The Complete Mitochondrial Genome of the Onychophoran *Epiperipatus biolleyi* Reveals a Unique Transfer RNA Set and Provides Further Support for the Ecdysozoa Hypothesis

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Onychophora (velvet worms) play a crucial role in current discussions on position of arthropods. The ongoing Articulata/Ecdysozoa debate is in need of additional ground pattern characters for Panarthropoda (Arthropoda, Tardigrada, and Onychophora). Hence, Onychophora is an important outgroup taxon in resolving the relationships among arthropods, irrespective of whether morphological or molecular data are used. To date, there has been a noticeable lack of mitochondrial genome data from onychophorans. Here, we present the first complete mitochondrial genome sequence of an onychophoran, *Epiperipatus biolleyi* (Peripatidae), which shows several characteristic features. Specifically, the gene order is considerably different from that in other arthropods and other bilaterians. In addition, there is a lack of 9 tRNA genes usually present in bilaterian mitochondrial genomes. All these missing tRNAs have anticodon sequences corresponding to 4-fold degenerate codons, whereas the persisting 13 tRNAs all have anticodons pairing with 2-fold degenerate codons. Sequence-based phylogenetic analysis of the mitochondrial protein-coding genes provides a robust support for a clade consisting of Onychophora, Priapulida, and Arthropoda, which confirms the Ecdysozoa hypothesis. However, resolution of the internal ecdysozoan relationships suffers from a cluster of long-branching taxa (including Nematoda and Platyhelminthes) and a lack of data from Tardigrada and further nemathelminth taxa in addition to nematodes and priapulids.

Introduction

Onychophorans are a small group of terrestrial invertebrates, which are predominantly found in soil, leaf litter, or rotten wood, and show a disjunct distribution dating back to the Gondwana landmass (Brinck 1957; Monge-Najera 1995; Reid 1996). Together with Tardigrada (water bears), Onychophora are commonly regarded as the next relatives of Arthropoda (Chelicerata, Myriapoda, Crustacea, and Hexapoda) (Giribet et al. 2001; Nielsen 2001; Kusche et al. 2002; Mayer et al. 2004; Regier et al. 2005; Vitkova et al. 2005; Hejnol and Schnabel 2006). However, there are alternative views placing Onychophora close to Chelicerata (Ballard et al. 1992; Strausfeld et al. 2006) or uniting Tardigrada and Nematoda (Park et al. 2006).

Traditionally, the onychophoran body plan has been regarded as a mosaic of anatomical characters found in both annelids and arthropods (Snodgrass 1938; Storch and Ruhberg 1993; Nielsen 1998). The study of onychophorans, therefore, might yield valuable information for resolving the persisting Articulata/Ecdysozoa controversy (Scholtz 2002; Giribet 2003; Jenner and Scholtz 2005; Philippe et al. 2005; Pilato et al. 2005; Mayer 2006a; Schmidt-Rhaesa 2006). One of the major implications of the Ecdysozoa hypothesis is that the "annelid-like" characters of onychophorans must represent homoplasies, that is, either convergent features of Onychophora and Annelida or ancestral features of protostomes or even bilaterians. Recent anatomical and embryological studies have shown that some of the major putative correspondences between Onychophora and Annelida are ambiguous and, accordingly, do not support the monophyly of Articulata (Mayer 2006a). Moreover, recent insights into the onychophoran head development (Eriksson and Budd 2000; Eriksson et al. 2003) and extensive paleontological record (Whittington 1978; Ramsköld and Chen 1998; Bergström and Hou 2001; Budd 2003; Maas et al. Forthcoming 2007) are better compatible with the Ecdysozoa hypothesis rather than with the Articulata concept (Giribet 2003). Nevertheless, additional approaches are required to further clarify and improve our understanding of arthropod evolution.

To date, the study of mitochondrial genomes has yielded significant insights into the evolution of major metazoan groups and their genomes (Boore 1999; Helfenbein and Boore 2004; Papillon et al. 2004; Valles and Boore 2006). Besides the sequence information, the mitochondrial gene order provides a useful character set for phylogenetic implications (Boore et al. 1995). Despite an extreme genome shuffling in some taxa (Machida et al. 2002), the identification of specific gene rearrangements in the mitochondrial genomes has led to a robust support of hitherto uncertain groupings and the establishment of novel sister-group relationships (e.g., Boore et al. 1998).

In contrast to the wealth of mitochondrial genomic data from various arthropods, only a small subset of mitochondrial genes has been scrutinized from Onychophora (Boore et al. 1995, 1998; Gleeson et al. 1998; Trewick 2000). Phylogenetic analyses of large data sets on the position of Arthropoda within Bilateria, thus, either are restricted to other data sources (Giribet et al. 2001; Mallatt et al. 2004; Mallatt and Giribet 2006) or exclude the Onychophora (Webster et al. 2006). In order to close this gap and to understand how the onychophoran mitochondrial genome compares to that of other arthropods and bilaterians, we sequenced the complete mitochondrial DNA from *Epiperipatus biolleyi* (Onychophora, Peripatidae). Here, we compare the onychophoran mitochondrial gene order with that in other Bilateria and perform a sequence-based phylogenetic analysis, which contributes to the ongoing Articulata/Ecdysozoa debate.

Key words: Onychophora, mitochondrial genome, mitochondrial gene order, tRNA import, Arthropoda.

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Table 1
PCR Primer Sequences and Corresponding Annealing Temperatures

Primer Name	Primer Sequence (5′–3′)	Temperature (°C)	Reference
Crust-12f	CAGCAKYCGCGGTTAKAC	45	Podsiadlowski and Bartolomaeus (2005)
Crust-12sr	ACACCTACTWTGTTACGACTTATCTC	45	Podsiadlowski and Bartolomaeus (2005)
Crust-nd4f	TTGAGGTTAYCAGCCYG	50	Podsiadlowski and Bartolomaeus (2005)
Crust-nd4r	ATATGAGCYACAGAAGARTAAGC	50	Podsiadlowski and Bartolomaeus (2005)
L329-ND2	GGWGCHGCHCCNTTWCATTTTTG	45	Yamauchi et al. (2004)
H1410-ND2	AGTGCCWACTATWCCWGMTCA	45	Yamauchi et al. (2004)
L1384-CO1	GGTCAACAAATCATAAAGATATTGG	45	Yamauchi et al. (2004)
H2043-CO1	TAAACTTCAGGGTGACCAAAAAATCA	45	Yamauchi et al. (2004)
L1564-CO1	ATGGTWATACCGATTWTRATTGG	45	Yamauchi et al. (2004)
H2619-CO1	GGTATWCCWGCKAGWCCTAAGAAATGTTG	45	Yamauchi et al. (2004)
L3020-CO2	ATTTTTTTYCATGAYCATGC	45	Yamauchi et al. (2004)
H3514-CO2	CCACAAATTTCKGAACATTGWCCATAAAA	45	Yamauchi et al. (2004)
L3542-CO2	GGNCAATGTTCAGAAATTTGTGG	45	Yamauchi et al. (2004)
H4375-A6	GCDATCATGTTDGCDGMWAGTCG	45	Yamauchi et al. (2004)
L4672-CO3	GGWCTWGTBAAATGGTTTCA	45	Yamauchi et al. (2004)
H5244S-CO3	GCTTCAAATCCWAMGTGGTG	45	Yamauchi et al. (2004)
L11197-ND1	GCTAGATATATHAGTTTGTCATADCG	45	Yamauchi et al. (2004)
H11892-ND1	GGWTATATTCAGATTCGWAAGGGDCC	45	Yamauchi et al. (2004)
L10061-CYB	GGATTWTTTTTAGCKATRCATTACAC	45	Yamauchi et al. (2004)
H-10699-CYB	GCAAATAGAAAATATCATTCWGGTTG	45	Yamauchi et al. (2004)
L12167S-16S	CGGTCTGAACTCAGATCATG	45	Yamauchi et al. (2004)
H12663-16S	CGCCTGTTTACCAAAAACAT	45	Yamauchi et al. (2004)
Ebio-cox1f	GTTGTTGCTATCTCTGCCTGTGTTGG	55	This study
Ebio-cox2r	AAACAGGTAATACCGTTCAAATCATCT	55	This study
Ebio-cox2f	GGTGAATTCCGTTTGTTAGATGTTG	55	This study
Ebio-cox2r	GGGCTAATCTACTATGAAAAAAAG	55	This study
Ebio-cox3f	AGGAAATTATATTCAGGGAGTTCA	54	This study
Ebio-cobf	ATTGCCCAATCCAAAGCACC	54	This study
Ebio.cobr	TTTGGAATACTAATGTGATACCAAGTA	56	This study
Ebio-nad1r	TGAATTCGTGGTTCATATCC	54	This study
Ebio-nad1f	GATAATAAGATAAGGGCGAGAC	54	This study
Ebio-12Sf	AAAAATCAAGATAAGTCGTAACATAGT	54	This study
Ebio-12Sr	GAGGAAATGCCATATCAGGTGC	54	This study
Ebio-cox1r	AAATTCCTTTGTAAAAGTTAATACCGT	54	This study

NOTE.—All primer pairs that were used to amplify fragments of the mitochondrial genome of *Epiperipatus biolleyi* (Onychophora, Peripatidae) are listed. Long PCR products were sequenced using a primer walking strategy.

Materials and Methods

Animals, DNA Extraction, Polymerase Chain Reaction, and Sequencing

A specimen of *E. biolleyi* (Bouvier 1902) was obtained from cultures established by one of the authors (G.M.). The original specimens were collected as described previously (Mayer 2006b). DNA was extracted with the DNeasy tissue kit (Qiagen, Hilden, Germany) following manufacturers protocols. Initial polymerase chain reaction (PCR) was done with Eppendorf HotMaster *Taq* using short-range primer pairs crust-nd4f/r, crust-12Sf/r, S6(nad2), S8(cox1), S10(*cox1*), S13(*cox2*), S15(*cox2/atp6*), S18(*cox3*), S24(*nad3–nad5*), S38(*cob*), S41(*nad1*), S43(*rrnL*), and S48(*rrnS*) of previously described primer sets (Yamauchi et al. 2004; Podsiadlowski and Bartolomaeus 2005). Primer sequences and corresponding annealing temperatures are provided in table 1. PCR conditions: initial denaturation (94 °C, 1 min) was followed by 40 cycles of denaturation (94 °C, 30 s), primer annealing (45–50 °C, 30 s), and elongation (68 °C, 90 s). PCR was completed by final elongation (68 °C, 90 s). After sequencing of these initial PCR products, species-specific primer pairs were designed and combined in various ways to obtain PCR products bridging the gaps, thereby using Takara LA *Taq* (TaKaRa) and a protocol for a long range PCR; initial denaturation (94 °C, 1 min) was followed by 40 cycles of denaturation (94 °C, 30 s), primer annealing (50–55 °C, 30 s), and elongation (65 °C, 7 min). PCR was completed by final elongation (65 °C, 3 min). Resulting long PCR products were sequenced by a primer walking strategy. All sequencing was done using a Beckman–Coulter CEQ 8000 capillary sequencer and the DTCS quick start kit (Beckman–Coulter, Fullerton, CA). Sequences were analyzed and assembled using CEQ software and Bioedit (Hall 1999). Identity of protein-coding and rRNA genes was confirmed by Blast searches and by comparison with arthropod gene alignments. The tRNA search was carried out with tRNAscan-SE (Lowe and Eddy 1997) and by eye inspection of otherwise "noncoding" sequences.

Phylogenetic Analysis

Phylogenetic analysis was performed to clarify the position of Onychophora among Bilateria. Amino acid sequences from 11 protein-coding genes (all excepting *atp8* and *nad4L*, which have the shortest and highly divergent sequences) of several metazoan taxa (table 2) were aligned using ClustalW (Thompson et al. 1994), as implemented in

Table 2
Taxa Used in the Phylogenetic Analysis, Their Taxonomy and GenBank Accession Numbers

Species	Phylogenetic Position	Accession Number
Epiperipatus biolleyi	Onychophora–Peripatidae	NC_009082
Limulus polyphemus	Chelicerata–Xiphosura	NC_003057
Heptathela hangzhouensis	Chelicerata–Araneae	NC_005924
Ixodes hexagonus	Chelicerata–Acari	NC_002010
Centruroides limpidus	Chelicerata–Scorpiones	NC_006896
Nymphon gracile	Chelicerata–Pycnogonida	NC_008572
Scutigerella causeyae	Myriapoda–Symphyla	NC_008453
Scutigera coleoptrata	Myriapoda–Chilopoda	NC_005870
Lithobius forficatus	Myriapoda–Chilopoda	NC_002629
Narceus annularus	Myriapoda–Diplopoda	NC_003343
Thyropygus sp.-DVL2001	Myriapoda–Diplopoda	NC_003344
Tricholepidion gertschi	Hexapoda–Zygentoma	NC_005437
Petrobius brevistylis	Hexapoda–Archaeognatha	NC_007689
Pollicipes polymerus	Crustacea–Cirripedia	NC_005936
Daphnia pulex	Crustacea–Phyllopoda	NC_000844
Triops cancriformis	Crustacea–Phyllopoda	NC_004465
Penaeus monodon	Crustacea–Decapoda	NC_002184
Squilla empusa	Crustacea–Stomatopoda	NC_007444
Priapulus caudatus	Priapulida	NC_008557
Cephalothrix rufifrons	Nemertea	EF140788
Phoronis psammophila	Phoronida	AY368231
Terebratulina retusa	Brachiopoda	NC_000941
Haliotis rubra	Mollusca–Gastropoda	NC_005940
Katharina tunicata	Mollusca–Polyplacophora	NC_001636
Lumbricus terrestris	Annelida–Clitellata	NC_001677
Platynereis dumerilii	Annelida–Polychaeta	NC_000931
Urechis caupo	Annelida–Echiura	NC_006379
Flustrellidra hispida	Bryozoa	NC_008192
Paraspadella gotoi	Chaetognatha	NC_006083
Ascaris suum	Nematoda	NC_001327
Caenorhabditis elegans	Nematoda	NC_001328
Trichinella spiralis	Nematoda	NC_002681
Paratomella rubra	Platyhelminthes–Acoela	AY228758
Fasciola hepatica	Platyhelminthes–Trematoda	NC_002546
Paragonimus	Platyhelminthes–Trematoda	NC_002354
Taenia solium	Platyhelminthes–Cestoda	NC_004022
Leptorhynchoides thecatus	Acanthocephala	NC_006892
Balanoglossus carnosus	Enteropneusta	NC_001887
Saccoglossus kowalevskii	Enteropneusta	NC_007438
Florometra serratissima	Echinodermata–Crinoidea	NC_001878
Paracentrotus lividus	Echinodermata–Echinoidea	NC_001572
Branchiostoma lanceolatum	Chordata–Acrania	NC_001912
Xenoturbella bocki	Xenoturbellida	NC_008556
Acropora tenuis	Cnidaria–Anthozoa	NC_003522
Metridium senile	Cnidaria–Anthozoa	NC_000933

Bioedit version 7.01 (Hall 1999), under default settings. Ambiguously aligned regions were omitted using Gblocks version 0.91b (Castresana 2000), using default settings, except for changing "allowed gap positions" to "with half."

Maximum parsimony (MP) analysis was performed with PAUP* version 4.0b10 (Swofford 1993), 1,000 bootstrap replicates were performed, each with 10 replicates with random addition of taxa. Maximum likelihood (ML) analysis was done with Treefinder (Jobb 2007). We performed 10 different analyses with random taxon order for obtaining the best tree and, in addition, 100 bootstrap replicates. Model selection was done with Prottest version 1.3 (Abascal et al. 2005). According to the Akaike information criterion, the mtArt + G + I model performed best and was chosen for ML analysis. Bayesian inference (BI) was done with MrBayes version 3.1.2 (Huelsenbeck and Ronquist 2001). As the mtArt model is not implemented in this software, we used the second best scoring model, mtRev + G + I for the Bayesian analysis. BI was run with 8 chains of 1,000,000 generations, sampling a tree every 1,000 generations; the first 200 trees were omitted as burn-in and the remaining 800 were used to calculate Bayesian posterior probabilities (BPP).

Results and Discussion

Organization of the Mitochondrial Genome

The complete mitochondrial genome of *E. biolleyi* (GenBank accession number: DQ666064) consists of 14,411 bp and contains genes for 13 protein subunits and 2 ribosomal RNAs, as is usual for most bilaterian animals, whereas only 13 tRNA genes are present (fig. 1; table 3). The major noncoding region (ncr), the putative mitochondrial control region, is located between *trnK* and *trnI*. Genes are encoded on both strands. Nucleotide composition analysis of single genes reveals a strand bias (positive CG skew on [+] strand genes and negative CG skew for [−] strand genes, table 3), similar to that found in most other mitochondrial genomes of arthropods (Hassanin 2006). The onychophoran mtDNA shows only few correspondences to the gene order of the putative basal pattern in Arthropoda (Staton et al. 1997), which is represented by the mitochondrial genome of *Limulus polyphemus* (fig. 2). The priapulid mitochondrial gene order is very similar to the arthropod ground pattern because only 1 large inversion distinguishes the 2 gene orders (Webster et al. 2006), giving further support for the *Limulus* pattern as putative arthropod ground pattern. Like in arthropods, priapulids, and nematodes (Boore 1999; Webster et al. 2006), but in contrast to annelids (Valles and Boore 2006), protein-coding genes are found encoded on both strands of the mitochondrial genome in *E. biolleyi*. Compared with *L. polyphemus*, the protein-coding genes *nad5*, *nad4*, *nad4L*, *cob*, and *nad6*, as well as the ribosomal gene *rrnL* are found at deviating positions and on the opposite strand. Furthermore, the mitochondrial control region (ncr) and 11 out of 13 tRNA genes are located at relative positions different from those in *L. polyphemus* (fig. 2).

Of outstanding interest is the position of the genes *trnL(UUR)* and *nad1* in the onychophoran mtDNA, which are adjacent to each other (figs. 1 and 2). Because the same arrangement is also found in Myriapoda, Chelicerata, Tardigrada, Annelida, Echiura, and Mollusca (Boore et al. 1995, 1998), this pattern most likely represents an ancestral feature (plesiomorphy) of Panarthropoda. This is in contrast to Crustacea and Hexapoda, in which *trnL(UUR)* is placed between *cox1* and *cox2*. Our data, accordingly, confirm a previous conclusion that the position of *trnL(UUR)* between *cox1* and *cox2* represents a derived feature (autapomorphy) supporting the monophyly of Pancrustacea (Tetraconata), a taxon uniting Crustacea and Hexapoda (Boore et al. 1998) to the exclusion of Chelicerata and Myriapoda.

Although the mitochondrial gene order varies among multicellular animals and within single bilaterian taxa (e.g., Mollusca, see (Dreyer and Steiner 2004; Akasaki et al. 2006; Valles and Boore 2006), constant relative positions

Table 3
Organization of the Mitochondrial Genome of *Epiperipatus biolleyi* (Onychophora, Peripatidae)

Gene	Strand	Position Number	Size (nt)	CG-Skew	Start Codon	Stop Codon	Intergenic Nucleotides
trnI	−	1–48	48				17
trnF	−	66–134	69				−2
trnY	−	133–197	65				6
trnN	+	204–263	60				18
nad6	−	282–788	507	−0.278	ATG	TAA	a
rrnS	−	789–1392[a]	604	−0.133			a
rrnL	+	1393–2597[a]	1,205	0.698			a
nad4L	+	2598–2888	291	0.530	ATG	TAG	0
nad4	+	2889–4244	1,356	0.373	ATG	TAG	28
trnW	−	4273–4338	66				30
trnQ	−	4369–4427	59				−7
nad2	+	4421–5422	1,002	0.598	ATG	TAA	0
cox1	+	5423–6952	1,530	0.291	ATG	TAA	1
cox2	+	6954–7631	678	0.371	ATG	TAA	43
atp8	+	7675–7833	159	0.419	ATT	TAA	−7
atp6	+	7827–8504	678	0.326	ATG	TAA	−1
cox3	+	8504–9289	786	0.301	ATG	TAA	1
nad3	+	9291–9642	352	0.357	ATG	T–	18
trnM	+	9661–9720	60				10
cob	−	9731–10870	1,140	−0.274	ATG	TAA	2
nad1	−	10873–11799	927	−0.225	ATA	TAA	−24
trnL(UUR)	−	11776–11845	70				9
trnC	+	11855–11916	62				0
trnH	+	11917–11976	60				16
nad5	+	11993–13672	1,713	0.320	ATG	TAG	−6
trnE	−	13667–13718	52				6
trnD	+	13725–13792	68				16
trnK	+	13809–13871	63				540
Noncoding		13872–14411	540				

[a] Start and end positions of rRNA genes not determinable by sequence data alone.

of certain protein-coding and ribosomal genes are found throughout a wide range of animal groups. For example, a block of *nad5*, *nad4*, and *nad4L*, coding on the same strand (sometimes interrupted by tRNA genes, fig. 2), is found in organisms as diverse as arthropods (Staton et al. 1997), molluscs (Boore and Brown 1994), brachiopods (Helfenbein et al. 2001), nematodes (Lavrov and Brown 2001), priapulids (Webster et al. 2006, 2007), and most deuterostomes (Boore 1999). Due to its conserved nature, this pattern might have been inherited from a common bilaterian ancestor. The same holds true for a block consisting of *cox1*, *cox2*, *atp8*, *atp6*, and *cox3*, all coding on the same strand and for adjacent *nad6* and *cob* on the same strand (with the exception of Deuterostomia) and adjacent *rrnL* and *rrnS* on the same strand (fig. 2). However, in *E. biolleyi* 3 of these 4 conserved blocks of adjacent genes are disrupted by other protein-coding and rRNA genes (figs. 1 and 2). Regarding all the mentioned outgroup taxa, we suggest that most (if not all) changes in the gene order of this species are specific to Onychophora or at least to an onychophoran subtaxon including *E. biolleyi*. The greatly modified arrangement of the mitochondrial genes in *E. biolleyi*, thus, does not reflect the overall conservative nature of the onychophoran anatomy. However, studies on the mitochondrial genomes throughout the Onychophora might disclose a diversity of gene orders useful for phylogenetic considerations. Such diversity is likely because the major onychophoran subgroups have a long history and have been separated for at least 100 Myr since the breakup of Gondwana (Brinck 1957; Monge-Najera 1995; Reid 1996).

A Unique Set of Transfer RNA Genes

Although representatives of Porifera (Lavrov et al. 2005), Placozoa (Dellaporta et al. 2006), and Choanoflagellata (Burger et al. 2003) bear 24–25 tRNA species in their mitochondrial genomes, almost all other animals have a set of 22 tRNAs (fig. 3). Among these 22 tRNAs, 2 species (recognizing the codons UUR and CUN) transfer leucine and 2 species (recognizing AGN and UCN) transfer serine, whereas all the other amino acids are handled by only 1 tRNA species each. Only few metazoan taxa show less than 22 tRNA genes in their mitochondrial genomes. Chaetognatha and Cnidaria represent 2 extreme examples of tRNA gene reduction in their mtDNA because they retained only 1 (tRNA-Met; Helfenbein et al. 2004) or 2 tRNA species, (tRNA-Met and tRNA-Trp; Pont-Kingdon et al. 1998; Medina et al. 2006), respectively. A permanent import of the missing (but essential) tRNA molecules from the cytosol into the mitochondria must be assumed in each case of the tRNA reduction and, indeed, has been demonstrated in some plants (Dietrich et al. 1992; Delage et al. 2003).

Despite an extensive search with the tRNAscan-SE software (Lowe and Eddy 1997) and by eye inspection, we detected only 13 tRNA genes in the mitochondrial genome of *E. biolleyi* (figs. 3 and 4). Notably, all of the recognized tRNAs show anticodons corresponding to the 2-fold degenerate codons (2-tRNAs), whereas all the missing tRNA species would pair with the 4-fold degenerate codons (4-tRNAs). We exclude the possibility that the missing tRNA genes might be located on any additional

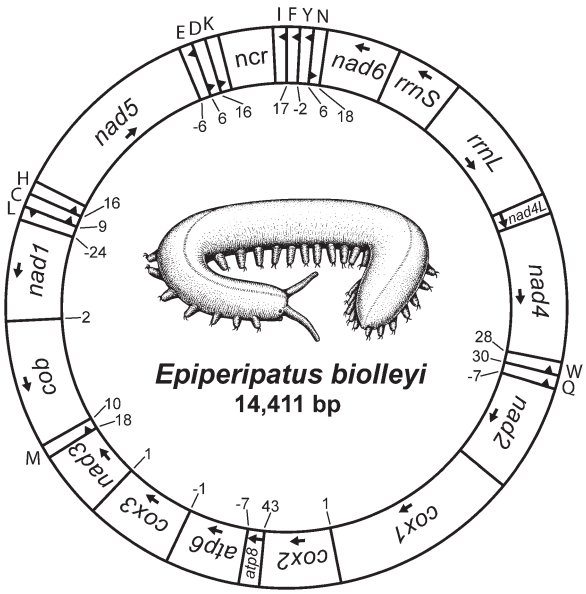

FIG. 1.—Organization of the mitochondrial genome of *Epiperipatus biolleyi* (Onychophora, Peripatidae). Transfer RNA genes are depicted by their corresponding 1-letter amino acid code. Numbers at gene borders indicate the number of noncoding (positive values) or overlapping nucleotides (negative values) between 2 adjacent genes. Arrows and arrowheads indicate orientation of genes either on (+) strand (clockwise) or (−) strand (counterclockwise). Gene sizes only roughly approximated; ncr, noncoding or putative mitochondrial control region.

genomic elements in the mitochondria of *E. biolleyi*, for example, as described in the nematode *Globodera pallida* (Gibson et al. 2007). It is very unlikely that exactly this specific set of genes has been removed from the major mtDNA molecule by chance. Instead, we suggest that these genes have been completely obliterated from the *E. biolleyi* mtDNA and are substituted by corresponding tRNAs encoded by nuclear DNA. In case of such complete reduction, the missing tRNA species have to be imported permanently from the cytosol into the mitochondria in order to facilitate the translation of essential proteins in these organelles.

The exclusive reduction of 4-tRNAs and the retention of a complete set of 2-tRNAs in the mtDNA of *E. biolleyi* (figs. 3 and 4) raise the fundamental question of whether there are any differences in the transmembrane transport or recognition mechanisms between the 2 tRNA groups. Because an accidental reduction of the one group and a simultaneous retention of the other seem unlikely, we suggest that there must be a distinguishing feature between them, although it has not been discovered yet. Detailed studies on the pathways of tRNA import mechanisms from the cytoplasm into the mitochondria are desirable to clarify this issue.

Phylogenetic Analysis

We performed a phylogenetic analysis with concatenated amino acid sequences of 11 protein-coding genes from 43 bilaterian species and 2 cnidarians as an outgroup (fig. 5). In the resulting ML tree (the best tree from a likelihood analysis), Bilateria are divided into 4 large groups: 1) Deuterostomia + *Xenoturbella*, 2) Arthropoda + Onychophora + Priapulida (or Ecdysozoa, excluding Nematoda), 3) Annelida, Mollusca, Nemertea, Brachiopoda, and Phoronida (Lophotrochozoa, excluding Platyhelminthes and Acanthocephala), and 4) an assemblage of long-branching taxa (Platyhelminthes, Acanthocephala, Nematoda, Acoela, Chaetognatha, and Bryozoa). The monophyly of the first

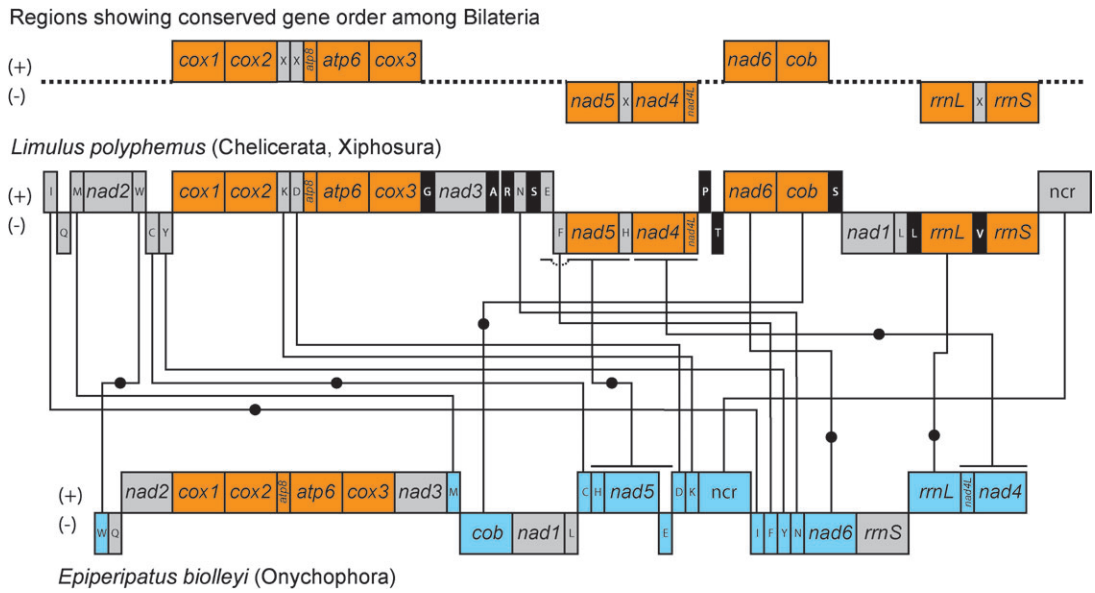

FIG. 2.—Comparison of linearized mitochondrial gene orders in Bilateria, Arthropoda, and *Epiperipatus biolleyi* (Onychophora, Peripatidae). As a representative of the ground pattern of Arthropoda, the mitochondrial gene order of *Limulus polyphemus* (Chelicerata, Xiphosura) has been chosen (see text). Transfer RNA genes are depicted by their corresponding 1-letter amino acid code. Genes shaded in blue have different positions in *E. biolleyi* and *L. polyphemus*. Transfer RNA genes missing in *E. biolleyi* are shaded in black in *L. polyphemus*. Regions shaded in red indicate gene blocks that are conserved in many triploblastic animals and, thus, might represent the ground pattern of Bilateria. Black circles indicate switches in gene position between (+) and (−) mtDNA strands.

	U			C			A			G		
U	UUU	Phe	F	UCU	Ser	S	UAU	Tyr	Y	UGU	Cys	C	U
	UUC	Phe	F	UCC	Ser	S	UAC	Tyr	Y	UGC	Cys	C	C
	UUA	Leu	L	UCA	Ser	S	UAA	stop	*	UGA	Trp	W	A
	UUG	Leu	L	UCG	Ser	S	UAG	stop	*	UGG	Trp	W	G
C	CUU	Leu	L	CCU	Pro	P	CAU	His	H	CGU	Arg	R	U
	CUC	Leu	L	CCC	Pro	P	CAC	His	H	CGC	Arg	R	C
	CUA	Leu	L	CCA	Pro	P	CAA	Gln	Q	CGA	Arg	R	A
	CUG	Leu	L	CCG	Pro	P	CAG	Gln	Q	CGG	Arg	R	G
A	AUU	Ile	I	ACU	Thr	T	AAU	Asn	N	AGU	Ser	S	U
	AUC	Ile	I	ACU	Thr	T	AAC	Asn	N	AGC	Ser	S	C
	AUA	Met	M	ACA	Thr	T	AAA	Lys	K	AGA	Ser	S	A
	AUG	Met	M	ACG	Thr	T	AAG	Lys	K	AGG	Ser	S	G
G	GUU	Val	V	GCU	Ala	A	GAU	Asp	D	GGU	Gly	G	U
	GUC	Val	V	GCC	Ala	A	GAC	Asp	D	GGC	Gly	G	C
	GUA	Val	V	GCA	Ala	A	GAA	Glu	E	GGA	Gly	G	A
	GUG	Val	V	GCG	Ala	A	GAG	Glu	E	GGG	Gly	G	G

FIG. 3.—Degenerate genetic code of the invertebrate mitochondrial DNA (code no. 5) and its coverage by the tRNA genes in *Epiperipatus biolleyi* (Onychophora, Peripatidae). Each codon is accompanied by its corresponding amino acid (depicted by a 3-letter code). Codons represented in black boxes lack a corresponding tRNA gene in the mitochondrial genome of *E. biolleyi*.

3 groups is in accordance with the most common view of the metazoan phylogeny, built upon the analyses of various molecular data sets (Halanych 2004; Philippe et al. 2005; Mallatt and Giribet 2006; Webster et al. 2007). The fourth group, however, might be an artificial assemblage due to the long-branch attraction. Especially Platyhelminthes, Acanthocephala, and Nematoda show branches, which are extremely long in comparison to the other lophotrochozoan or ecdysozoan taxa (fig. 5). Although some of these long-branching taxa are usually included into one of the large bilaterian groups (Platyhelminthes and Acanthocephala into Lophotrochozoa and Nematoda into Ecdysozoa) (Halanych 2004; Philippe et al. 2005; Mallatt and Giribet 2006), the phylogenetic positions of Bryozoa, Chaetognatha, and Acoela are still discussed controversially. As these taxa cannot be included in any of the major taxa with certainty, at least some of them might represent basal branches in the bilaterian tree (Haszprunar 1996; Ruiz-Trillo et al. 2002; Telford et al. 2003; Helfenbein et al. 2004; Papillon et al. 2004; Marletaz et al. 2006; Matus et al. 2006; Waeschenbach et al. 2006).

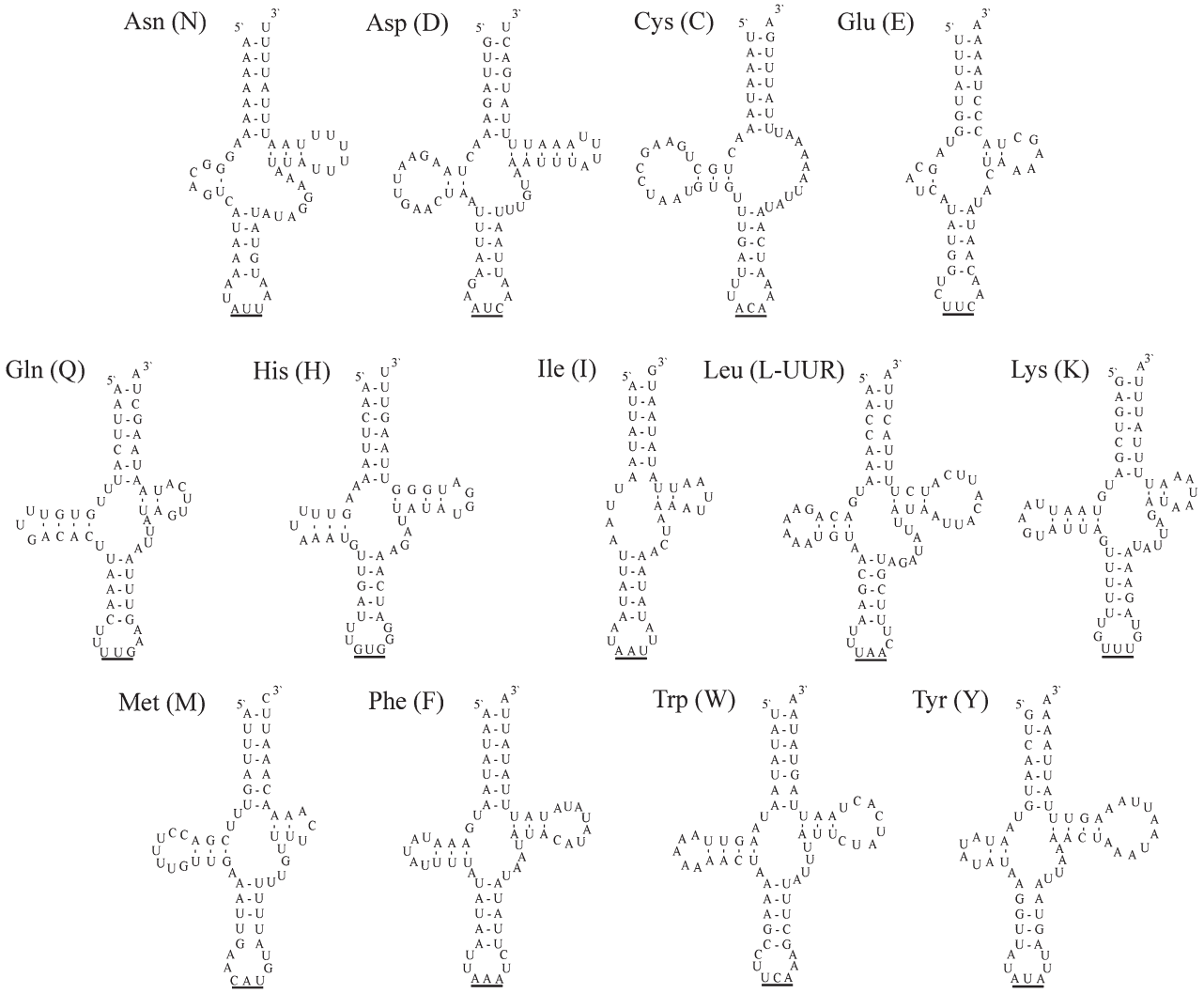

FIG. 4.—Plots of the putative secondary structures of mitochondrial transfer RNAs from *Epiperipatus biolleyi* (Onychophora, Peripatidae). Anticodon sequence is underlined.

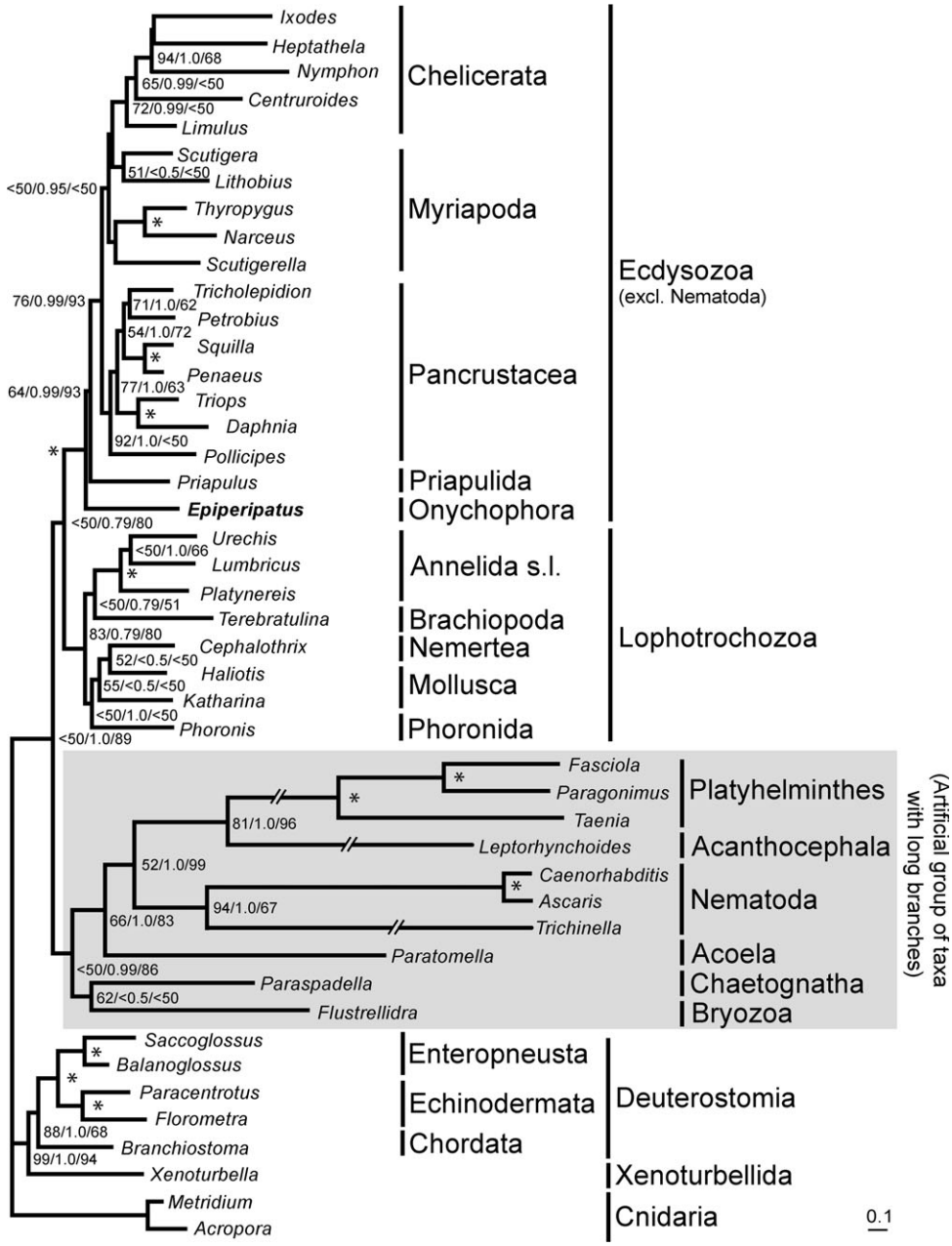

FIG. 5.—Bilaterian relationships: best tree from the ML analysis (−ln = 90078). Concatenated amino acid alignments from 11 protein-coding genes were used for the analysis (from *Paratomella* and *Cephalothrix*, only a subset of sequences was available; mtArt + G + I). Numbers next to nodes represent 1) support from ML bootstrapping (%), 2) BPP, and 3) Maximum Parsimony bootstrap percentage (from left to right); asterisks indicate that all 3 numbers have reached the maximum values (100/1.0/100). Branch lengths reflect substitutions per site (scale bar). See table 2 for full species names and GenBank accession numbers of sequence data.

The assemblage of Onychophora, Priapulida, and Arthropoda is strongly supported by high maximum likelihood bootstrap percentage (MLBP: 100) and maximum parsimony bootstrap percentage (MPBP: 100), as well as by high BPP (BPP: 1.0), whereas the sister group relationship between these 3 groups is unresolved. Furthermore, there is only a weak support for arthropod monophyly (MPBP: 76, BPP: 1.0, MPBP: <50), but there is no hint for an affinity of Onychophora to Chelicerata, as proposed recently on the basis of the brain anatomy (Strausfeld et al. 2006). Among arthropods, there is a good support for a monophyletic Pancrustacea in both ML and BI analyses (MPBP: 92, BPP: 1.0, MPBP: <50), a moderate support for a monophyletic Chelicerata (MPBP: 72, BPP: 0.99, MPBP: <50), and no support for the monophyly of Myriapoda. The clustering of Myriapoda and Chelicerata only got support from BI (MPBP: <95, BPP: 0.95, MPBP: <50).

In summary, our data provide a robust support for a close relationship of onychophorans, arthropods, and priapulids. A close relationship of priapulids and arthropods has been demonstrated recently by an analysis of the mitochondrial gene order and by a phylogenetic analysis using

expressed sequence tag data from various eukaryotes (Webster et al. 2006, 2007). The inclusion of an onychophoran in our analysis of the mtDNA sequences did not improve the resolution within the Ecdysozoa, possibly because of a lack of data from Tardigrada, Nematomorpha, and other nemathelminth taxa. Moreover, the long branches of the nematode mitochondrial genomes led to an artificial position of Nematoda far away from the other ecdysozoan taxa. However, the placement of Onychophora close to arthropods indicates that the analyzed sequences of the protein-coding genes in *E. biolleyi* are less derived than the strongly rearranged mitochondrial gene order. An increased taxon sampling for Onychophora and other Ecdysozoa, therefore, might contribute to a better resolution of the basal arthropod and ecdysozoan relationships.

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