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Mitochondrial protein phylogeny joins myriapods with chelicerates

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The animal phylum Arthropoda is very useful for the study of body plan evolution given its abundance of morphologically diverse species and our profound understanding of *Drosophila* development[1]. However, there is a lack of consistently resolved phylogenetic relationships between the four extant arthropod subphyla, Hexapoda, Myriapoda, Chelicerata and Crustacea. Recent molecular studies[2–4] have strongly supported a sister group relationship between Hexapoda and Crustacea, but have not resolved the phylogenetic position of Chelicerata and Myriapoda. Here we sequence the mitochondrial genome of the centipede species *Lithobius forficatus* and investigate its phylogenetic information content. Molecular phylogenetic analysis of conserved regions from the arthropod mitochondrial proteome yields highly resolved and congruent trees. We also find that a sister group relationship between Myriapoda and Chelicerata is strongly supported. We propose a model to explain the apparently parallel evolution of similar head morphologies in insects and myriapods.

The basal diversification of arthropod lineages, which date back into the late Cambrian period is still unclear. Morphological analyses[5,6] all suggest a monophyletic Arthropoda within which insects and myriapods are most closely related. Controversy, however, continued over whether insects, myriapods and crustaceans form a second major subclade, Mandibulata, on the basis of the shared derived possession of mandibles[5] or whether crustaceans are a sister group to chelicerates on the basis of the occurrence of biramous appendages in representatives of both groups[6]. Several independent molecular studies provided strong support for arthropod monophyly, a monophyletic Hexapoda, Myriapoda and Chelicerata, and, most significantly, a sister group relationship between insects and crustaceans (Pancrustacea) (for a review see ref. 7). Although they ruled out the possibility of insect/myriapod or crustacean/chelicerate sister clades, previous molecular studies did not resolve relationships between myriapods, chelicerates and Pancrustacea[2–4]. Mitochondrial gene order rearrangements were initially interpreted to support a monophyletic Mandibulata[8], but were later re-interpreted to further corroborate the Pancrustacea clade[2].

Complete mitochondrial genome sequences can be informative at deep phylogenetic levels[9]. We therefore investigated their potential use for arthropod phylogeny. As examples of mitochondrial genomes are known from all arthropod subphyla except myriapods, we determined the complete mitochondrial genome sequence of the centipede *Lithobius forficatus*. The *Lithobius* mitochondrial genome is 15,437 base pairs (bp) (details will be given elsewhere). Gene content and arrangement correspond to that of conservatively evolving arthropod mitochondrial genomes with two exceptions. Most crustacean and insect mitochondrial genomes differ from *Lithobius* with regard to the position of the transfer RNA$^{Leu(UUR)}$ gene, which in crustaceans is located between the COXI and COXII genes and in *Lithobius* between the tRNA$^{Leu(CUN)}$ and ND1 genes. This is consistent with the previous demonstration that the COXI/tRNA$^{Leu(UUR)}$/COXII arrangement is a synapomorphy of the Pancrustacea[2].

Another difference concerns the position of the tRNA^{Cys} gene, which in most arthropods resides between tRNA^{Trp} and tRNA^{Tyr} (Fig. 1), but in *Lithobius* it lies within the non-coding region of the

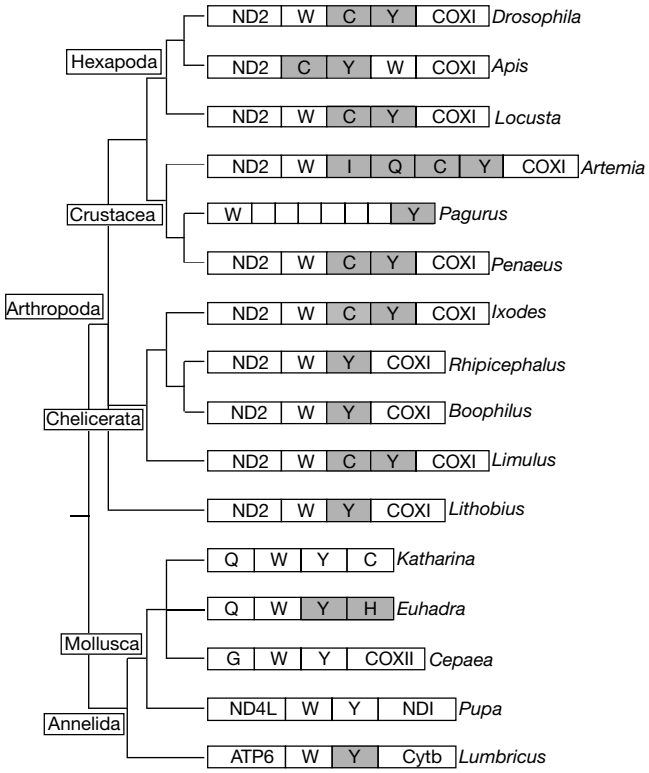


Figure 1 Phylogenetic distribution of tRNA^{Cys} arrangements in arthropod mitochondrial genomes. The relative location of tRNA^{Trp} (W), tRNA^{Cys} (C) and tRNA^{Tyr} (Y) is shown for representative arthropod and outgroup species with similar arrangements. Multiple coding units separating tRNA^{Trp} and tRNA^{Tyr} in *Pagurus* are indicated by boxes. Transcription units in clear boxes code from left to right, those in shaded boxes code from right to left. The mollusc *Euhadra herklotsi* is the only non-arthropod species known so far in which tRNA^{Trp} and tRNA^{Tyr} are neighbours in opposite coding orientation, as in *Lithobius*. In a few non-arthropod species tRNA^{Trp} and tRNA^{Tyr} are next to each other, although in the same coding orientation. Re-examining non-annotated regions in published mitochondrial genome sequences, we found that the annelid species *Lumbricus terrestris* has coding probability for a second tRNA^{Tyr}, which could result in a *Lithobius*-like tRNA^{Trp} and tRNA^{Tyr} arrangement (U.W.H., unpublished observation). This possibility, however, awaits confirmation by tRNA transcript analysis.

mitochondrial genome. Further exceptions are the honeybee *Apis mellifera*, the decapod *Pagurus longicarpus* and the tick species *Rhipicephalus sanguineus* and *Boophilus microplus*; these, however, represent lineages with exceptionally high rearrangement rates. In *Lithobius*, the $tRNA^{Trp}$ and $tRNA^{Tyr}$ genes lie directly next to each other in opposite coding directions, so we asked whether this or the $tRNA^{Trp}/tRNA^{Cys}/tRNA^{Tyr}$ arrangement is ancestral for arthropods. No non-arthropod mitochondrial genome is known to exhibit a $tRNA^{Trp}/tRNA^{Cys}/tRNA^{Tyr}$ arrangement, but the *Lithobius*-like $tRNA^{Trp}/tRNA^{Tyr}$ arrangement also appears to be rare (Fig. 1)[10]. The inference of character state polarity is further confounded by considerable positional variation of the respective tRNAs across species. The tick species *Rhipicephalus sanguineus* and *Boophilus microplus* share the $tRNA^{Trp}/tRNA^{Tyr}$ arrangement with *Lithobius*. This is due to parallel evolution, as indicated by the $tRNA^{Trp}/tRNA^{Cys}/tRNA^{Tyr}$ arrangement shared by the closely related tick species *Ixodes hexagonus* and the more distantly related horseshoe crab *Limulus polyphemus*. The $tRNA^{Typ}/tRNA^{Cys}/tRNA^{Tyr}$ arrangement is therefore with high certainty ancestral for chelicerates (Fig. 1). Thus, parallel evolution and the high evolutionary mobility of these particular tRNAs make the use of these tRNAs unreliable for deep-level cladistic analysis.

To explore the phylogenetic signal in mitochondrial protein sequences, we generated an alignment (18P2560) 2,560 amino acid sites long from conserved regions of 12 mitochondrial proteins from *Lithobius* and additional arthropod taxa. Annelid, mollusc and vertebrate species were added for outgroup comparison. Pairwise relative rate tests revealed that several species including the locust *Locusta migratoria*, the decapod *Pagurus longicarpus*, the branchiopod species *Artemia franciscana*, the tick species *Ixodes hexagonus* and *Lithobius* exhibited significantly accelerated substitution rates. Furthermore, four species significantly departed from the average amino-acid composition in the alignment (Table 1). Nonetheless, maximum-likelihood mapping indicated a high phylogenetic information content in the alignment (Fig. 2a). Tree reconstruction with maximum-parsimony, distance and maximum-likelihood methods converged on a number of strongly supported clades (Fig. 3). Well established clades such as monophyletic Vertebrata, Eutrochozoa (*Lumbricus* and *Katharina*), Arthropoda, Decapoda (*Pagurus* and *Penaeus*) and Branchiopoda (*Artemia* and *Daphnia*), Chelicerata and Hexapoda were recovered with high branch-support values. Most basal nodes within arthropods were also consistently resolved. Decapods were strongly supported as a sister clade to insects, suggesting a paraphyletic Crustacea as recently noted[11,12]. Although the maximum-likelihood tree included a monophyletic Pancrustacea, branch-support analysis yielded little resolution with regard to the position of the Branchiopoda. The most striking result was a strong support for a sister group relationship between the myriapods and chelicerates with branch-support values equalling those of well established clades such as Chelicerata or Hexapoda.

To assess the impact of alignment site choice, we repeated tree estimation with alignments built from more stringently selected protein regions. The shortest alignment included 1,528 sites (18P1528), which exhibited an average maximum-likelihood distance two times lower than in the 18P2560 alignment, demonstrating considerable restriction to more slowly evolving sites (Table 1). This was associated with improved homogeneity of amino acid composition across taxa (Table 1). Maximum-likelihood mapping revealed a slight decrease in phylogenetic information content, which, in part, must be due to the reduction of sequence sample size (Fig. 2). Tree estimation yielded well resolved topologies, which were largely congruent with the results obtained with the 18P2560 alignment, the only difference being increased support for a monophyletic Pancrustacea (Fig. 3). These results suggest that the high resolution in the mitochondrial trees derives from the most slowly evolving protein regions. The consistent strong support for a monophyletic Myriapoda/Chelicerata demonstrates a robust phylogenetic signal for this clade in the mitochondrial proteins.

The support for a monophyletic Pancrustacea is conspicuously lower in the mitochondrial trees than in the nuclear ribosomal trees[4], but the opposite applied to the support for the chelicerate/myriapod clade. This discrepancy is probably due to the combined effect of differences in taxon sampling and gene-specific fluctuations in the conservation of phylogenetic signal. Indeed, the support for a monophyletic Pancrustacea is much stronger in 28S than in the 18S nuclear ribosomal DNA sequences[3].

It is essential to include closely related outgroup species to root the basal relationships of a phylogeny correctly. Recent studies suggest arthropods to be part of a higher clade, Ecdysozoa, of moulting animals including nematodes[13]. Although a monophyletic Ecdysozoa is not entirely consistently supported[14,15], we considered the possibility that nematode mitochondrial sequences could

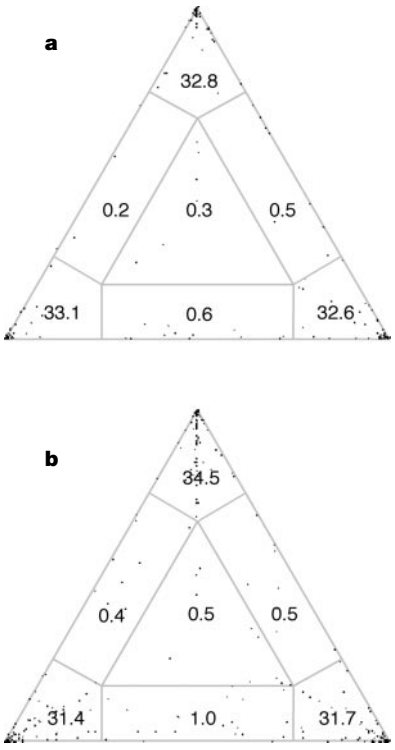
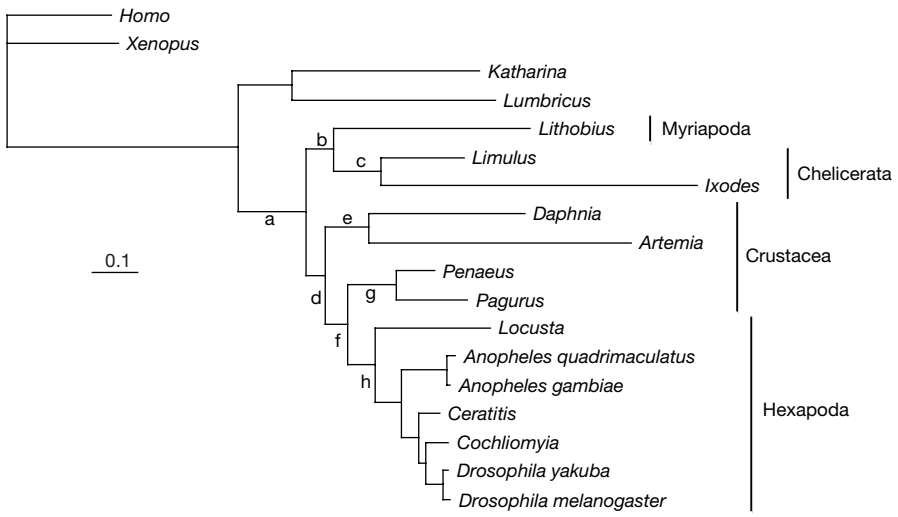


Figure 2 Visualization of phylogenetic information content by maximum likelihood mapping. **a**, The 18P2560 alignment. **b**, The 18P1528 alignment. Maximum-likelihood trees for the total of 3,060 possible quartet combinations are mapped according to ref. 16. Corner regions contain the partition of fully resolved quartet trees, lateral regions contain the partition of partially resolved quartet trees, and the centre region contains the partition of completely unresolved quartet trees. For both alignments, more than 95% of all possible quartets are fully resolved, indicating high phylogenetic information content.

Table 1 Comparison of multiple alignment features

	18P2560	18P1528
Percentage of constant sites	31.7	46.1
α	0.46	0.39
Average maximum-likelihood distance	0.77	0.33
Species with significant amino-acid bias	*Daphnia* *Ixodes* *Xenopus* *Homo*	*Ixodes*

Comparison of long (18P2560) and short (18P1528) multiple alignments with respect to percentage of constant sites, rate heterogeneity across sites as indicated by the α parameter, average maximum-likelihood distance (substitutions per site) in pairwise species comparisons and partition of species with significantly deviating amino acid composition.



	a	b	c	d	e	f	g	h
MP	100/97	83/84	65/54	40[1]/92	70/92	57/38[4]	100/100	91/98
NJ	100/100	100/96	100/81	17[2]/88	99/86	100/97	100/100	100/100
QP	91/94	99/99	100/99	46[3]/57	99/97	95/84	100/97	99/96

Figure 3 Phylogram of best maximum-likelihood tree with 18P2560 alignment (ln(likelihood) = −42925.32). Bar represents 0.1 substitutions per site. Branches with letters have branch support values (BP) given below the tree for maximum parsimony (MP), neighbour-joining (NJ) and the maximum-likelihood-based quartet puzzling method (QP)[28]. Left numbers refer to 18P2560 alignment, right numbers to 18P1528 alignment. Superscript numbers indicate branches that are not included in bootstrap majority rule consensus trees: 1, Branchiopoda placed at the base of arthropods with BP = 57; 2, Branchiopoda placed at the base of the arthropods with BP = 82; 3, Branchiopoda placed at the base of the arthropods with BP = 53; 4, monophyletic Crustacea supported with BP = 53.

represent a more adequate outgroup than the choice of the protostome and deuterostome sequences. Although nematode mitochondrial sequences are problematic for phylogeny reconstruction owing to dramatically accelerated substitution rates, a chelicerate/myriapod sister group clade was robust when nematodes were included in tree estimation (see the Supplementary Information). Nonetheless, it will be important to examine the effect of slowly evolving sequences from ecdysozoan taxa, particularly onychophorans, on the rooting of the arthropod mitochondrial tree.

A close link between myriapods and chelicerates has never, to our knowledge, been considered from a morphological perspective. We note, however, that the same grouping is tentatively supported in various analyses of nuclear ribosomal genes[4,16,17]. In addition, recent analyses of arthropod haemocyanin and *Hox* gene sequences point to a close relationship between chelicerates and myriapods[18,19]. Independent molecular data thus provide consistent support for a chelicerate/myriapod sister group relationship, arguing against a monophyletic Mandibulata. Future research is needed to examine the possibility of morphological synapomorphies for a chelicerate/myriapod clade.

Another important question is how similar head appendage arrays evolved in insects and myriapods, given the closer relationship of the latter to chelicerates. One possible scenario is that head segmentation and appendage differentiation in extant myriapods, insects and crustaceans is ancestral for arthropods. Chelicerate head morphology must then have evolved from a myriapod-like head morphology. Such evolutionary transformation is not inconceivable given that *Drosophila* head appendages have retained the potential to develop into primitive leg structures[20]. Nonetheless, the presence of largely undifferentiated postoral head appendages in primitive representatives of Trilobites and other extinct arthropods argues against this idea[21]. This is more consistent with the alternative possibility that the arthropod ancestor possessed a head with largely undifferentiated appendages from which myriapod and insect head morphologies evolved in parallel. Recent comparisons of *Hox* gene expression revealed that arthropods share mechanisms of homeotic control of head segment specification even between groups as divergent as chelicerates and insects[22,23]. This implies that the myriapod and insect heads evolved from a common developmental grid despite an apparently more distant phylogenetic relationship. Their similarity may thus be the result of shared developmental constraints and parallel functional adaptation. Future comparative studies of arthropod head patterning should therefore reveal more similarities between the evolutionarily more closely related crustaceans and insects than myriapods. □

Methods

Sequence analysis

Total DNA was isolated from a specimen of the centipede species *Lithobius forficatus* collected in the garden of the Zoological Department of the University of München (Germany). A 538-bp portion of the large subunit ribosomal RNA gene (16S rDNA) was amplified by standard polymerase chain reaction (PCR) using universal primers (16SA: 5′-CGC CTG TTT ATC AAA AAC AT-3′; 16SB: 5′-CCG GTT GAA CTC AGA TCA-3′) and sequenced. The complete genome was then amplified using the Expand Long Template PCR System (Roche Biochemicals) with the primers HPK16Saa (32mer), 5′-ATG CTA CCT TTG CAC RGT CAA GAT ACY GCG GC-3′, and HPK16Sbb (34 mer), 5′-CTT ATC GAY AAA AAA GWT TGC GAC CTC GAT GTT G-3′. Cycling settings included one cycle of 2 min at 92 °C for initial denaturation, followed by 30 cycles of 10-s denaturation at 92 °C, 30-s annealing at 65 °C, and 13-min elongation at 68 °C. During the last 20 cycles, elongation times were increased for 20 s per cycle. The reaction was finished with a 20-min final elongation step at 68 °C. A single 15.5-kb-long PCR fragment was purified and used as a template for secondary PCR reactions. *Eco*RI or *Xba*I restriction fragments were cloned and sequenced in both directions on an ABI310 automated sequencer (Perkin Elmer) Overlaps between restriction fragment clones were confirmed by direct sequencing of PCR products spanning these regions. Protein-coding genes were identified by similarity of predicted amino-acid sequence with known mitochondrial protein sequences. The annotated sequence has been submitted to the EMBL data bank (accession number: AJ270997).

Phylogenetic analysis

Complete mitochondrial genome sequences were retrieved from GenBank from the following arthropod species: fruitfly *Drosophila yakuba* (X03240), fruitfly *Drosophila*

melanogaster (U37541), mosquito *Anopheles quadrimaculatus* (L04272), mosquito *Anopheles gambiae* (L20934), medfly *Ceratitis capitata* (CCA242872), *Cochliomyia hominivorax* (AF260826), locust *Locusta migratoria* (X80245), honey bee *Apis mellifera* (L06178), brine shrimp *Artemia franciscana* (X69067), water flea *Daphnia pulex* (AF117817), shrimp *Penaeus monodon* (AF217843), hermit crab *Pagurus longicarpus* (AF150756), horseshoe crab *Limulus polyphemus* (AF216203), tick *Ixodes hexagonus* (AF081828), tick *Rhipicephalus sanguineus* (AF081829). For outgroup comparison, sequences were retrieved for the annelid *Lumbricus terrestris* (U24570), the mollusc *Katharina tunicata* (U09810), the nematodes *Caenorhabditis elegans* (X54252), *Ascaris suum* (X54253), *Trichinella spiralis* (AF293969) and *Onchocerca volvulus* (AF015193), and the vertebrate species *Homo sapiens* (J01415) and *Xenopus laevis* (M10217). Additional sequences were analysed for gene arrangements: *Boophilus microplus* (AF110613), *Euhadra herklotsi* (Z71696), *Cepaea nemoralis* (U23045) and *Pupa strigosa* (NC_002176).

Multiple alignments were prepared for all putative protein sequences using Clustal W[24] at default settings. Consistent with previous studies[25], preliminary analyses revealed obvious tree estimation artefacts due to extremely accelerated substitution rates or protein composition bias in the nematode species, the honeybee *Apis mellifera* and the tick species *Rhipicephalus sanguineus* and *Ixodes hexagonus*. With the exception of *Ixodes hexagonus* all of these taxa were therefore excluded from further analyses, reducing the total number of species considered to 18. Sequence alignment was repeated and inspected by eye for sufficient levels of sequence conservation, which resulted in the exclusion of the ATPase 8 gene (see Supplementary Information for single protein alignments). We used Gblocks[26] to extract regions of defined sequence conservation from the gene specific alignments and generate a single file of concatenated conserved regions. Default settings yielded the 18P2560 alignment. Modified parameter settings for generating the 18P1528 alignment were: minimum number of sequences for a conserved position: 15; maximum number of contiguous nonconserved positions: 2; minimum length of a block after gap cleaning: 5. Alignments can be retrieved from the EBI webserver (ftp://ftp.ebi.ac.uk/pub/databases/embl/align) under accession numbers ALIGN_000111 and ALIGN_000112. Maximum-likelihood mapping was carried out as described in ref. 16. Pairwise relative rate tests were carried out with the Hy-Phy program package[27]. Protein composition homogeneity test and maximum likelihood tree estimation was carried out using the TREE-PUZZLE program[28] applying the mtREV24 sequence evolution model for mitochondrial proteins[29] and a four rate approximated gamma distribution of among-site rate heterogeneity. Maximum-likelihood trees were determined by likelihood ratio tests between competing topologies. Maximum-parsimony tree reconstruction and neighbour-joining analysis with Dayhoff PAM matrix distances were performed using the respective algorithms implemented in Phylip 3.5 (ref. 30). Non-parametric bootstrapping analyses were based on 100 replicate data sets.

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Supplementary information is available from *Nature*'s World-Wide Website (http://www.nature.com) or as paper copy from the London editorial office of *Nature*.

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Arthropod phylogeny based on eight molecular loci and morphology

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The interrelationships of major clades within the Arthropoda remain one of the most contentious issues in systematics, which has traditionally been the domain of morphologists[1,2]. A growing body of DNA sequences and other types of molecular data has revitalized study of arthropod phylogeny[3–7] and has inspired new considerations of character evolution[8,9]. Novel hypotheses such as a crustacean–hexapod affinity[4,10–12] were based on analyses of single or few genes and limited taxon sampling, but have received recent support from mitochondrial gene order[13], and eye and brain ultrastructure and neurogenesis[14,15]. Here we assess relationships within Arthropoda based on a synthesis of all well sampled molecular loci together with a comprehensive data set of morphological, developmental, ultrastructural and gene-order characters. The molecular data include sequences of three nuclear ribosomal genes, three nuclear protein-coding genes, and two mitochondrial genes (one protein coding, one ribosomal). We devised new optimization procedures[16,17] and constructed a parallel computer cluster with 256 central processing units[18] to analyse molecular data on a scale not previously possible. The optimal 'total evidence' cladogram supports the crustacean–hexapod clade, recognizes pycnogonids as sister to other euarthropods, and indicates monophyly of Myriapoda and Mandibulata.

Based on morphological evidence, neontological[1,5,6] and palaeontological[2] hypotheses regarding deep divergences within Arthropoda differ in the monophyly of Mandibulata (arthropods with mandibles: crustaceans, myriapods and hexapods) versus