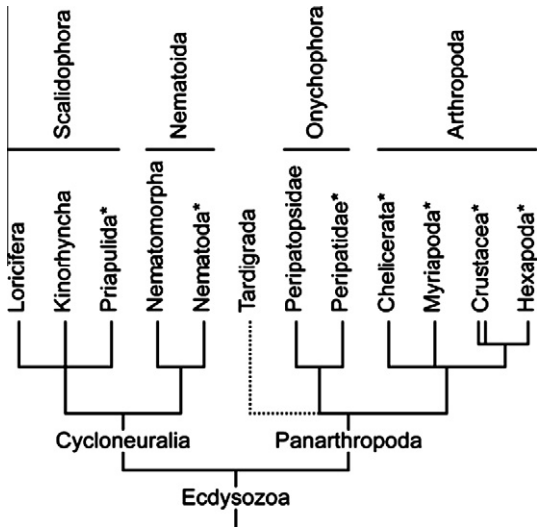

Fig. 1. Phylogenetic relationships of major animal groups within Ecdysozoa (combined from various sources: Schmidt-Rhaesa et al., 1998; Nielsen, 2001; Dunn et al., 2008; Edgecombe, 2009; Hejnol et al., 2009). The phylogeny of Scalidophora is unresolved. The position of Tardigrada is uncertain (dotted 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)*.