A new spiralian phylogeny places the enigmatic arrow worms among gnathiferans

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Chaetognaths (arrow worms) are an enigmatic group of marine animals whose phylogenetic position remains elusive, in part because they display a mix of developmental and morphological characters associated with other groups [1,2]. In particular, it remains unclear whether they are sister group to protostomes [1,2], one of the principal animal superclades or whether they bear a closer relationship with some spiralian phyla [3,4]. To address the phylogenetic position of chaetognaths and to refine our understanding of relationships among spiralians is essential to comprehend fully character changes during bilaterian evolution [5]. To tackle these questions, we generated new RNA-seq datasets for ten chaetognath species, compiling an extensive phylogenomic dataset that maximizes data occupancy and taxonomic representation. We employed inference methods that consider rate and compositional heterogeneity across taxa, to avoid limitations of earlier analyses [6]. In this way, we greatly improved the resolution of the protostome tree of life. We find that chaetognaths cluster together with rotifers, gnathostomulids, and micrognathozoans within an expanded Gnathifera clade, and that this clade is the sister-group to other spiralians [7,8]. Our analysis shows that several previously proposed groupings are likely due to systematic error and we propose a revised organization of Lophotrochozoa with three main clades: Tetraneuralia (molluscs and entoprocts), Lophophorata (brachiopods, phoronids and ectoprocts) and a third unnamed clade gathering annelids, nemerteans and platyhelminthes. Considering classical morphological, developmental, and genomic characters in light of this topology indicates secondary loss as a fundamental trend in spiralian evolution.

Results and Discussion

A phylogenomic resolution of the bilaterian tree of life

The neat tripartite classification of bilaterian animals into deuterostomes, ecdysozoans, and lophotrochozoans imparts considerable uncertainty, especially involving lineages comprising the lophotrochozoan superclade and the relationships among them [9]. To accurately reconstruct bilaterian relationships, we extracted 1174 single-copy orthologues present in available metazoan complete genomes and transcriptomic datasets, combining existing datasets with newly generated sequences (Methods). We aimed for a balanced taxonomic sampling by retaining four to six species for each phylum to maximize taxonomic representation, giving priority to species with the highest orthologue recovery, and deliberately focusing on the slowest evolving lineages to minimize the impact of long branch attraction (LBA). We evaluated the impact of taxonomic sampling by analysing five distinct supermatrices that included varying numbers of fast evolving species, and species with marked compositional biases, as well as the use of alternative rooting taxa (Figure 1C and Methods). We clipped the data matrices for potential contamination and stretches of mistranslated amino acids that can occur in transcriptome data. We also employed several tree inference methods and models known to show distinct robustness toward model violation and reconstruction artefacts. In particular we used both site-homogeneous models assuming gene partitions (LG4X+R), as well as site-heterogeneous models (CAT+GTR) for which we restricted the analyses to a set of

markers selected for their lower mutational saturation [10,11]. We also examined how efficiently these different models account for compositional heterogeneity across taxa using 'posterior predictive analyses' (PPA), which revealed that compositional deviation is affecting model fit (Figure 2B and C, Figure S3) [6,10]. To reduce the impact of composition heterogeneity, we also performed tree reconstruction using site-heterogeneous models after recoding our datasets in the six broader Dayhoff amino acid functional categories (Dayhoff6) [12].

Using these alternative methods and datasets, we recover the major well-accepted bilaterian clades, obtaining strong support for protostome, ecdysozoan and a clade of non-ecdysozoan protostomes that include spiralian and lophotrochozoan taxa, as well as verifying the monophyly of all represented animal phyla (Figure 1A,2A) [9]. Unexpectedly, the monophyly of deuterostomes does not always receive maximal support, particularly in Dayhoff6 recoded datasets (CAT+GTR, Figure 1C, 2A), with the occasional earlier divergence of Ambulacraria (echinoderms and hemichordates) relative to chordates, a topology that has been reported previously [13]. Although this question is not the primary focus of our analyses, we found a preferential association of the Xenacoelomorpha group (Xenoturbella and acoelomorph flatworms) with Ambulacraria [14] when more sophisticated molecular evolution models are used (CAT+GTR and Dayhoff6 recoding) and when the fastest evolving acoel flatworm species are excluded. With simpler site-homogeneous models, however, Xenacoelomorpha remain branched as the sister group of bilaterians [15]. We note that acoels show a diverging amino acid composition, which could have impacted earlier studies (Figure S3). In sum, our analyses support the 'new view' of animal phylogeny, but also illustrate the impact of reconstruction method on the obtained trees.

Chaetognaths join an extended Gnathifera clade

Chaetognaths are a major zooplankton group that has long been a challenge for both morphology- and molecular-based phylogenetics. They display a mosaic of morphological and developmental characters, presenting a secondary blastopore opening reminiscent of deuterostomes, while possessing two ventral nerve cords and a circum-oesophageal brain classically associated with protostomes [1,3]. Interestingly, Cambrian fossil deposits such as the Burgess Shale contain chaetognath representatives that are remarkably similar in body organisation to present days forms [16]. At the molecular level, early evidence from ribosomal RNA rejected deuterostome affinities of chaetognaths [17], and later attempts using multigene datasets indicated a more likely association with protostomes [1–3]. However, these studies did not agree on chaetognath branching within protostomes, suggesting that they could either be the sister-group of other protostomes [1,2] or represent an early lineage within spiralians [3,4]. Another recent study pointed toward a possible association of chaetognaths with some gnathiferan taxa, but with limited support and discrepancies between analyses [18].

Strikingly, we find that chaetognaths are united with rotifers, gnathostomulids and the recently described micrognathozoans in a well-supported clade using multiple tree inference strategies and taxonomic sampling (Figure 1A and 2A). In particular, our results were unchanged when we excluded the fastest evolving species of gnathostomulid (*Gnathostomula sp.*) and some rotifer species (Figure 1A and C, 2A, S1 and S2). Since rotifers present a derived amino acid content compared to other bilaterian taxa (Figure 2B), we generated a set of trees after Dayhoff6 recoding, which alleviates compositional heterogeneity across taxa [6,12] and still obtained strong support for a Gnathifera clade including chaetognaths (Figure 1A).

The Gnathifera clade was originally proposed based on pharyngeal hard parts and protonephridial structures [19] found to be shared by Rotifera, Gnathostomulida [7] and later Micrognathozoa as well [20]. Our phylogenetic analyses expand this clade to include chaetognaths, which is corroborated by additional morphological and molecular characters. First, the presence of a complex jaw apparatus with hardened parts in chaetognaths is compatible with the primary morphological character defining this group (Figure 1B) [7]. The association of chaetognaths with Gnathifera was originally suggested on this base in the second edition of Nielsen (2001) but not in the subsequent third edition published ten years later [19]. The chaetognath grasping spines, the *mastax* of rotifers and jaws of gnathostomulids possibly share a composite organisation at the ultrastructural level with alternating layers of material opaque or dense to electrons disposed in tubular fashion [21,22]. Such an organisation is absent from other spiralians (*e.g.* annelids, molluscs) [22]. Second, several authors have suggested that chaetognaths might share an unusual intracellular mode of cuticle formation with rotifers and acanthocephalans, although this was never confirmed [23]. These observations require further investigation as chaetognaths are notable for their multilayered epidermis [24]. Finally, the

recent discovery in the rotifer *Brachionus plicatilis* of a plausible orthologue of a Medpost Hox gene would constitute a remarkable molecular synapomorphy for an extended Gnathifera clade including chaetognaths [25]. This class of Hox genes shows intermediate residues between median and posterior Hox, and was originally considered to be specific to chaetognaths [26].

The relationships within the newly extended Gnathifera clade are more elusive. Micrognathozoans robustly group with rotifers, corroborating morphology and previous molecular studies [8,20]. Gnathostomulids sometimes branch as the sister group to other gnathiferans (Figure 1A), while in other trees they are closer to Rotifera and Micrognathozoa (Figure S1) or even chaetognaths (Figure S2). Our broad chaetognath sampling also provides some insights into the intraphyletic relationships of chaetognaths. Eukrohniidae are positioned as the sister-group to other chaetognath species, which supports the paraphyly of Phragmophora. In contrast, Aphragmophora is monophyletic with an early divergence of Krohnittidae, and a paraphyletic *Sagitta* genus due to the nested position of the monospecific genus *Pterosagitta* [27,28]. Although sampling of additional taxa would be required to examine the details of chaetognath taxonomy, the recovered relationships are in broad agreement with previous schemes [27,28].

Revised spiralian relationships

Our phylogenetic reconstructions also suggest a new scheme for relationships among non-ecdysozoan protostome taxa, a notoriously difficult problem [9]. Many early phylogenomic studies recovered a 'Platyzoa' clade that collected many morphologically-simple fast-evolving lineages, including platyhelminthes, rotifers, gastrotrichs, gnathostomulids and others [4]. However, recent studies instead found this assembly to be paraphyletic, leading to the proposal of a Rouphozoa clade that unites platyhelminthes and gastrotrichs, sometimes closely related to a clade consisting of entoprocts and ectoprocts (Polyzoa) [5,8,18]. This latter topology receives support in some of our analyses, particularly the ones relying on site-homogeneous and empirical mixture (C20) models (Figure S1 and S2), but an improved taxonomic sampling, the usage of a site-heterogenous model and the reduction of missing data rejects the Rouphozoa (Figure 1A,2A). More sophisticated infinite mixture models (CAT-GTR), however, suggest a novel view of spiralian relationships. We find three distinct spiralian subclades (Figure 1A,2A): (i) entoprocts group with molluscs to recover the Tetraneuralia clade, previously proposed based on muscle system and larval characteristics [29]; (ii) a monophyletic Lophophorata that includes brachiopods, phoronids and ectoprocts (but not Entoprocta), possibly associated with gastrotrichs and (iii) a new unnamed clade that gathers nemerteans and platyhelminthes with annelids. Platyhelminthes are very fast evolving and show marked compositional deviation compared to other bilaterians (Figure 2B and S3). Hence, we particularly scrutinized both the recoded dataset (Figure 1A,1C) and analyses restricted to the slowest evolving available Platyhelminthes (Figure 2C and 'strin' dataset), which both confirmed this new and as yet unnamed clade. The association of platyhelminthes and nemerteans supports a century-old view mostly based on the abundant parenchyma between body wall and internal organs ('Parenchymia'). Similarities of ciliary band organisation in the Götte's larva of platyhelminthes and the *pilidium* larva of nermerteans have been noted as possible synapomorphies for Parenchymia, as well as the shared absence of chitin in both groups. However, the Parenchymia clade has not been supported by recent molecular phylogenies [19,30]. Similarly, the association of annelids and nemerteans has previously been argued based on similarities in their circulatory systems under the name Vermizoa [31].

A reappraisal of spiralian character orientation

Our new spiralian phylogeny argues for a reappraisal of the evolution of a number of clade-defining traits [19,32]. The respective branching of annelids, molluscs and platyhelminthes in our trees indicates a common origin for spiral cleavage and trochophore larvae, followed by subsequent loss in lophophorates (Figure 3) [33]. The observed position of nemerteans also corroborates observations in paleonemerteans, suggesting that a trochophore-like larva is likely the ancestral condition in nemerteans [34]. In our extended Gnathifera, spiral cleavage and larval stage are absent in chaetognaths and rotifers, which are both direct developers, while gnathostomulids have only been briefly mentioned as undergoing possible spiral cleavage in a 1969 publication [35]. Until future investigation clarifies the ancestral cleavage type in Gnathifera, for instance by applying lineage tracing or 4D microscopy techniques, it seems reasonable to continue using the name 'Spiralia' to refer to the clade formed by all non-ecdysozoan protostomes, which itself is subdivided in Gnathifera and Lophotrochozoa. Under this scheme, platyhelminthes and gastrotrichs would be considered members of the Lophotrochozoa clade, although they were not originally included. If future characterization

of embryonic development in gnathiferans – especially gnathostomulids– were to reject the hypothesis that spiral cleavage is ancestral to these taxa, then the name Spiralia, as a synapomorphy-based name, would become a synonym of the clade referred to here as Lophotrochozoa. In that case, a new name would be needed for the non-ecdysozoan protostome clade comprised of Gnathifera and the restricted Spiralia. Here, we propose that such a clade could be named Gnathospiralia following the same associative reasoning as was used for naming the Lophotrochozoa [33,35].

The distribution of morphological characters shows a patchy distribution across Spiralia, consistent either with repeated character losses from a complex ancestor or repeated character acquisition [5,36] (Figure 3). This remark also applies to genome evolution with traits such as intron positions, gene families, and genome architecture shared among bilaterians but experiencing dramatic loss in some lineages [37] (Figure 3). Earlier claims that the relative simplicity encountered in the previously proposed 'Platyzoa' assemblage could be indicative of a simple acoelomate bilaterian ancestor are therefore questioned [5] (Figure 3). Finally, some clades proposed here, such as Annelida with Parenchymia or Gastrotricha with Lophotrochozoa, do not have acknowledged synapomorphies that could be used as a basis for a name, which pleads for further investigation.

Conclusion

Our phylogenetic analyses support the inclusion of chaetognaths in the Gnathifera clade, which strengthens the importance of this subdivision of the animal tree of life. Our analyses reject the previously proposed 'Platyzoa' assemblage that gathered fast evolving lineages. Some of its members (*e.g.*, rotifers and gnathostomulids) are incorporated in Gnathifera; others (*e.g.*, Platyhelminthes) are now members of the Lophotrochozoa clade. The inclusion of chaetognaths, a coelomate phylum with a complex nervous system among Gnathifera, implies that subsequent character loss took place in the other members of this group, or that chaetognaths independently evolved a number of traits, such as condensed nervous system or deuterostomic development [36,38]. Further study of genomes and development in gnathiferans is essential to better understand the ancestral condition in this clade, in protostomes and in bilaterians.

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Author contributions

F.M., K.T.C.A.P., N.S. and D.S.R. designed the study, K.T.C.A.P., F.M. and T.G. collected and identified animal samples. F.M. and N.S. prepared and sequenced samples. F.M. performed phylogenetic analyses, and wrote the manuscript. F.M., D.S.R. and K.T.C.A.P revised the manuscript.

Declaration of interests

Nothing to declare

Figures

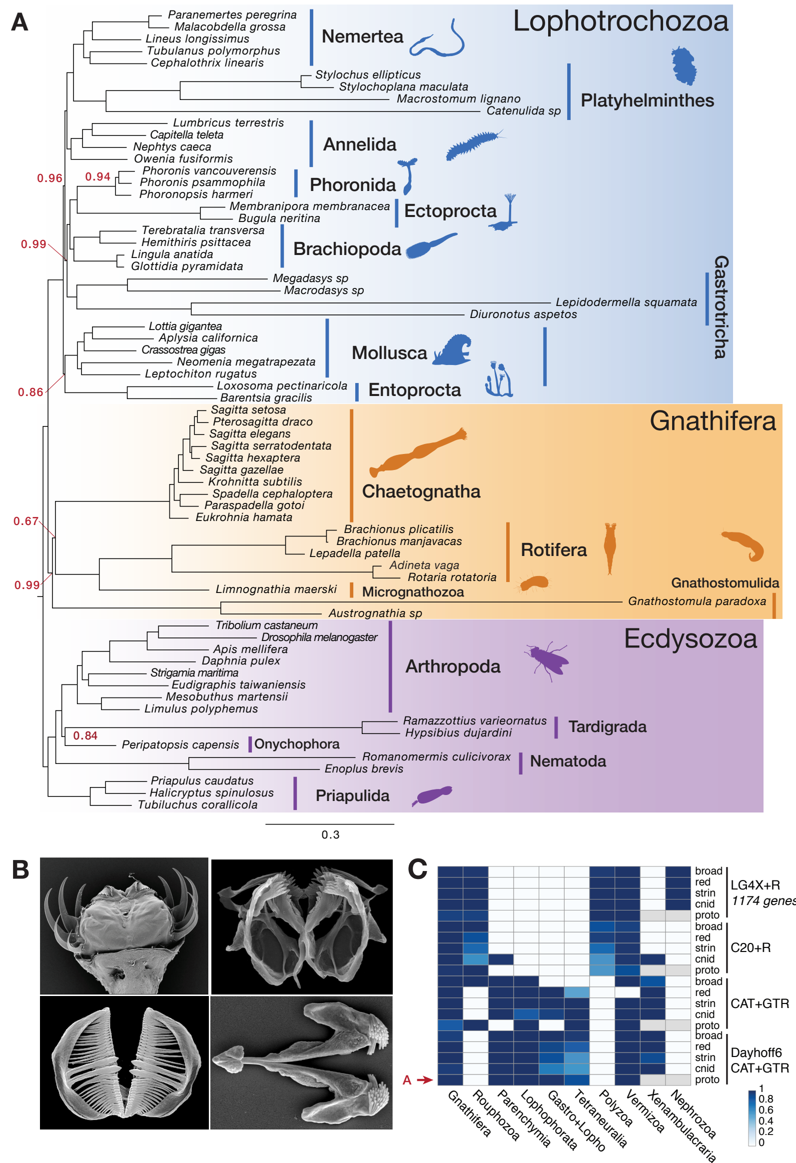

Figure 1. Chaetognaths are members of the Gnathifera clade together with rotifers, micrognathozoans and gnathostomulids. (A) Protostome phylogeny reconstructed with Phylobayes using CAT+GTR and Dayhoff6 recoding scheme. All nodes but the ones labelled with red numbers show maximal posterior probability (silhouettes from Phylopic). (B) Pictures of jaw apparatus in gnathiferas: clockwise, chaetognaths, rotifers, micrognathozoans and gnathostomulids (adapted from [64]). Image credit: M.V. Sørensen (Natural History Museum of Denmark). (C) Summary of support values obtained using distinct taxonomic sampling, reconstruction methods (site-homogenous and heterogenous) and recoding scheme (see methods and online dataset). For the detail of clade and topologies names, see methods. 'Gastro+Lopho' means Gastrotriches with Lophophorata. See supplemental information for detailed trees and Figure S1 and S2 for selected maximum-likelihood trees.

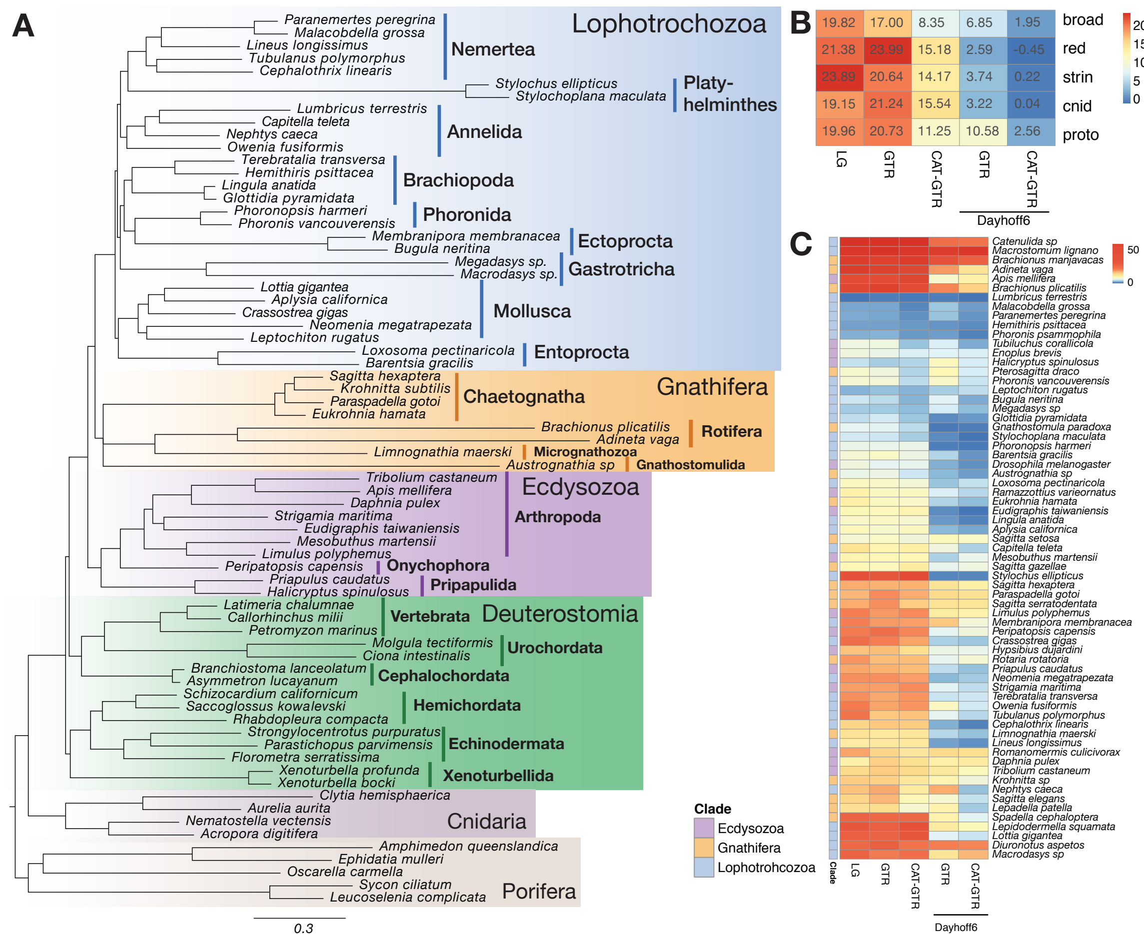

Figure 2. Impact of compositional heterogeneity across taxa on bilaterian phylogenetic reconstruction. (A) Bilaterian phylogeny reconstruction with Phylobayes using the CAT+GTR model using only taxa with slowest evolutionary rates and steady deviating amino acid composition. All posterior probabilities are maximal (B and C) Z-scores statistics of posterior predictive analyses (PPA) to assess compositional heterogeneity (B) Global scores for different datasets and models. (C) detail of z-score for each species in the 'proto' datasets of Figure 1. Species order was derived k-means clustering based on PPA z-score. See Figure S3 for PCA analysis and PPA results on all taxa.

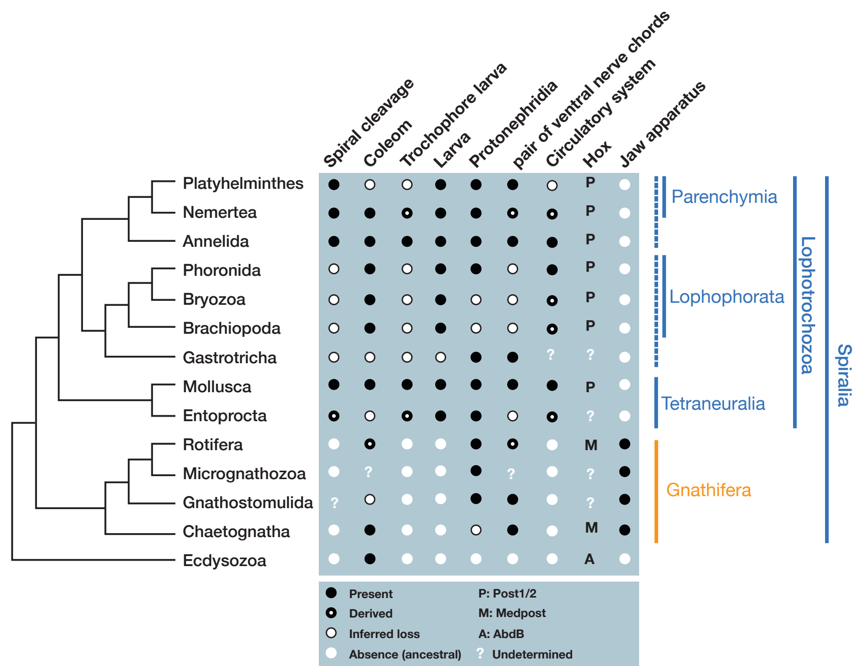

Figure 3. Distribution of morpho-developmental characters in spiralians. Morphological characters of interest are mapped along the proposed topology. In the legend, derived means that the character homology has been a subject of debate in the literature, and inferred loss indicates a possible character loss following a parsimony reasoning, but other scenarios are not excluded. Accepted clade names are indicated on the right, along with novel unnamed spiralian clades (dashed line).

STAR METHODS

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and data should be directed to and will be fulfilled by the Lead Contact, Ferdinand Marlétaz (ferdinand.marletaz@gmail.com).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Sample collection

Samples from multiple chaetognath species were collected during the Atlantic Meridional Transect cruises 22 and 24 in the Atlantic Ocean, in the Gullmarfjord (Sweden), in Amakusa (Japan) and in Marseille (France) (details about collection locations are given in Table S2). Chaetognaths were examined under a microscope while alive, identified, and preserved in RNAlater (Sigma or Invitrogen) and kept frozen. Reference samples were preserved in 4% formaldehyde solution to verify species identifications.

METHOD DETAILS

Sample preparation for sequencing

RNA was extracted using the RNAeasy micro or mini kits (Qiagen) after homogenisation with the Tissuelyser device (Qiagen). RNA integrity was verified on an Experion instrument (Biorad), and RNA-seq polyA+ libraries were prepared using the TruSeq RNA kit (Illumina) at the WTCHG (Oxford) and Ovation RNA-seq system (NuGen) at SQC (OIST, Japan). Libraries were sequenced on Illumina instruments (HiSeq2000 and HiSeq4000). Detail and accession numbers for each sample are provided on Table S1.

Transcriptome assembly and filtering

We processed newly sequenced samples and datasets downloaded from the NCBI Short Read Archive (SRA) similarly. After trimming reads with Sickle , transcriptomes were assembled with Trinity (v2.3.2) using default parameters and a k-mer of 25 [39]. Open reading frame (ORF) for each transcript was determined using Trans-decoder [40] using a blast against a version of Swissprot restricted to metazoan taxa as clue (e-value 10-5). For newly sequenced samples, we sometimes noticed occurrence of cross-contamination during the sequencing process. To remove transcripts derived from mis-barcoded reads, we followed the approach described in [48]. To do this, we estimated the read counts of each transcript demultiplexed as one sample for each other sample sequenced on the same lane and excluded the transcripts with (1) two times more coverage in another sample than the one they belonged to, and (2) a coverage less than 2x in their sample of origin. Python implementation of this approach is available together with other scripts used in this study at http://github.com/fmarletaz/phylogenomics/.

Gene family reconstruction and homology search

We used OMA (v1.0.5) to reconstruct a set of single-copy orthologous gene families [41] from proteomes derived from 20 metazoan genomes (Table S1) including a set of proteins derived from the *Paraspadella gotoi* genome (in preparation). To avoid performing clustering on transcript isoforms, transcripts were clustered using Corset [42] and we picked the most highly expressed transcript for each cluster, after measuring expression with Kallisto (v0.43.1) [42,43]. We retained all families with 16 representatives or more, yielding a set of 1174 single-copy orthologues suitable to infer metazoan phylogeny. For each marker, we generated a protein alignment using Msaprobs (v0.9.7) [44] and built a hmm profile using hmmbuild of the hmmer package (v3.1b2). We then searched the collection of translated transcriptome using these hmm profiles using hmmsearch (e-value 1e-1). To eliminate any wrongly assigned sequence, we performed a reciprocal Blast search (e-value $1e^{-9}$) against the proteomes and excluded any sequence whose best hit did not belong to the corresponding orthologous group.

Supermatrix assembly, marker selection and taxonomic sampling

To assemble a comprehensive phylogenomic dataset for bilaterians, we incorporated our novel transcriptomes from chaetognaths and we included transcriptomic data from previously published studies, in particular [5,8,15]. Some taxa are sometimes represented in databases by a large number of RNA-seq datasets. To take into account the computational limitations, our philosophy was to assemble a dataset with a balanced number of taxa in each phylum (4-6 species), to minimize missing data, and to preferentially retained the slowest evolving species. Our initial dataset includes 103 taxa (Figure 1C, Figure S1) and we alternatively analysed datasets with subsets of 83, 70 and 65 taxa (Figure 1C).

Proteins extracted from transcriptomes and genomes were independently aligned using Msaprobs (v0.9.7) [44] . To detect and remove mis-translated regions, we used Hmmclean with a threshold of 20 (Hervé Philippe, personal communication). Hmmclean compares each sequence to a Hidden Markov Model profile derived from the alignment without this sequence and removes highly divergent stretches of amino acid. Alignments were further trimmed with BMGE to exclude blocks of highly variable misaligned residues with a maximum gap rate of 0.9 (-g option) [46]. After these steps, a ML tree was reconstructed for each individual marker alignment with RAxML (v8.2.4) assuming a LG+Γ_4 model [45]. To further exclude possible residual contaminations, we calculated the median absolute deviation of the distance to the root for all taxa, and we excluded those showing a 20-fold higher distance than this deviation, leading to the exclusion of 104 sequences in total. To perform marker gene stratification, we calculated the saturation for each of them as the linear regression coefficient between the ML p-distance and the percentage identity for each pair of taxa.

Phylogenetic analyses

Concatenation of all 1174 genes yielded a 416,663 amino acid supermatrix with 34.59% missing data. We analysed the whole matrix using a per-gene partition scheme and a LG4X+R model in IQ-TREE (v1.6.2) [47]. Support values were estimated by ultrafast bootstrapping for 1000 replicates with the UFBoot option to account for model violation (-bnni -bb 1000) (Figure 1C, S1 and S2). Such an alignment is computationally too expensive to examine using a site-heterogenous model. Therefore, we selected the 267 marker genes with the lowest levels of saturation computed as explained before. This yielded a 74,014 positions matrix (Table S3). This matrix was examined using Phylobayes-mpi assuming a CAT+GTR+Γ_4 model with 4 chains running for more than 5000 generations and the first 1500 cycles discarded as burn-in [10]. Total computation represented more than 9 weeks of computation with 50 cores per chain.To evaluate the possible impact of compositional heterogeneity across taxa, we performed a CAT+GTR+Γ_4 analysis after recoding the data in broad protein categories using Dayhoff6 scheme (Figure 1 and Table S3). Convergence was assessed using the 'bpcomp' command and by visually inspecting parameters values for the multiple runs. Alternatively, to approximate the CAT model in a maximum-likelihood framework, we also applied IQTREE with a C20 mixture of profiles, the LG matrix of exchange rates and freerates heterogeneity (LG+C20+R4+FO) (Table S3). Composition heterogeneity was asses using a posterior predictive analysins in Phylobayes-mpi on bayesian samples for alternative datasets and models of evolution [10]. A Z-score was used as a measure of the deviation to the null-hypothesis (homogenous compositional distribution) across replicates of PPA; the higher the absolute z-score, the stronger the deviation.

We examined trees with several taxonomic sampling: 'broad' corresponded to most exhaustive datasets (103 taxa), 'red' (reduced) excluded taxa showing strongest compositional biases and fastest evolutionary rate (83 taxa), 'strin' (stringent) excluded further taxa (70 taxa), 'cnid' used only cnidarians (and not sponges) as an outgroup (65 taxa) and 'proto' included all protostome taxa (67 taxa). Observed topologies as detailed in Figure 1C are the following: Rouphozoa (Platyhelmintha + Gastrotricha), Parenchymia (Nemertea+Platyheminthes), Lophophorata (Brachiopoda + Phoronida + Ectoprocta [Bryozoa]), Gastrotriches with Lophophorata (Gastro+Lopho), Tetraneuralia (Mollusca+Entoprocta), Polyzoa (Ectoprocta [Bryozoa] + Entoprocta), Vermizoa (Annelida + Nemertea disregarding platyhelminthes branching), Xenambulacraria (Xenacoelomorpha + Ambulacraria) and Nephrozoa (for Xenoacoelomorpha sister-group of other bilaterians) (Table S3).

DATA AND SOFTWARE AVAILABILITY

The alignments, phylogenetic trees, bayesian samples have been deposited as a zenodo dataset (doi: 10.5281/zenodo.1403005). Datasets are available on Zenodo as https://doi.org/10.5281/zenodo.1403005.

The sequencing reads for newly sequenced species have been deposited under the bioproject PRJNA487918 (https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA487918) with the accession SRR7754742-SRR7754750 and SRR8149062.

Supplemental tables

Table S1. List of species included in the analyses with accessions numbers and respective marker representativity. Related to Figure 1.

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