Improved resolution of recalcitrant nodes in the animal phylogeny through the analysis of genome gene content and morphology

Juravel, Ksenia[1]; Porras, Luis[1]; Höhna, Sebastian[1,2]; Pisani, Davide[3]; Wörheide, Gert[1,2,4, *]

[1] Department of Earth and Environmental Sciences, Paleontology & Geobiology, Ludwig-Maximilians-Universität München, Richard-Wagner-Str. 10, 80333 München, Germany

[2] GeoBio-Center, Ludwig-Maximilians-Universität München, Richard-Wagner-Str. 10, 80333 München, Germany

[3] School of Biological Sciences and School of Earth Sciences, University of Bristol, UK.

[4] SNSB-Bayerische Staatssammlung für Paläontologie und Geologie, Richard-Wagner-Str. 10, 80333 München, Germany

* corresponding author email: woerheide@lmu.de

Abstract

An accurate phylogeny of animals is needed to clarify their evolution, ecology, and impact on shaping the biosphere. Although multi-gene alignments of up to several hundred thousand amino acids are nowadays routinely used to test hypotheses of animal relationships, some nodes towards the root of the animal phylogeny are proving hard to resolve. While the relationships of the non-bilaterian lineages, primarily sponges (Porifera) and comb jellies (Ctenophora), have received much attention since more than a decade, controversies about the phylogenetic position of the worm-like bilaterian lineage Xenacoelomorpha and the monophyly of the "Superphylum" Deuterostomia have more recently emerged. Here we independently analyse novel genome gene content and morphological datasets to assess patterns of phylogenetic congruence with previous amino-acid derived phylogenetic hypotheses. Using statistical hypothesis testing, we show that both our datasets very strongly support sponges as the sister group of all the other animals, Xenoacoelomorpha as the sister group of the other Bilateria, and largely support monophyletic Deuterostomia. Based on these results, we conclude that the last common animal ancestor may have been a simple, filter-feeding organism without a nervous system and muscles, while the last common ancestor of Bilateria might have been a small, acoelomate-like worm without a through gut.

Introduction

Large multi-gene amino acid sequence (phylogenomic) datasets promised to achieve the phylogenetic resolution (Rokas et al., 2003) needed to accurately understand the evolution of life (Gaucher et al., 2010). These phylogenies enable inferences about the phenotype, physiology, and ecology of common ancestors of clades (Cannon et al., 2016; Schierwater et al., 2016), and to test hypotheses about the emergence of key innovations such as the nervous- and digestive systems (Haszprunar, 2016; Marlow & Arendt, 2014).

However, modeling the evolution of amino acid sequences is difficult (Philippe, Brinkmann, Lavrov, et al., 2011; Tihelka et al., 2021). Deep metazoan phylogenies reconstructed from alternative amino acid datasets, or even the same amino acid dataset analysed using different substitution models (Cannon et al., 2016; Philippe et al., 2019; Pisani et al., 2015; Whelan et al., 2015), as well as using different taxon samplings of the ingroup (Dunn et al., 2008; Pick et al., 2010) and the outgroup (Pisani et al., 2015; Whelan et al., 2015), are frequently incongruent. This acknowledged model- and data dependency of phylogenomic analyses underpins the phylogenetic instability observed towards the root of the animal tree (e.g., Dunn et al., 2014).

Although the sister group of all animals is well established - the Choanoflagellata, a group of single-celled and sometimes colonial collared and flagellated eukaryotes (King et al., 2008), three nodes towards the root of the animal tree are proving difficult to resolve using multi-gene amino acid datasets, hindering progress in understanding early animal evolution (Jékely & Budd, 2021).

The first recalcitrant node in the animal tree concerns its first offshoot, and the discussion largely centers around the question of whether sponges (Porifera) or comb jellies (Ctenophora) are the sister group of all the other animals (Dohrmann & Wörheide, 2013; Telford et al., 2016). This controversy impinges on our understanding of the last common ancestor of Metazoa (Ros-Rocher et al., 2021), and despite receiving much attention for more than a decade (e.g., Dunn et al., 2008; Feuda et al., 2017; Li et al., 2021; Philippe et al., 2009; Pick et al., 2010; Pisani et al., 2015; Redmond & McLysaght, 2021; Ryan et al., 2013; Shen et al., 2017; Simion et al., 2017; Telford et al., 2016; Whelan et al., 2015, 2017), it is not yet resolved.

Two other recalcitrant nodes have more recently been identified from alternative analyses of amino acid datasets that affect our understanding of the root of the Bilateria (all bilaterally symmetrical animals, including humans).

The first node involves the position of the worm-like Xenacoelomorpha, a bilaterian clade that unites the Acoelomorpha and Xenoturbellida (Philippe, Brinkmann, Copley, et al., 2011). With a few exceptions (Rouse et al., 2016), Xenacoelomorpha are millimeter-sized and primarily benthic or sediment dwelling bilaterians devoid of a true body cavity and an anus. Xenacoelomorpha has been recovered in different positions in the animal tree: as the sister group of all other bilaterian animals (Nephrozoa) (Cannon et al., 2016; Rouse et al., 2016), or as the sister group of the Ambulacraria (Echinodermata+Hemichordata) constituting the clade Xenambulacraria (Kapli & Telford, 2020; Philippe et al., 2019).

The second node concerns the Deuterostomia, one of the two main bilaterian lineages ("Superphyla"). Bilateria have long been split into two lineages, the Protostomia (Ecdysozoa + Lophotrochozoa) and the Deuterostomia (traditionally: Chordata + Ambulacraria [= Hemichordata + Echinodermata]), based on the different origins of the mouth and other features during development (e.g., Hyman, 1959). However, recent phylogenomic studies challenged the monophyly of Deuterostomia (Kapli et al., 2021; Marlétaz et al., 2019) and recovered paraphyletic deuterostomes in conjunction with Xenambulacraria. This combination of results, if confirmed, would have substantial implications for our understanding of the last common ancestor of all Bilateria, which might then have been a fairly large organism, with pharyngeal gill slits and other traits previously thought to represent apomorphies of Deuterostomia (see Kapli et al., 2021 for an in-depth discussion).

Accordingly, a stable resolution of the relationships of the Xenacoelomorpha with reference to the deuterostomes is key to correctly infer the condition of the last common ancestor of the Bilateria – a small and simple organism if Xenacoelomorpha are the sister group to the Nephrozoa, or a larger and much more complex organism if Xenambulacraria is correct and Deuterostomia is not monophyletic.

Considering that phylogenomic analyses are model- and data dependent, we must employ rigorous approaches to select between phylogenetic hypotheses. One way is to use model fit- and model adequacy tests to discriminate between alternatives, favoring those derived using

the best-fitting and most adequate model(s) (Feuda et al., 2017; Pisani et al., 2015). Alternatively, simulations can be used to compare alternative tree topologies and their chance of being inferred under different models (Kapli & Telford, 2020). Finally, independent data sources can be used to "triangulate" conflicting hypotheses (Munafò & Davey Smith, 2018).

Here we use two independent data types, genome gene content ("gene content") data and morphology, to evaluate alternative hypotheses of animal relationships that emerged from previous analyses of amino acid sequence data and investigate their relative consilience (Campbell et al., 2011; Rota-Stabelli et al., 2011). We focus on the three recalcitrant nodes mentioned above: the relative relationships of sponges and comb jellies with respect to the other animals, the relationships of the Xenacoelomorpha within the Bilateria, and the monophyly of the Deuterostomia.

The phylogenetic analysis of gene content data relies on the proteomes derived from fully sequenced genomes and converts the presence or absence of gene families in the genomes of the terminals into a binary data matrix (Leclère et al., 2019; Pett et al., 2019; Pisani et al., 2015; Ryan et al., 2013). We constructed separate datasets for "Homogroups" (homologous gene families) and "Orthogroups" (orthologous gene families). The former include homologous proteins that are predicted to be inherited from a common ancestor and can contain orthologs, xenologs, and out-paralogs, whereas the latter contains only proteins predicted to be inherited from a common ancestor and separated by a speciation event (see Methods for details).

We assembled a large number of new gene content datasets (see Methods, Fig. 1) to extensively test the effect of different parameter combinations when identifying homogroups and orthogroups, because this crucial step remains a challenge (Frech & Chen, 2010; Lunter et al., 2008) and may influence the outcome of the downstream phylogenetic analysis (Natsidis et al., 2021).

We also compiled different datasets to extensively evaluate other potential sources of error, such as the so-called "long branch attraction" (LBA) artifact (Felsenstein, 1978) (see Methods, Fig. 1). LBA occurs when two (or more) long branches in a phylogenetic tree group together without true relationship, generating "phylogenetic artifacts" (Philippe, Brinkmann, Lavrov, et al., 2011). Previous gene content analyses have focussed on the root of the animals. Accordingly, here we primarily focus our LBA assessment on the Xenacoelomorpha by performing taxon exclusion experiments in an approach similar to Philippe et al. (2019).

Additionally, we carefully collated a new 770-character morphological data matrix. As a starting point we built on the classical work of Peter Ax (Ax, 1996) that was systematised by Deline et al. (2018), and introduced additional information from two other reputable datasets (Goloboff et al., 2009; Peterson & Eernisse, 2001) to build our new data matrix. The coding of the base set was updated with the current interpretation of the morphology of groups such as Ecdysozoa and Xenacoelomorpha. In order to avoid artifacts caused by the lack of character comparability across the tree, we utilised two different coding strategies: non-additive and reductive coding (see Methods for details). Because the non-additive coding may be affected by taxa with many uncertain states, we ran the analyses with a reduced outgroup set, retaining only the Choanoflagellata, the sister group of animals. Other taxa exclusion experiments include runs without the taxa that showed problematic behaviour in the gene content analyses, the longest branches in the morphological trees, and parts of Xenacoelomorpha to check robustness.

Altogether, our results provide very strong support for the view that Xenacoelomorpha is the sister group of the rest of the Bilateria (Nephrozoa hypothesis). Monophyletic Deuterostomia is also largely supported. With respect to the sister group of all other animals, our results are fully consistent with sponges being the sister group of the rest of the animals (Porifera-sister hypothesis).

Results

Genome gene content data analyses

47 genome-derived proteomes were used to initially generate and analyse a total of 190 gene content datasets of different taxon samplings and parameter combinations (see Methods and data repository for details). The datasets were partitioned into several groups due to the different approaches applied (see below), all taxon sub-samplings and different parameter combinations were done in parallel for homologous gene families (“homogroups”) and orthologous gene families (“orthogroups”) (Pett et al., 2019) (Fig. 1). To assess the reproducibility of the results, the construction and analysis of the different datasets was performed twice (for results of the replicated analyses see Supp. Fig. 5; see the data repository for a more detailed explanation).

To test whether the specific phylogenetic relationships of the Xenacoelomorpha with reference to Deuterostomia were affected by LBA, different taxon sampling experiments,

based on a core taxon set of 40 species, were performed by defining three groups of datasets (Fig. 1): the "Opi" (Opisthokonta) group that consisted of all the datasets scoring a complete set of 47 taxa, including full outgroups. The "Aco" group consisted of all datasets that excluded *Xenoturbella* from the Opi dataset, and the "Xen" group consisted of all datasets that excluded the Acoelomorpha from the Opi dataset. Opi, Aco, and Xen included datasets with different parameter combinations for orthogroups and homogroups, resulting in 120 datasets in total (Fig. 1, see Methods for details).

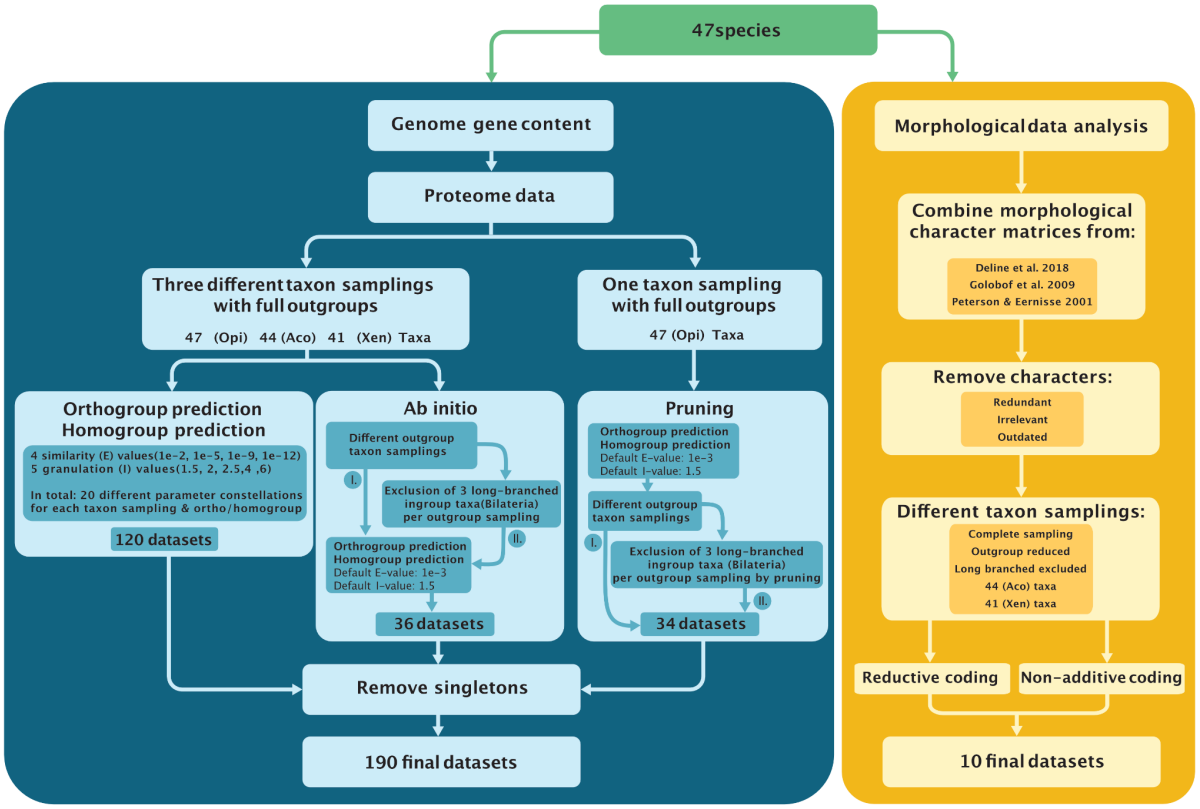

Figure 1: Concise graphical illustration of the methodology and workflow used for the creation of the different datasets analysed. *Left/Blue*: Genome Gene Content; *Right/Yellow*: Morphology (See data repository for the illustration of the complete steps of the gene content dataset creation).

With the same aim of LBA detection, additional 70 datasets were generated where distant outgroups (i.e., Fungi, Ichthyosporea) and the long-branched in-group (bilaterian) species *Caenorhabditis elegans* (Nematoda), *Pristionchus pacificus* (Nematoda), *and Schistosoma mansoni* (Platyhelminthes) were excluded and different methods were used to construct the datasets. Datasets were assembled using two strategies. First, the "*ab initio*" strategy carried out the whole homo/orthogroup prediction *de novo* on the reduced taxon samplings. Second, the "pruning" strategy pruned taxa from the full Opi homo/orthogroup data matrices which were constructed using default E (similarity) and I (granulation) values (Fig. 1, see Methods

for details). The *ab initio* vs. pruning dataset constructions aimed to assess the effect of those two approaches on the dimensions (gene family number) on the resulting datasets and phylogenies estimated from them.

The resulting topologies from the individual analysis were inspected manually (see Methods, Supp. Tables 2, 3 and Supp. Fig. 1). Additionally, Total Posterior Consensus Trees (TPCT; Supp. Data 4) were calculated for different datasets that summarise all trees sampled (after convergence) from all analyses with the exact same taxon sampling in a single Majority rule consensus tree. These trees are referred to as TPCT Opi (Fig. 2, Genome gene content), TPCT Opi-homo and Opi-ortho (Supp. Fig. 2, Fig. 5 A-B), TPCT Aco-homo and Aco-ortho (Supp. Fig. 3, Fig 5. C-D), and TPCT Xen-homo and Xen-ortho (Supp. Fig. 4, Fig. 5 E-F). Support for different hypotheses was then examined using statistical hypothesis testing (Bergsten et al., 2013; Kass & Raftery, 1995) (see Supp. Fig. 9-10).

Genome gene content further supports Porifera as the sister group of the other animals. Our new datasets provided the opportunity to investigate the most likely sister group of all other animals. In all 190 analyses, sponges emerged as a monophyletic group, and the TPCT Opi (Fig 2, genome gene content) indicates that the support across all analyses with a full taxon sampling is high with a Posterior Probability (PP) of 0.99 for Porifera as the sister group of the rest of the animals, with overwhelmingly strong statistical hypothesis test support (see Supp. Figs. 9, 10; Supp. Table 5). Ctenophora invariably emerged as the sister group of all the animals except sponges in the TPCTs; however, the support for this node is more variable in the different TPCTs derived from homogroups and orthogroups (PP=0.55-0.99; Supp. Figs. 2–4). The variable level of support indicates that some analyses found Ctenophora to be placed elsewhere in the tree.

Three alternative topologies were found for the placement of the Ctenophora when Porifera branched first (Supp. Fig. 1C, 2–5): Placozoa branches off before Ctenophora, the relationships between Ctenophora and Placozoa are not resolved, or Placozoa emerges as the sister group of Ctenophora. These appear in very low numbers of trees, mostly derived from homogroup-based datasets (see Suppl. Table 3 for details). In some cases, Placozoa emerges as the sister group of all animals (Supp. Table 3). Finally, Cnidaria appears as the sister group of the Bilateria in all analyses (PP=0.99).

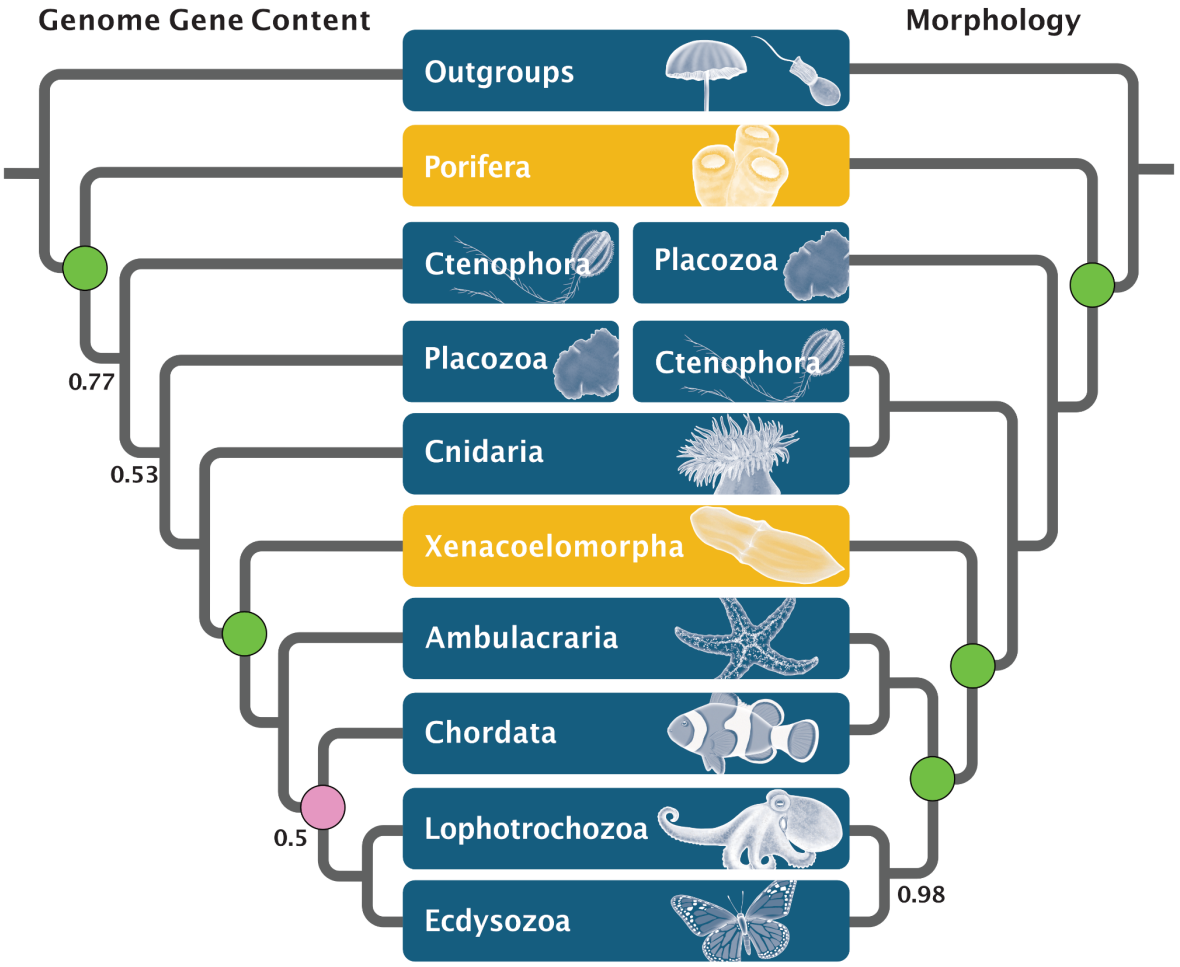

Figure 2: **Reconstruction of animal phylogeny with 47 species (Opi taxon sampling) based on gene content datasets (TPCT) and morphological data.** *Left*: Total consensus tree of >10.5 million individual tree samples from analyses using datasets of homogroups and orthogroups of all the different E- and I-values for genome gene content (for details see Materials and Methods, see Supp. Data 1 for details of analytical settings). *Right*: morphology-based phylogeny based on the non-additive coding scheme.
Note the different position of Ctenophora. Second to branch off in gene content and sister group to Cnidaria in morphology (i.e., Coelenterata) analyses. The monophyly of Deuterostomia is strongly supported by morphology but around 50% by gene content datasets.
Posterior probabilities lower than 0.99 are indicated on both phylogenies.
Statistical hypothesis tests of focal nodes: *Green circle* = node is strongly supported in the majority of tests conducted; *Purple circle* = node is not strongly supported in the majority of tests conducted (see Suppl. Figs 6, 9, 10 for details).

Genome gene content supports Xenacoelomorpha as the sister group of the other Bilateria

The 47-genomes Opi dataset included five Xenacoelomorpha species and the full outgroup taxon sampling (Fig. 1, see Methods). The Xenacoelomorpha were recovered as the highly-supported sister group of the rest of the Bilateria (Fig. 2, Genome gene content), consistent with the Nephrozoa hypothesis, irrespective of whether homogroups or orthogroups were used, and with different granularity values and different outgroup sampling.

Statistical hypothesis tests provided very strong support for the Nephrozoa hypothesis in 96% of the Opi, Aco and Xen datasets (Supp. Figs. 9-10). Similarly, datasets in the Aco group (those in which *Xenoturbella* was excluded) placed Acoelomorpha as the sister group of the rest of the Bilateria (both based on homogroups and orthogroups, Supp. Fig. 3). The overwhelming majority of the 41-genome datasets in the Xen group (those where Acoelomorpha were excluded) also resolved *X. bocki* as the sister group of the rest of the Bilateria (Supp. Fig. 3, 5, Suppl. Table 3). Finally, in the TPCT Opi-ortho, deuterostome paraphyly is supported but with low posterior probability (PP=0.77). Statistical hypothesis test support for deuterostome monophyly is strong from most Opi, Aco and Xen homogroup datasets, but not so from orthogroup datasets (See Supp. Figs. 9, 10).

Parameter changes affect mainly the final topologies in homogroup-based predictions. Different Similarity (E) and Granulation (I) values were used to construct the gene content datasets and evaluate their influence on dataset construction and downstream phylogeny estimation. Parameter changes resulted in final matrices with different numbers of characters, but always in the range of 20,000 to 80,000 genes (Supp. Table 2, 3). The choice of E-values did not significantly affect matrix reconstruction, but by contrast, the choice of granularity and whether homo- or orthogroups were used when defining matrices had significant but predictable effects.

It was expected that Orthogroup-based datasets contain a larger number of characters than the corresponding homogroup-based datasets (Supp. Fig. 1 A, B), because homogroups include multiple orthogroups. However, higher granulation values resulted in the identification of a higher number of smaller homo- and orthogroups, which translated into matrices with more characters. In datasets Opi, Aco, and Xen, the lower I-values resulted in phylogenies favoring the Porifera-sister hypothesis, Xenacoelomorpha as the sister group of the Nephrozoa, and monophyletic Deuterostomia (see Supp. Fig. 1C).

Phylogenies based on homogroups exhibit more variability in the resulting tree topologies than phylogenies based on orthogroups. However, while the overwhelming majority were consistent with the Porifera-sister hypothesis, 11.1% of all trees showed Placozoa as the sister group of all the other animals. From all analyses that showed Porifera-sister, less than 25% of datasets with high I-values placed *X. bocki* within Deuterostomia (see Supp. Fig. 1C and Supp. Table 3). Up to 75% of datasets have consistent support for the Nephrozoa hypothesis, independent of granulation values.

Paraphyletic Deuterostomia appears in around 25% of the trees with high granulation values (Supp. Fig. 1C), while in the rest of the treatments it appears in less than 25% of the trees. The variability of the phylogenies obtained with high granulation values is also reflected in the statistical hypothesis tests performed, where high granularity of homogroups did not support any of the tested constraints (Supp. Data 5). The prediction of homo- or orthogroups appears to affect the support for deuterostome paraphyly; orthogroups favor it, while homogroup-based datasets do not (Supp. Fig. 1-4).

The Porifera-sister hypothesis is robust to outgroup sampling as indicated by their very strong statistical hypothesis test support (see Supp. Table 5). The Nephrozoa hypothesis received very strong statistical hypothesis test support for the reduced outgroup sampling datasets (see Suppl. Table 5). All reduced taxon-sampling phylogenies where Porifera branched first supported monophyletic Deuterostomia (Supp. Fig. 1C).

The different taxon exclusion schemes showed high variations in the number of characters in the final data matrices (Supp. Fig. 1A). However, only minor topological changes were observed in phylogenies reconstructed with different numbers of characters, compared to the phylogeny displayed in Fig. 2 (Genome gene content). *Xenoturbella bocki* was only recovered in an intra-nephrozoan location three times, all three were from the Holozoa datasets (Supp. Table 5).

Morphological data analyses

The morphological data sets constructed here are the first to include the state of the art knowledge about the shared characters across Xenacoelomorpha. Two different coding schemes, i.e., non-additive and reductive coding (Methods; Fig. 1, Supp. Data 1) were applied to the morphological dataset. In addition to the different coding schemes, four taxon exclusion experiments were performed: a version with a reduced outgroup, where all the non-metazoan outgroups except the choanoflagellates were excluded from the taxon sampling, two matrices with the 41 and 44 taxon samplings (see above) and a set without the three taxa with the longest morphological branches (dataset name Morphology Long Branches, MLB) in the previous analyses (*Ixodes scapularis* [Arthropoda], *Danio rerio*, *Gallus gallus* [both Chordata]). All ten analyses resulted in similar topologies (see data repository for details). The analysis of the non-additive matrices exhibits heterogeneous branch lengths and high node support across the phylogeny (Fig. 2, Morphology). The phylogeny resulting from the datasets applying reductive coding has lower node support, with

three polytomies in the ingroup (within echinoderms, chordates and the sponge classes) (Supp. Fig. 7).

The only notable difference between the results of these analyses are the relationships within Porifera. In all phylogenies, sponges branched off first (Fig. 3 Morphology, Supp. Fig. 7). However, in the reductive-coding datasets, sponges are paraphyletic, with demosponges branching off first and the Homoscleromorpha and Calcarea in a polytomy with the rest of the animals. In both datasets, Placozoans branched off next and are the sister group of the traditional Eumetazoa (PP=1.0 for non-additive coding, and PP=0.89 for reductive coding). Within eumetazoans, ctenophores are the sister group of the Cnidaria (Coelenterata) (PP=1.0 for non-additive coding, and PP=0.65 for reductive coding).

The hypothesis that Xenacoelomorpha is the sister group of the Nephrozoa is fully supported in the non-additive coded dataset (Supp. Fig. 6) and the outgroup-reduced reductive coded dataset (Supp. Fig. 8), but slightly less supported in the complete sample reductive-coded phylogeny (PP=0.9) (Supp. Fig. 7). The internal relationships of Bilateria show monophyletic Nephrozoa, Deuterostomia, Protostomia, Ecdysozoa, and Lophotrochozoa in all the coding schemes applied.

The statistical hypothesis tests found strong to very strong support for the topology displayed in Fig. 2 (Morphology) in the three different morphological analyses (Supp. Fig. 6). The Nephrozoa hypothesis and the Porifera-sister hypothesis have consistent very strong support. Deuterostome monophyly has strong support in the reductive coding, but very strong support in the non-additive coding (see Supp. Table 5 for the exact values).

Statistical hypothesis tests support monophyletic Deuterostomia

Although the gene content TPCT displayed in Fig. 2 shows paraphyletic Deuterostomia, this tree topology received only lower support (PP=0.5). Statistical hypothesis tests (Supp. Fig. 10, and details above) showed that monophyletic Deuterostomes was consistently and very strongly supported in the majority of datasets analysed, except for orthogroup taxon sampling Opi with granulation values other than the default value of 1.5, and homogroup taxon sampling Opi with higher granulation values of 4 and 6, as well as taxon sampling Xen with a granulation value of 6. The statistical hypothesis tests of the morphological data (Supp. Fig. 6) provided strong to very strong support for monophyletic Deuterostomes.

Discussion

We analysed new genome gene content datasets constructed under various settings and with various taxon samplings, and novel morphological character matrices. In contrast to primary sequence-based phylogenies, the use of gene content in phylogenetics is a comparably recent development (Leclère et al., 2019; Pett et al., 2019; Pisani et al., 2015; Ryan et al., 2013) and has been advocated to complement amino acid phylogenomic analyses (Dunn et al., 2014). This approach relies on the correct estimation of the underlying ortho- and homogroups, which is affected by the tool- and parameter choices (Remm et al., 2001).

In order to understand the effect of different parameter combinations on the prediction of ortho- and homogroups in gene content-based phylogenies, we tested a variety of similarity (E) and granulation (I) values. The differences in the numbers of characters in our datasets, as parameters change, is consistent with the observation that the identification and delimitation of gene families is difficult (Frech & Chen, 2010; Lunter et al., 2008). However, we observed good congruence across datasets over the topology in Fig. 2 (Genome Gene Content), indicating that errors induced by ortho- and homogroup misidentifications were negligible (contra Natsidis et al., 2021).

Potential biases can be induced in the results of gene content analyses when the available genomes are fragmented. While we strived to use high quality genomes only, some were still fragmented, and even recent “chromosome-level” genome assemblies can not guarantee a complete and unfragmented set of the gene content of a species. For example, the genome of *Ephydatia muelleri*, not available at the time we assembled our data set in 2018, is dispersed over 1419 scaffolds, even though about 84% of it was contained in the 24 largest scaffolds, encompassing 22 of the 23 chromosomes (Kenny et al., 2020). Virtually complete chromosome scale genome assemblies of non-bilaterians are only now starting to appear, i.e., the ctenophore *Hormiphora californensis*, where 99.47% of the genome are contained in 13 scaffolds (Schultz et al., 2021).

While the ascertainment bias correction introduced and used in the gene content analyses of Pisani et al. (2015) and Pett et al. (2019) accounts for unobserved genes in all species, no correction currently exists to account for unobserved genes in individual species, the type of bias that may be induced by incomplete genomes. However, we used ortholog and homolog identification methods that are standard in the field (see Methods) and those do not rely on complete genes, but assess the given sequence. Nonetheless, developing additional

corrections to account for potential errors introduced during *in silico* genome assembly and annotation could be a fruitful avenue for future research.

Considerable attention was given to the investigation of putative long-branch attraction artifacts (LBA) that might have caused a placement of Xenacoelomorpha at the root of Bilateria and the sponges at the root of the animals. To achieve this goal we performed taxon exclusion experiments, similar to Pisani et al. (2015) and Philippe et al. (2019). Based on our tests, where we do not see taxa changing position as the ingroup and the outgroup are subsampled, we suggest that the placement of Porifera and Xenoacoelomorpha in our trees does not seem to be affected by LBA.

Based on multi-gene alignments, several studies showed that the evolutionary model used can affect the inferred topologies (e.g., Feuda et al., 2017; Kapli & Telford, 2020; Li et al., 2021; Redmond & McLysaght, 2021; Simion et al., 2017; Whelan et al., 2015). For the burgeoning field of the phylogenetic analysis of gene content data, model development is still limited. Pett et al. (2019) applied both the Dollo model, in which, if applied to gene content data, each gene family may be gained only once on a tree. They also applied a reversible binary substitution model, in which a gene family may be gained more than once on a tree. Both models recovered identical topologies, but the reversible binary substitution model, also used here, was shown to have the best fit for this type of data. In any case, additional and more biologically realistic evolutionary models need to be developed to analyse this type of data that may show better fit and adequacy.

The independently estimated phylogeny from the morphological dataset is fully consistent with the results from the gene content analyses concerning the placement of Porifera and Xenacoelomorpha. A notable difference concerns the position of Ctenophora, which appears as the sister group of Cnidaria, forming the classic Coelenterata (Zhao et al., 2019) (Fig. 2, Morphology). Deuterostomes are recovered as monophyletic in the morphology-based phylogeny, different from their paraphyly as recovered in a few gene content analyses.

Our genomic and morphological results agree with each other, with previous genome content analyses (Pett et al., 2019; Pisani et al., 2015), and with phylogenetic trees of amino acid datasets supporting the Nephrozoa (Cannon et al., 2016; Rouse et al., 2016) and Porifera-sister hypotheses (Feuda et al., 2017; Kapli & Telford, 2020; Nosenko et al., 2013; Philippe et al., 2009; Pick et al., 2010; Pisani et al., 2015; Redmond & McLysaght, 2021; Simion et al., 2017). Our results on the other hand are in disagreement with studies that

identified Ctenophora as the sister of all the other animals (Chang et al., 2015; Dunn et al., 2008; Hejnol et al., 2009; Li et al., 2021; Moroz et al., 2014; Ryan et al., 2013; Whelan et al., 2015, 2017), and Xenambulacraria (Bourlat et al., 2006; Kapli et al., 2021; Kapli & Telford, 2020; Philippe, Brinkmann, Copley, et al., 2011; Philippe et al., 2019).

Nonetheless, irrespective of the arrangement of the lineages towards the root of the animal tree, the transition to animal multicellularity from a unicellular last common ancestor was marked by an expansion of a preexisting genetic toolkit to enable multicellularity (Sebé-Pedrós & de Mendoza, 2015). The functionalities necessary for this transition, such as cell adhesion, were already present in the closest protist relatives of animals, the Choanoflagellata (Ros-Rocher et al., 2021). Additionally, new protein domains evolved in the Urmetazoan that enabled more complex traits (Adamska et al., 2007; Marlow & Arendt, 2014; Nichols et al., 2006; Radha et al., 1996), for example novel signaling pathways, such as tyrosine kinases signal transduction cascades (Radha et al., 1996) and many components of *Wnt* pathway (Nichols et al., 2006), and transcription factors, such as the common glutamate GABA-like receptors (Müller, 2001).

In any case, our conciliated results allow for addressing more conclusively questions about early animal evolution. If we accept that sponges are the sister group of the rest of the animals (Fig. 2), it can not be excluded that the last common animal ancestor (the Urmetazoan) may have been a sponge-like organism that fed using choanocyte-type cells (Nielsen, 2008). However, the homology of the collar apparatus in the Choanoflagellata, the sister group of animals, with the one of the choanocyte in sponges is currently disputed (Mah et al., 2013; Pozdnyakov & Karpov, 2013; Sogabe et al., 2019). In spite of that, whatever the true phenotype and metabolic capacities (Mills et al., 2018) of this urmetazoan were, the key innovations required for animal multicellularity must have happened along the stem lineage towards this urmetazoan. Furthermore, if the Porifera-sister hypothesis is correct, the last common ancestor of animals might have lacked most recognizable metazoan cell types and organ systems, despite having the capacity to transit between different cell states similar to stem cells (Sogabe et al., 2019).

If we accept that Xenacoelomorpha is the sister group of the rest of the Bilateria (Nephrozoa) and Deuterostomia is monophyletic, the urbilaterian (the last common ancestor of Bilateria) was most likely an acoelomate worm (Cannon et al., 2016). This contrasts scenarios (e.g., Balavoine & Adoutte, 2003) that posit a very complex urbilaterian that could have possessed

a coelom, metameric segmentation, and many other bilaterian organ systems. The most notable feature of the urbilaterian would be the lack of any ultrafiltration organs or cell types (Cannon et al., 2016; Perea-Atienza et al., 2015). This lack has been argued to be primary because most xenacoelomorphs are predators and a system for nitrogen excretion is very beneficial for animals with protein-rich diets (Haszprunar, 2016). Other notable aspects would be the presence of a blind stomach without an anus and their simple gonads which would have been more similar to those of non-bilaterians. Nevertheless, the high morphological disparity present within extant xenacoelomorphs introduces some uncertainty about the plesiomorphic status of many features. Their nervous systems, for example, are extremely varied (Jondelius et al., 2019) and the presence of eyes in their last common ancestor can not be established with confidence (Haszprunar, 2016).

Elucidating the origin of bilaterians is also fundamental for our understanding of the early history of our biosphere. The precise sequence of character acquisition is important because it can be correlated with the appearance of more complex body plans and new metazoan ecological guilds such as burrowers and grazers. For example, in the early Cambrian fossil record, it has been postulated that the rising abundance of burrowing bilaterian animals led to the decline of the dominant Precambrian bacterial mats and an initial diversification of ecological interactions – the "agronomic revolution" (Seilacher, 1999).

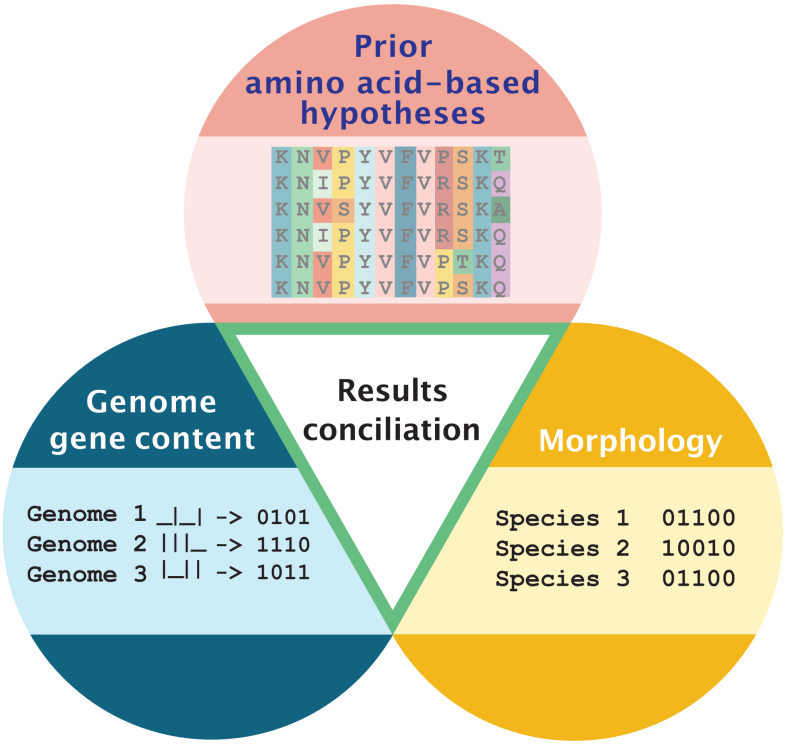

Figure 3: Illustration of the different data sources used in this study to conciliate results. In circles are the different data sources. *Top/Red*: This data (amino acid sequence-based multi gene alignments) is not used here but the competing hypotheses about the relationships towards the root of the animal tree of life assessed in this study are derived from previous publications that used this data type. *Left/Blue*: Genome gene content. This data is used here. *Right/Yellow*: Morphological characters. This data is used here.
Middle triangle: The outcome of independent sources of information allows the conciliation of the results.

In summary, we independently analysed two lines of evidence, i.e., novel gene content and morphological data matrices, and investigated the robustness of different parameter constellations, including taxon sampling, on the resulting phylogenies. Our results provide further evidence to resolve recalcitrant nodes in the animal phylogeny.

With reference to the root of the animals, where the debate is quite mature, and many contributions from different fields exist (Dohrmann & Wörheide, 2013; Dunn et al., 2008; Feuda et al., 2017; Hejnol et al., 2009; Kapli & Telford, 2020; Li et al., 2021; Moroz et al., 2014; Nosenko et al., 2013; Philippe et al., 2009; Pick et al., 2010; Pisani et al., 2015; Redmond & McLysaght, 2021; Ryan et al., 2013; Simion et al., 2017; Telford et al., 2016; Whelan et al., 2015, 2017; Zhao et al., 2019), our results further strengthen the view that sponges are the sister group of all the other animals. However, resolving the exact relationships of the Ctenophora and Placozoa with respect to the Cnidaria and the Bilateria remains a future challenge.

With reference to the phylogenetic placement of the Xenoacoelomorpha, our analyses favour and further strengthen the Nephrozoa hypothesis. However, the debate on the placement of the Xenoacolemorpha is much less developed (Bourlat et al., 2006; Cannon et al., 2016; Kapli et al., 2021; Kapli & Telford, 2020; Philippe, Brinkmann, Copley, et al., 2011; Philippe et al., 2019; Rouse et al., 2016), with some key new hypotheses (e.g., the non-monophyly of Deuterostomia) recently emerging (Kapli et al., 2021; Marlétaz et al., 2019). Clearly, more studies, using different datasets and methods, as well as the development of more sophisticated evolutionary models for the analysis of gene content data, are necessary to more firmly establish the relationships at the root of the Bilateria.

3. Methods

Data set creation

1. *The general strategy for assembly of the genome gene content datasets*

Publically available proteomes derived from full genome sequences of 47 species were collected in 2018 (Supp. Table 1), representing 17 phyla, to create a balanced taxon sampling across animal phyla, supplementing the taxon sampling of Pett et al. (2019). The collection of proteomes also included non-metazoan outgroups sampled across Opisthokonta (Fungi + Ichthyosporea + Choanoflagellates + Metazoa; Supp. Data 1).

The core taxon set in the datasets included 40 species (bold in Supp. Table 1), from which additional taxon samplings were created. The 47-species Opisthokonta (Opi) taxon set contained the full set of species, and is the largest genome gene presence/absence dataset to date. Two additional taxon sets (see Fig. 1; Supp. Data 1, 2) with different taxon samplings of the Xenacoelomorpha were assembled adding species to the 40-species core set: a 44-species dataset that had four Acoelomorpha species and no *Xenoturbella bocki* (specified with "Aco" in the dataset name) and a 41 species dataset that had only *X. bocki* and no Acoelomorpha (specified with "Xen" in the dataset name). The rationale behind this taxon-pruning approach was to test for long-branch attraction artifacts in the ingroup (following Philippe et al. (2019)) that may impact the relationships of the Xenacoelomorpha. Each taxon sampling was analysed based on (1) homogroups, i.e., a dataset including homologous proteins that are predicted to be inherited from a common ancestor. They can include orthologs, xenologs, and out-paralogs, and (2) orthogroups, i.e., a dataset containing proteins predicted to be inherited from a common ancestor and separated by a speciation event.

Datasets were constructed using different parameters of similarity (E-value) in DIAMOND and granulation (Inflation parameter; I) in the MCL algorithm. Granulation affects the cluster size, i.e., the number of the predicted clusters (gene family content). Small I-values indicate coarse-grained clustering resulting in larger clusters, and large values a fine-grained clustering, chopping the big clusters into smaller fractions (Enright et al., 2002). Increasing the inflation parameter (I) leads to further splitting of the most significant clusters, therefore more and smaller clusters.

For all species in the dataset where only coding sequences (CDS) were available, transdecoder (Haas, 2017) was used to extract the best possible prediction of open reading

frames (ORF) and corresponding proteins. All proteomes were analysed using a general approach similar to Pett et al. (2019), but with different tools. A homology search of the individual proteomes against each other was conducted with a combination of four different E-values. The search was performed using Diamond v0.9.22.123 (Buchfink et al., 2015) for the E-values of 1e-2, 1e-5, 1e-9, and 1e-12. To obtain orthogroups, we used OrthoFinder v2.3.7 (Emms & Kelly, 2015) with the Diamond option. To establish the homogroup datasets, we used homomcl (Pett et al., 2019) with a Diamond search. MCL v14-137 (Enright et al., 2002) was used to cluster the different gene sets with five I parameters: 1.5 (default), 2, 2.5, 4, and 6 (Ballesteros & Sharma, 2019; van Dongen & Abreu-Goodger, 2012). Similar to Pett et al. (2019), we applied a correction for the ascertainment bias by removing all singletons (i.e., sequences that appear to be present in only one genome) from each presence/absence matrix (gene groups represented by single species). Both homogroup and orthogroup datasets therefore do not contain any single species homo- or orthogroups (singletons), i.e., proteins need to be shared by at least two species. The final matrices of homogroup/orthogroup presence/absence for phylogenetic analyses were generated with custom python and BASH scripts. For the dataset naming convention used here, see Supp. Table 4.

All steps of the analysis (dataset construction, phylogenetic analyses) were performed twice to ensure reproducibility, resulting in a total of 380 different datasets analysed.

1) ***Datasets to test for long-branch attraction artifacts (LBA)***

Using the default E-value of 1e-3 and I-value of 1.5 in OrthoFinder, Diamond, and MCL, we further tested the outcome of different species combinations. The complete taxon sampling of the 47 Opisthokonta (Opi) species and the two subsets Aco and Xeno, were used to construct further reduced datasets for two different approaches (see Fig. 2). These are divided into two sub-categories to test for putative long-branch attraction artifacts by either outgroup taxa exclusion or by excluding long-branched ingroup taxa from the taxon sampling.

Outgroup taxa exclusion

We tested the effect of reducing certain taxa in two possible methods: first analyzing the data after reducing the taxa from start to end and second by reducing the data from an

already pre-analysed matrix. The latter can significantly reduce the complexity of the analyses.

1) All outgroups but the Choanoflagellates, the sister group of the Metazoa, were successively excluded from the full 47-species Opisthokonta (Opi) taxon set, and a new OrthoFinder search was conducted to create three different taxon sets, namely ii) Ichthyosporea + Choanoflagellata + Metazoa (= Holozoa; dataset prefix Holo), and iii) Choanoflagellata + Metazoa (= Choanozoa; dataset prefix Cho) (Torruella et al., 2012), see Supp. Data 1 for more details.

2) All outgroups but the Choanoflagellates were pruned from the whole taxon set above. However, the initial character matrix derived from the full Opi dataset was used (no new OrthoFinder search), deleting new singletons and orphans (that resulted from taxon deletion) instead of re-running OrthoFinder. We refer to this approach as pruning see Supp. Data 1 for more details.

Exclusion of long-branched ingroup taxa

3) The long-branched species *Caenorhabditis elegans* (Nematoda), *Pristionchus pacificus* (Nematoda), and *Schistosoma mansoni* (Platyhelminthes) were excluded from each of the different taxon sets described above. The complete analysis of ortho- and homogroups estimation was rerun from start to end (*ab initio*). The datasets analysed were Opi-homo/ortho-Ab, Hol-homo/ortho-Ab, and Cho-homo/ortho-Ab, where Ab refers to *ab initio*; see Supp. Data 1 for more details.

4) The long-branched species *Caenorhabditis elegans* (Nematoda), *Pristionchus pacificus* (Nematoda), and *Schistosoma mansoni* (Platyhelminthes) were excluded from the final matrix of 47 species together with the outgroups, but without re-running the complete analysis of ortho- and homogroups estimation from start to end, creating three more datasets: Opi-homo/ortho-P, Hol-homo/ortho-P, and Cho-homo/ortho-P, where P refers to *pruning*; see Supp. Data 1 for more details.

These experiments of different taxon-samplings for homogroups and orthogroups resulted in 70 additional datasets analysed and phylogenies estimated. For a full illustrated explanation of the different datasets created, see Fig. 1 (main manuscript) and Figure "All_graph.p" of the data repository in folder "Additional information".

Phylogenetic analysis based on genome gene content data matrices

All matrices generated were analysed with the MPI version of RevBayes v1.0.14 (Höhna et al., 2016). The reversible binary substitution model (Felsenstein, 1992; Ronquist et al., 2012) was used for phylogenetic analysis, as it was found to have the best fit to gene content data in Pett et al. (2019) (for details see Supp. Data 6). Each run was conducted with four replicated MCMC runs of 50,000 to 80,000 generations to achieve full convergence. Convergence of the four runs was assessed with bpcomp and tracecomp of PhyloBayes v4.1c (Lartillot et al., 2009). An ESS value >300 and bpdiff values <0.3 were used as thresholds to indicate convergence.

Majority rule consensus trees were calculated with bpcomp of PhyloBayes v4.1c (Lartillot et al., 2009) for each dataset and i) from the individual four MCMC runs of each of the matrices that achieved convergence; ii) from all posterior trees from all converged MCMC runs of homo- and orthogroup datasets, all different E-value (similarity) and granulation value (I) constellations with the same taxon samplings. The resulting phylogeny thus represents the total majority rule consensus tree of all posterior trees (TPCT).

For a detailed methodological explanation of Total Posterior Consensus Tree (TPCT) see Supp. Data 4.

The final trees were visualized with Figtree v1.4.4 (A. Rambaut, 2012), all the trees were rooted with the most distant outgroup (Supp. Table 1).

Phylogenetic analysis based on morphological characters

The taxon sampling of the morphological data matrix was tailored to be identical to the 47-taxon Opi gene content dataset to make the results fully comparable (see data repository). The set of 770 morphological characters is a curated combination of three different previously published datasets: 1) Dataset 1 (Goloboff et al., 2009) was used due to its broad eukaryotic sampling, including some fungi and non-metazoan holozoans needed for the coding of the outgroups. 2) Dataset 2 (Deline et al., 2018) represented the animal backbone as the most comprehensive and exhaustive source of general animal morphological characters. 3) Dataset 3 (Peterson & Eernisse, 2001) was added because it included more up-to-date interpretations of some morphological features. Although Dataset 2 (Deline et al., 2018) is an extensive dataset, it is based on the classical work of Peter Ax from 1996 (Ax, 1996) and, consequently, some well-established changes in the scoring of some characters were needed.

For example, characters regarding cuticles and molting not known at the time of Ax work to define the Ecdysozoa (Schmidt-Rhaesa et al., 1998) were coded independently for "nemathelminthes" and arthropods in the original dataset.

The final character list analysed here (Supp. Data 3) was constructed by first combining the character lists of the publications as mentioned above. Then, the combined list was manually checked, and some characters were removed based on four criteria: 1) characters that were redundant (i.e., that reference the same information); 2) characters that only make reference to the specific morphology of clades that were not included in the sample; 3) highly debated characters where the homology was uncertain and has been questioned through independent lines of research, like the homology of "articulatan" (the classical grouping of annelids and arthropods) features (Schmidt-Rhaesa et al., 1998); and 4) characters that would have to be coded as unknown for most taxa because we are coding at the species level (i.e., reproductive, developmental and molecular).

In addition to the full 47 taxa set, four taxon sampling experiments were performed by pruning taxa from the full taxon samplings similar to the gene content analyses: two datasets without the two problematic/unresolved echinoderms and a subsample of Xenacoelomorpha (only Xenoturbella and only Acoelomorpha, respectively); a dataset without long branches observed in preliminary morphological analyses (*Danio rerio*, *Gallus gallus*, *Ixodes scapularis*); and lastly a dataset excluding all outgroups except the two choanoflagellates.

All morphological data matrices are available in the data repository. We analysed the morphological data matrices in MrBayes (Huelsenbeck & Ronquist, 2001). We used a Markov *k* (Mk) model, where *k* represents the number of states for a character, to model transitions between character states ((Huelsenbeck & Ronquist, 2001; Lewis, 2001)). Additionally, we assumed that only variable characters (Mkv model) were used and therefore applied the commonly used ascertainment bias correction ((Huelsenbeck & Ronquist, 2001; Lewis, 2001)). We ran two replicate MCMC analyses with two million iterations per chain for each dataset. The reductive-coded Opi and Aco sets were run for 10 million because they had not fully converged after the initial two million generations. We checked for convergence using Tracer v1.7.1 (Andrew Rambaut et al., 2018).

Hypothesis testing

We used posterior odds (Bergsten et al., 2013; Kass & Raftery, 1995) to test statistical support for three competing hypotheses: (1) the Porifera-sister vs Ctenophora-sister hypotheses, (2) Nephrozoa vs Xenambulacraria hypotheses, and (3) Deuterostome monophyly vs Deuterostome paraphyly. Specifically, we computed the statistical support in favor of the null model M_0 over the alternative model M_1. Following standard statistical practice (Kass & Raftery, 1995), we used the log-posterior odds of larger than 1 as substantial support, larger than 3 as strong support, and larger than 5 as very strong support. For a detailed explanation of the statistical hypothesis tests carried out see Supp. Data 5.

Code availability

All data and code necessary to reproduce results are available in a public repository https://github.com/PalMuc/triangulation

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