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A congruent solution to arthropod phylogeny: phylogenomics, microRNAs and morphology support monophyletic Mandibulata

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While a unique origin of the euarthropods is well established, relationships between the four euarthropod classes—chelicerates, myriapods, crustaceans and hexapods—are less clear. Unsolved questions include the position of myriapods, the monophyletic origin of chelicerates, and the validity of the close relationship of euarthropods to tardigrades and onychophorans. Morphology predicts that myriapods, insects and crustaceans form a monophyletic group, the Mandibulata, which has been contradicted by many molecular studies that support an alternative Myriochelata hypothesis (Myriapoda plus Chelicerata). Because of the conflicting insights from published molecular datasets, evidence from nuclear-coding genes needs corroboration from independent data to define the relationships among major nodes in the euarthropod tree. Here, we address this issue by analysing two independent molecular datasets: a phylogenomic dataset of 198 protein-coding genes including new sequences for myriapods, and novel microRNA complements sampled from all major arthropod lineages. Our phylogenomic analyses strongly support Mandibulata, and show that Myriochelata is a tree-reconstruction artefact caused by saturation and long-branch attraction. The analysis of the microRNA dataset corroborates the Mandibulata, showing that the microRNAs miR-965 and miR-282 are present and expressed in all mandibulate species sampled, but not in the chelicerates. Mandibulata is further supported by the phylogenetic analysis of a comprehensive morphological dataset covering living and fossil arthropods, and including recently proposed, putative apomorphies of Myriochelata. Our phylogenomic analyses also provide strong support for the inclusion of pycnogonids in a monophyletic Chelicerata, a paraphyletic Cycloneuralia, and a common origin of Arthropoda (tardigrades, onychophorans and arthropods), suggesting that previous phylogenies grouping tardigrades and nematodes may also have been subject to tree-reconstruction artefacts.

Keywords: arthropod; phylogeny; Mandibulata; microRNA

1. INTRODUCTION

With over 1 million living species described and a rich 520 Myr fossil record, arthropods are the most species-rich clade of animals on Earth, accounting for nearly 80 per cent of animal biodiversity [1]. Four main euarthropod sub-phyla are recognized: Hexapoda (including insects); Crustacea (lobsters, water fleas and others); Myriapoda (e.g. millipedes and centipedes); and Chelicerata (including arachnids, horseshoe crabs and

possibly sea spiders). After many years of debate, a consensus has emerged that these four classes (or sub-phyla) form a monophyletic group called the Euarthropoda [2,3]. The relationships between the four euarthropod groups remain disputed, however, as is the validity of their close relationship to tardigrades (water bears) and onychophorans (velvet worms) in a more inclusive clade called Arthropoda (named Panarthropoda by Nielsen [4]).

Within the Euarthropoda, the main point of disagreement concerns the position of the myriapods, which were long thought to be most closely related to the hexapods [5]. Myriapods and hexapods notably share a distinctive head composed of five segments distinguished by their unique appendages—the antennal, intercalary

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(appendage-less), mandibular, and usually two pairs of maxillae (the second being the insect labium). Molecular data, however, have shown crustaceans, which differ in having a second antennal rather than an intercalary segment, to be the closest sister group of hexapods in a clade named Pancrustacea or Tetraconata [6,7]. When compared with chelicerates, the detailed similarities of the arrangement of head segments and associated appendages in Pancrustacea and myriapods strongly support their sister group relationship within a wider clade that has been named the Mandibulata in recognition of the similarity of their biting mouthparts (see the electronic supplementary material). Considering the complex shared features of myriapod and pancrustacean head morphology, it is surprising that the majority of published molecular phylogenetic analyses do not support the Mandibulata, instead placing the myriapods as the sister group of the chelicerates in an assemblage that has been named the Myriochelata or Paradoxopoda [8,9]. Molecular support for Myriochelata was initially obtained using large and small subunit rRNAs [10] and later Hox genes [8], mitochondrial protein-coding sequences [11] and combined datasets of both nuclear and mitochondrial genes [9]. Myriochelata was also supported by several phylogenomic analyses [12–15]. However, recently, a dataset of 62 nuclear protein-coding genes found support for Mandibulata [16]. Regier *et al.* [16] did not identify the factors underpinning the difference between their new results and those of previously published phylogenies that supported Myriochelata. Consequently, and in light of the varying results from these molecular samples, the Mandibulata versus Myriochelata controversy remains an open question.

Uncertainty in deep arthropod phylogeny has recently been reinforced as Mayer & Whittington [17] proposed various putative synapomorphies of the Myriochelata, including a revised character polarity for the well-studied neuro-developmental pattern [18], and the mechanism of dorsoventral patterning. Here, debate surrounds the ancestral conditions, specifically whether nervous tissue forms from immigration of single or clusters of cells, and whether or not the neuroectoderm invaginates in each developing segment.

In a similar conflict between molecules and morphology, arthropods share features including segmentation and appendages with tardigrades and onychophorans [1], yet a close relationship between these three phyla has not been clearly supported by molecular analyses. A close relationship between onychophorans and euarthropods is widely accepted, but affinities of tardigrades are less clear, to the extent that they have been linked with nematodes in several phylogenomic studies [13–15]. Recently, a mitogenomic study of the Ecdysozoa supported a monophyletic origin of these three groups, although support is model-dependent [19].

There are two explanations for the discrepancies between different molecular datasets and between molecules and morphology. First, morphology may mislead—mandibles might have evolved independently in pancrustaceans and myriapods or been lost in chelicerates; similarly, segmentation and legs may have appeared separately in arthropods, onychophorans and tardigrades. The second explanation is that some molecular data may be affected by errors—either stochastic (unlikely with

phylogenomic scale datasets) or systematic such as compositional bias or long-branch attraction (LBA) [20–22]. The possibility of systematic error is suggested by some datasets being equivocal regarding myriapod [7,9,19,23,24] or tardigrade affinities [12,19].

To resolve the phylogenetic relationships of the arthropods and their ecdysozoan outgroups, we present analyses of three independent datasets. The first is a phylogenomic dataset of 198 protein-coding genes, which includes new data from the pivotal myriapods. The second is a novel set of arthropod microRNAs (miRNAs), small non-coding regulatory genes implicated in the control of cellular differentiation and homeostasis. The third is a comprehensive dataset of 393 morphological characters, including the recently proposed morphological homologies of Myriochelata [17] and recent gene expression data [25] alongside new and traditional characters supporting the Mandibulata.

In addition, we have explored the nature of the conflict between molecular datasets supporting alternative arthropod phylogenies by assaying the potential effects of systematic error on our phylogenomic dataset using an experimental approach coupling targeted taxon-sampling, the use of alternative models of molecular evolution, and the analyses of subsets of slowly evolving sites extracted from our full dataset.

2. MATERIAL AND METHODS

Detailed description of methods used to generate novel expressed sequence tags and, miRNA datasets, to assemble and align sets of orthologous genes, and for phylogenetic analyses of phylogenomic and morphological datasets, are available in the electronic supplementary material.

3. RESULTS

(a) *Phylogenomic analyses support Mandibulata*

To elucidate the phylogenetic position of myriapods and the discrepancy between recent analyses [12,16], we first analysed a phylogenomic dataset of 198 genes (corresponding to 40 100 reliably aligned amino acid positions) from 30 taxa (see figure 1). The dataset contains new sequences from the centipede *Strigamia maritima*. Bayesian analysis using the CAT + Γ model in the software package PHYLOBAYES [26] supports monophyly of Mandibulata with a posterior probability (PP) of 0.92 and a non-parametric bootstrap support (BS) value of 79 per cent. A Bayesian analysis using an even larger sampling of 59 taxa and the mixed CAT-general time reversible (GTR) + Γ model corroborates these findings (see the electronic supplementary material, figures S1 and S2). Furthermore, our analysis supports the monophyly of Chelicerata (Pycnogonida plus Arachnida), a close relationship between Branchiopoda and Hexapoda, monophyly of Arthropoda (Euarthropoda, Tardigrada and Onychophora), and a paraphyletic origin of the Cycloneuralia (Nematoda more closely related to Arthropoda than to Scalidophora). These relationships are further addressed in §3e.

(b) *Myriochelata is the result of a LBA artefact*

Our results are in accordance with those of Regier *et al.* [16], but in contradiction of other phylogenomic studies

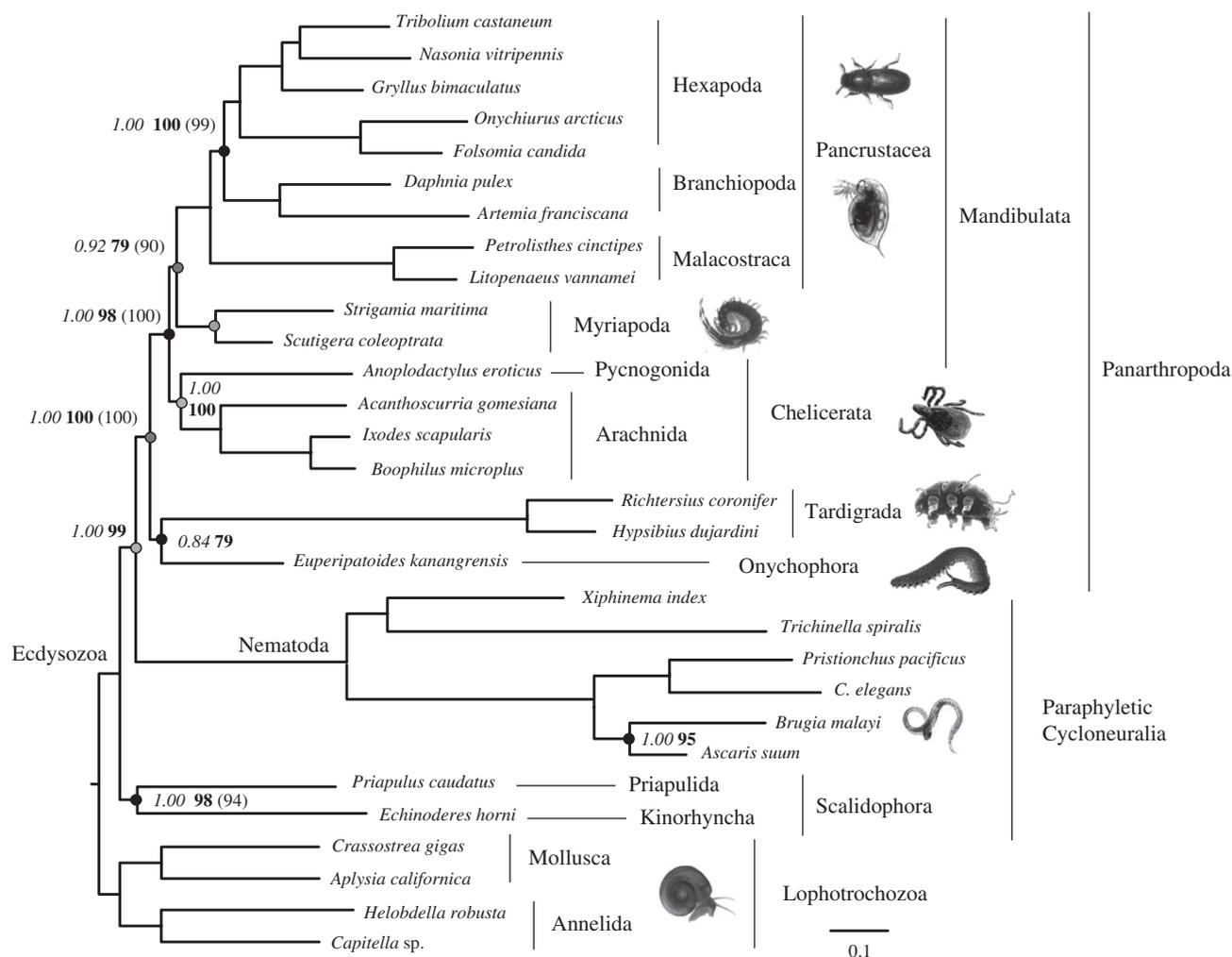


Figure 1. Phylogenomic analyses support Mandibulata, Arthropoda, Chelicerata and paraphyletic Cycloneuralia. Bayesian analyses using the CAT + Γ model. Values at nodes correspond to posterior probabilities (PP) (in italics) and bootstrap support (BS) from 100 pseudo-replicates (in bold); values in brackets are the BS for the same dataset reanalysed without the long branched Nematoda and Tardigrada lineages. Analyses support a monophyletic group of Mandibulata (Myriapoda, Hexapoda and Crustacea), a monophyletic group of Arthropoda (Euarthropoda, Tardigrada and Onychophora), monophyly of Chelicerata (Pycnogonida plus Euchelicerata) and a paraphyletic origin of the Cycloneuralia (Nematoda sister group of the Arthropoda). Where not shown, support values correspond to a PP of 1.00 and BS of 100 per cent. Images have been modified from <http://commons.wikimedia.org>.

[12,13,15]. We therefore explored whether systematic errors, in particular LBA, could have caused the discrepancy between our results and those of studies supporting Myriochelata. In this context, one notable aspect of the tree in figure 1 is the different branch lengths seen in various taxonomic groups. Pancrustacea have long branches in comparison to Myriapoda and Chelicerata, suggesting that in previous studies the fast evolving Pancrustacea could have been attracted towards the distant outgroup, resulting in the clustering of slowly evolving Myriapoda and Chelicerata owing to LBA. Because systematic errors, particularly LBA, become more apparent when the substitution model is unable to handle multiple substitutions correctly [14], we first asked how models such as Whelan and Goldman (WAG) + F + Γ and GTR + Γ —which assume homogeneity of the substitution process—fit our data. We find that WAG + F + Γ and GTR + Γ fit the data significantly less well than the heterogeneous CAT + Γ model (see the electronic supplementary material), and that this reduced

fit is matched by reduction in support for Mandibulata over Myriochelata (figure 2a and electronic supplementary material, figure S3a).

We next explored the possible effects of LBA using a strategy of different taxon sampling. Logically, if Myriochelata is the result of an LBA artefact, exaggerating this source of error by using long-branched or evolutionarily distant outgroups will result in more support for this artefactual clade. Conversely, the use of the shortest branched outgroups should reduce the effects of LBA and result in lower support for Myriochelata. Both of these predictions are supported; when we used either the most phylogenetically distant outgroup (Lophotrochozoa, figure 2b and electronic supplementary material, figure S3b) or the fastest evolving ecdysozoan outgroup (Nematoda, figure 2c and electronic supplementary material, S3c), support decreases for Mandibulata and the artefactual group of slow evolving Myriapoda and Chelicerata (Myriochelata, in grey) increases. Equally, removal of these distant outgroups

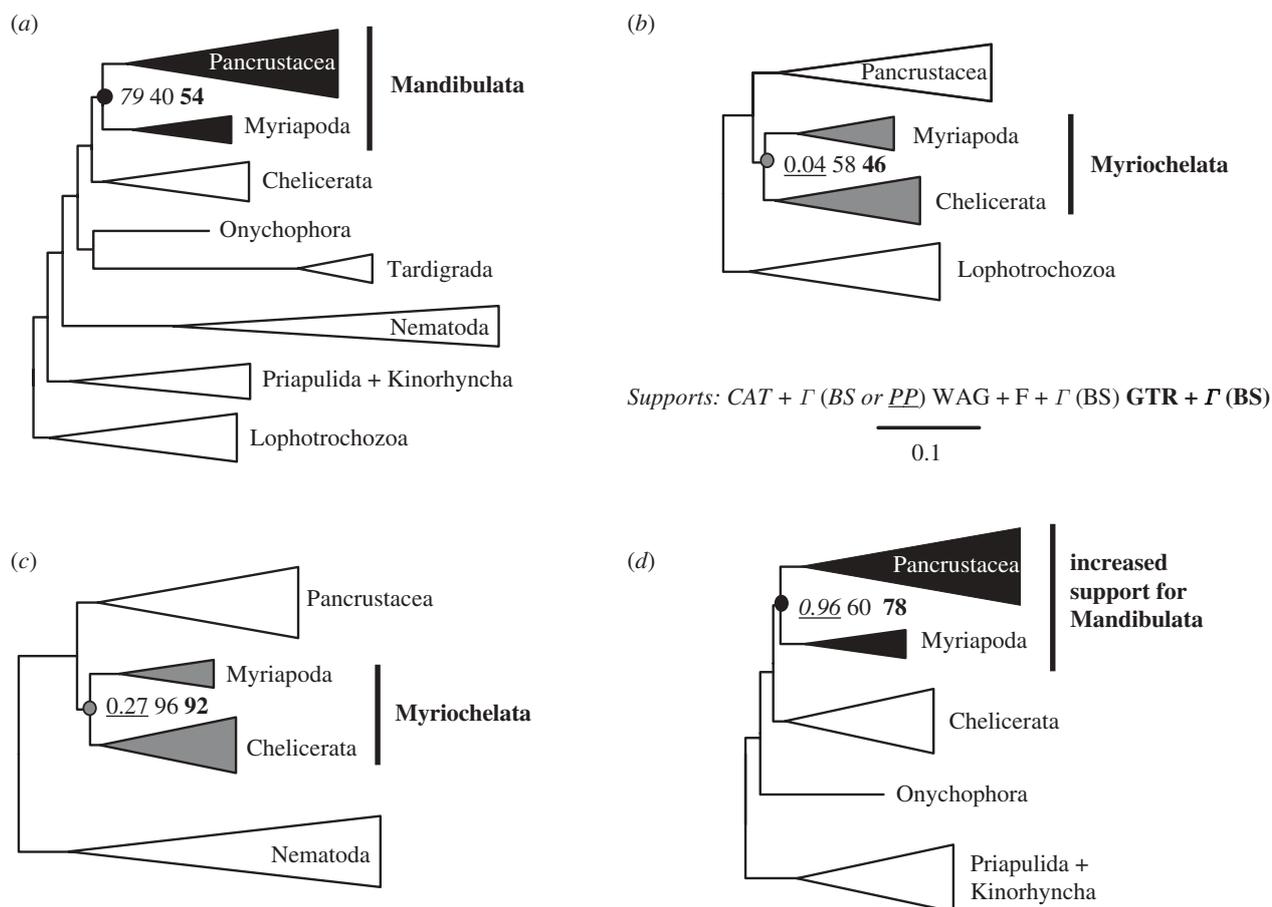


Figure 2. Taxon sampling and the artefactual nature of Myriochelelata. Phylogenetic analyses of our 198 gene dataset using different taxon samples and both Bayesian and maximum likelihood inference. (a) Use of the less well fitting WAG + F + Γ and GTR + Γ homogeneous models results in lower support for Mandibulata (black node and lineages) compared to the best fitting CAT + Γ model (figure 1). The tree depicted is from the Bayesian CAT + Γ analyses. (b) Phylogenetically distant Lophotrochozoa and (c) fast evolving Nematoda outgroups exert an LBA with the fast evolving Tetraconata lineage, thereby regrouping slow evolving Myriapoda and Chelicerata (Myriochelelata) (d) When using slowly evolving and phylogenetically close ecdysozoan outgroups, the support for Mandibulata increases. Trees b, c and d are the WAG + F + Γ maximum likelihood trees. Note that support for Mandibulata is high regardless of which outgroup is used when the dataset is analysed using best fitting model CAT + Γ , but significantly varies when using the less well fitting WAG + F + Γ and GTR + Γ models. Values at nodes are PPs from the Bayesian analyses using CAT + Γ model (PP in italics) BS from 100 replicates using the WAG + F + Γ (BS plain text) and GTR + Γ (BS in bold text) models. When not shown, the support is PP 1.00 and BS 100 per cent. Lineages have been collapsed for clarity with the length of triangles equal to the longest terminal branch in the collapsed lineage and stems are equal to the original length. Original trees with full support values are indicated in the electronic supplementary material, figure S3.

and their replacement with shorter branched taxa (e.g. Onychophora and Priapulida [27]) results in increased support for Mandibulata over Myriochelelata (figure 2d and electronic supplementary material, figure S3d). We also performed a bootstrap analysis (under CAT + Γ) excluding the fast evolving nematodes and tardigrades, which found 90 per cent support for Mandibulata. Notably, both Lophotrochozoa and Nematoda contain species with divergent amino acid composition (see the electronic supplementary material, table S1), supporting our inference that they represent less suitable outgroups [19].

Using our phylogenomic dataset, we have shown that conditions which reduce LBA result in the highest support for Mandibulata, whereas conditions that increase LBA result in increased support for Myriochelelata, implying the artefactual nature of the latter. We replicated these findings using the set of 150 genes of Dunn *et al.* [12],

hereafter ‘Dunn’. Reanalysis of a dataset using their original taxon sampling (of 16 ecdysozoans) resulted in strong support for Myriochelelata (figure 3a and electronic supplementary material, figure S4a) in accordance with their original analysis. To test if the difference between our phylogeny (which supports Mandibulata) and that of Dunn (which favoured Myriochelelata) is owing to taxonomic sampling we expanded their taxonomic representation to include all of our 30 taxa. Under these conditions, modest support for Mandibulata is obtained using the CAT + Γ model while support for Myriochelelata decreased under WAG + F + Γ and GTR + Γ (figure 3b and electronic supplementary material, figure S4b). However, when we remove fast evolving outgroups the support for Mandibulata increases significantly (figure 3c and electronic supplementary material, figure S4c). Removal of fast evolving characters (see the electronic supplementary material, figure S5a) also

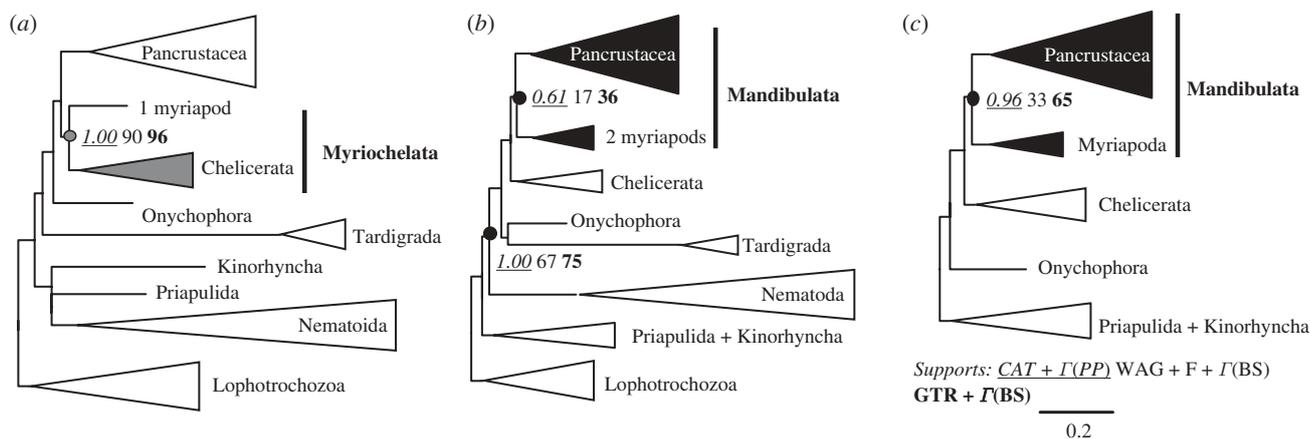


Figure 3. Support for Mandibulata from the gene set of Dunn *et al.* [12]. Bayesian and maximum likelihood analyses of the dataset of Dunn *et al.* [12]. (a) Using their original set of genes and taxa, Myriochelata is recovered with high support. (b) Using our taxon sampling (with the key addition of additional myriapod data) support for Myriochelata decreases and limited support for Mandibulata is recovered. (c) Support for Mandibulata increases when fast evolving or distant outgroups are excluded. Tree topologies correspond to the whole dataset Bayesian *CAT + Γ* trees. Values at nodes are PPs from the Bayesian analyses using *CAT + Γ* model (in italics and underscored) BS from 100 replicates using the WAG + F + Γ (plain text) and GTR + Γ (bold text) models. When not shown, the support is PP 1.00 and BS 100 per cent. Lineages have been collapsed for clarity with the length of triangles equal to the longest terminal branch in the collapsed lineage and stems are equal to the original length. Original trees with full support values are shown in the electronic supplementary material, figure S4.

results in support for Mandibulata instead of Myriochelata. Notably, even with identical taxonomic sampling our 198 gene set provides more support for Mandibulata than do the 150 genes of Dunn *et al.* (compare figures 2c and 3c). The difference may be partly explained by our dataset being larger and more complete (40 100 positions, 69% complete versus 18 829 positions, 61% complete), but also by the lower substitutional saturation of our genes (see the electronic supplementary material, figure S5b).

(c) *miRNAs corroborate Mandibulata, Euchelicerata and Myriapoda*

A useful way to test between the competing Mandibulata and Myriochelata phylogenetic hypotheses is to use an independent data source. We therefore explored the miRNA complements of key arthropod taxa using a combination of genomic sequence searches coupled with the generation and analysis of multiple small-RNA libraries. Novel miRNAs appear to have accumulated in animal genomes through time, and, although short, they show a level of sequence conservation exceeding that of ribosomal DNA [28], making it relatively easy to identify these novel miRNAs in descendant taxa. The apparent rarity of loss of miRNAs within evolutionary lineages coupled with the low likelihood of convergent evolution [29] makes miRNAs a valuable class of rare genomic characters in phylogenetics.

One miRNA, miR-965, had previously been found only in Pancrustacea and had been shown to be absent from the genome of the chelicerate *Ixodes scapularis* [28]. Importantly, we found reads of the mature miR-965 in the small RNA libraries of both myriapods (*Glomeris marginata* and *Scutigera coleoptrata*), and also in the genome of the centipede *S. maritima* (figure 4). Screening our miRNA libraries also showed that in addition to being absent from the genomic sequence of

the tick (*I. scapularis*), miR-965 could not be detected in the xiphosuran *Limulus polyphemus* or in the arachnid *Acanthoscurria chacoana*. Consequently, this distribution supports miR-965 as a genomic apomorphy (a rare genomic change) of the Mandibulata (figure 4). This same distribution is true of a second miRNA miR-282 that we have found only in insects, crustaceans and the centipedes *Strigamia* and *Scutigera*. miR-282 was not found in the *Glomeris* small RNA library and this may be because miR-282 is expressed at low levels in all Mandibulata sampled and the total number of reads and sequencing depth was relatively low in the *Glomeris* miRNA library.

In addition, upon screening the *L. polyphemus* and *A. chacoana* small-RNA libraries, we identified a novel chelicerate miRNA (Arthropod-Novel-1) that is not present in the Mandibulata, but is present in the genome of the tick *I. scapularis* (figure 4), and we thus suggest this miRNA to be a new genomic apomorphy for the Euchelicerata (Xiphosura and Arachnida). We have also identified a novel myriapod-specific miRNA (Arthropod-Novel-2) in the small-RNA libraries of *G. marginata* and *S. coleoptrata*, and in the genome of *S. maritima*, but not in the libraries or genomes of any other non-myriapod taxon analysed (figure 4). Further Myriapod-specific molecular synapomorphies have recently been described [30].

(d) *Updated morphological analyses support Mandibulata*

We assembled a large matrix of morphological data, which provides a third independent line of evidence in support of Mandibulata. While a number of possible morphological apomorphies of Myriochelata have recently been identified [17], inclusion of these characters in a cladistic analysis of 393 morphological characters still results in overall support for Mandibulata (Bremer support = 5)

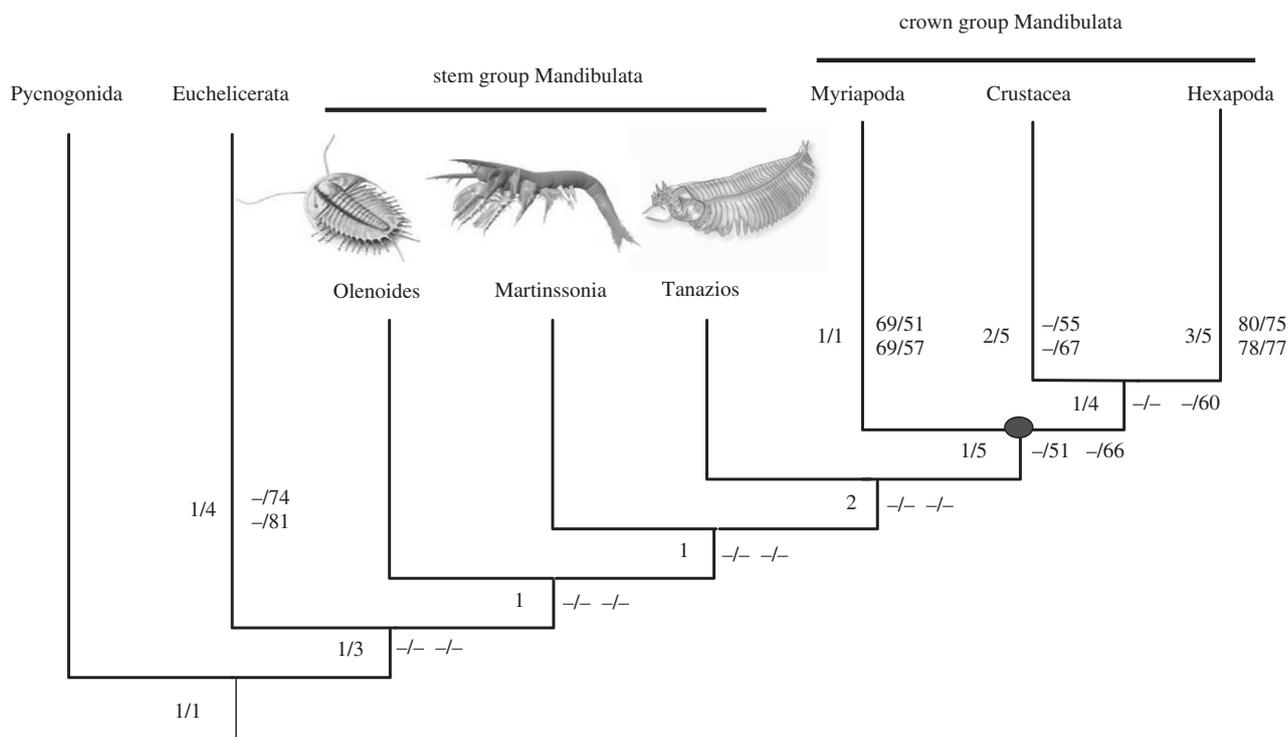


Figure 5. Morphology supports monophyly of crown Mandibulata. Summary cladogram of crown group euarthropod relationships based on morphological data (393 characters listed in the electronic supplementary material). Clades shown here are a strict consensus of shortest cladograms computed by TNT and PAUP*. Numbers to left of branches are Bremer support values; for extant taxa, values for analyses with (left) and without fossils (right) are separated by a slash. Numbers to right of branches are bootstrap (top) and jackknife (bottom) frequencies (indicated by a dash if less than 50%); values for analysis with and without fossils are separated by a slash. The fossils *Tanazios*, *Martinssonia*, and trilobites (*Olenoides*) are resolved progressively more stemward relative to the mandibulate crown group.

electronic supplementary material for a detailed compilation of morphological and developmental genetic characters).

(e) *Phylogenomic analyses support monophyletic Arthropoda, Chelicerata and Paraphyletic Cycloneuralia*

Most of our phylogenomic analyses support the monophyly of Arthropoda (euarthropods, tardigrades, onychophorans), either using our gene sampling (figure 1) or that of Dunn (figure 3*b*). The position of tardigrades is more unstable, varying from being sister to the onychophorans (figure 1 using CAT + Γ model) to being sister to a group of arthropods plus onychophorans (see the electronic supplementary material, figure S2 using the CAT + GTR model). Whereas the CAT + Γ model supports Arthropoda consistently, site-homogeneous WAG + F + Γ and GTR + Γ models tend to group tardigrades with nematodes (dotted arrows in the electronic supplementary material, figures S3 and S4). Our interpretation is that site-homogeneous models, which fit our data less well than the CAT model (see §2), are unable to overcome the effect of systematic errors responsible for the grouping of fast evolving nematodes and tardigrades.

All our phylogenomic analyses support a monophyletic origin of the chelicerates in which pycnogonids are sister to a group of arachnids plus Xiphosura. This finding is significant in light of recent debates over the position of the Pycnogonida, which some studies find to be the sister group to all other arthropods, a hypothesis known as

Cormogonida [23,32,33]. The possibility that systematic/stochastic errors were affecting the affinity of pycnogonids in previous studies is highlighted by their position being parameter-dependent in other studies [16,24,34].

Finally, all our phylogenomic analyses support a paraphyletic origin of the Cycloneuralia, with the Scalidophora (priapulids and kinorhynchs) sister to a group of nematodes plus arthropods. This is in accordance with ribosomal markers [23], but in contrast to previous phylogenomic studies [12,13], which instead supported monophyly of Cycloneuralia (Nematodoida + Scalidophora). Notably, when updating the gene selection of Dunn *et al.* [12] to our larger taxon sampling, a paraphyletic origin of the Cycloneuralia is recovered. Ultimately, the relationships of Nematodoida, Scalidophora and Arthropoda remain uncertain.

4. DISCUSSION

Arguably the strongest evidence of phylogenetic accuracy is the congruence of independent lines of evidence supporting the same tree topology [22,35]. In order to test current hypotheses of arthropod evolution, we have analysed three independent lines of evidence: a phylogenomic dataset of 198 genes, a new miRNA dataset and a large morphological dataset. All three datasets unambiguously support the monophyly of Mandibulata.

We have examined the possibility that previous molecular phylogenies supporting Myriocheilata might have been affected by systematic error and the robustness of the result from our phylogenomic dataset is supported by experiments designed to reduce the effects of

systematic errors. Increased taxon sampling, exclusion of outgroups with the longest branches, removal of the fastest evolving positions and the use of better evolutionary models systematically increase support for Mandibulata over Myriochelata.

The presence of miR-965 and miR-282 in Pancrustacea and in two groups of Myriapoda also represents compelling evidence in support of Mandibulata. These two miRNA are absent from both arachnids and horseshoe crabs as well as from all other Ecdysozoans for which the miRNA complement is known (nematodes and priapulid worms). As it is implausible for this miRNA to have been independently acquired in the different mandibulate lineages [29], we conclude that it constitutes a rare genomic change supporting Mandibulata. In light of congruence of these novel miRNA autapomorphies with other lines of evidence presented here (phylogenomics and morphology) and with the complementary findings of Regier *et al.* [16], we conclude that the most tenable position of the Myriapoda is as the sister group of the Pancrustacea within a monophyletic Mandibulata.

Our phylogenomic analyses suggest that studies which have grouped tardigrades with nematodes may have been similarly affected by LBA. When analysed using the CAT model, which has been shown to help in overcoming systematic errors [14], both our dataset and that of Dunn *et al.* [12] group Tardigrada with Euarthropoda and Onychophora in a monophyletic Arthropoda clade. Tardigrada are a sister group of the Onychophora in these trees, a topology which finds no support from a morphological point of view, but is in accordance with mitochondrial markers [36]. Furthermore, if the paraphyletic nature of the Cycloneuralia is correct, as supported by our phylogenomic analyses, this would suggest that the ancestral Ecdysozoa was cycloneuralian-like, possessing a circumpharyngeal brain and an introvert [37].

The Mandibulata, which includes insects, is by far the largest clade of animals on Earth, but the origin of this successful bodyplan in terms of the evolution of its development remains obscure. The picture from palaeontology is, however, somewhat clearer. Cambrian fossils that have been identified as a grade of stem-group mandibulates [38] indicate a crustacean-like *habitus* for basal members of the Mandibulata and may shed light on how the mandible common to these groups evolved. The limb on the third cephalic segment (the mandible homologue) in Cambrian stem-group mandibulates such as *Martinssonina* displays a stronger development of a movable, setose process at the limb base ('proximal endite'; [39]) than that on the adjacent limbs [40]. The more elaborated proximal endite used for food manipulation is viewed as a precursor to the fully differentiated coxal chewing surface in the mandibulate crown group [40]. Further studies of fossils and embryos in the light of what we suggest is a reliable phylogeny of arthropod classes should clarify the evolution of the mandibulate bodyplan [41], and consequently how anatomical novelties may have promoted their hugely successful radiation.

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