Mitigating anticipated effects of systematic errors supports sister-group relationship between Xenacoelomorpha and Ambulacraria.

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Summary.

Xenoturbella and the acoelomorph worms (Xenacoelomorpha) are simple marine animals with controversial affinities. They have been placed as the sister group of all other bilaterian animals (Nephrozoa hypothesis) implying their simplicity is an ancient characteristic [1, 2]; alternatively, they have been linked to the complex Ambulacraria (echinoderms and hemichordates) in a clade called the Xenambulacraria [3-5], suggesting their simplicity evolved by reduction from a complex ancestor. The difficulty resolving this problem implies the phylogenetic signal supporting the correct solution is weak and affected by inadequate modelling, creating a misleading non-phylogenetic signal. The idea that the Nephrozoa hypothesis might be an artefact is prompted by the faster molecular evolutionary rate observed within the Acoelomorpha. Unequal rates of evolution are known to result in the systematic artefact of long branch attraction which would be predicted to result in an attraction between long branch acoelomorphs and the outgroup pulling them towards the root [6]. Other biases inadequately accommodated by the models used can also have strong effects, exacerbated in the context of short internal branches and long terminal branches [7]. We have assembled a large and informative data set to address this problem. Analyses designed to reduce or to

emphasise misleading signals show the Nephrozoa hypothesis is supported under conditions expected to exacerbate errors and the Xenambulacraria hypothesis is preferred in conditions designed to reduce errors. Our reanalyses of two other recently published data sets [1, 2] produce the same result. We conclude that the Xenacoelomorpha are simplified relatives of the Ambulacraria.

Results

Assembling our data matrix

In order to provide the best chance of avoiding artefacts generated by data errors [7, 8] we assembled a new data set of 1,173 genes (350,088 amino acid positions) from a balanced and rich selection of 59 taxa with just 23.5% missing data, giving us a matrix that is larger and more complete than any previously used to examine the question. Our new matrix has been carefully curated to minimise potential errors from sources including contamination and non-orthology. Alongside existing data, it includes new gene predictions from 6 partial genomes and 4 new transcriptomes.

New predicted protein sets were derived from partial genomes of *Xenoturbella bocki*, *Symsagittifera roscoffensis*, *Meara stichopi*, *Nemertoderma westbladi*, *Pseudaphanostoma variabilis* and *Praesagittifera naikaiensis*; from new transcriptomes of *Xenoturbella bocki*, *Symsagittifera roscoffensis*, *Paratomella rubra* and *Isodiametra pulchra* and from published data available at the NCBI. To produce a balanced and computationally tractable data set we selected approximately equal numbers (6-8) of diverse species from the following clades: Xenacoelomorpha, Hemichordata, Echinodermata, Chordata, Lophotrochozoa, Ecdysozoa, Cnidaria and Porifera plus the placozoan *Trichoplax adhaerens*. We omitted members of Ctenophora due to their well-documented fast evolutionary rate [9]. From these original sets of predicted protein sequences, we used OMA to identify probable groups of orthologs covering the Metazoa [10, 11]. As OMA is rather stringent and can therefore omit valid

orthologs, we added some missing orthologs using the 42 pipeline (https://bitbucket.org/dbaurain/42/downloads/). These putative orthologs were then tested for possible cross contamination, non-orthology and other issues likely to affect accurate phylogenetic reconstruction (see methods). Our final data set contained 1,173 orthologous genes from 59 species of animals giving a total of 350,088 aligned amino acids.

Comparisons with existing recent data matrices

We compared our matrix to the two most recent studies addressing the question of the affinities of the Xenacoelomorpha in terms of data quality (percent of clades present in the concatenated tree that are also present in single gene trees) and quantity (number of amino acids present in the supermatrix: this number comes from the total number of amino acids in the matrix; if there were no missing data this would equal length of alignment multiplied by the number of species). Our dataset is among the largest and of the highest quality: our single-gene trees recover >50% on average of the expected clades, whereas the average for the other data sets is 29% (maximum 39% - See Figure S4D). This indicates that our dataset likely contains fewer erroneous data (e.g. contaminants, paralogs, frameshifts) than others and is therefore likely to contain more genuine phylogenetic signal: a prerequisite to infer phylogenies accurately [7, 9].

Analyses of our data using site heterogeneous models show limited support for Xenambulacraria

We analysed our complete matrix using a gene jackknife approach, which provides a conservative measure of clade support while being computationally tractable [9]. We used cross validation to compare the fit of different models of sequence evolution on all data sets and found that the CATGTR model was the best fitting in all cases. We therefore used the CATGTR model of PhyloBayes [12] with a gamma correction for between site rate variability to analyse 100 subsamples each containing ~90,000 positions from the complete data set.

We found weak support (60% jackknife support) for a monophyletic grouping of Xenacoelomorpha and Ambulacraria. The second best supported topology grouped Xenacoelomorpha with Protostomes (24% jackknife support) and Nephrozoa had 13% jackknife support. Other uncontroversial clades in the tree were reconstructed with strong support (Figure 1A,B). In common with some previously published results [13, 14], the relationships between Chordata, Xenambulacraria and Protostomia were unresolved - we did not reconstruct a monophyletic Deuterostomia (Chordata plus (Xen)ambulacraria).

Removing fast evolving Acoelomorpha reduces support for Nephrozoa

Our approach to testing the possible effects of systematic error is to consider situations in which we can predict whether, if the tree is influenced by artefacts, nodal support will increase or decrease using different subsets of data or analytical methods. Manipulations expected to strengthen artefactual signal (less adequate models or subsets of data with an exaggerated systematic bias) are expected to increase support for the artefactual topology and vice versa, while the genuine phylogenetic signal should remain unaffected. One established approach for dealing with LBA is to remove the fastest evolving members of the group of interest [6]. If the Nephrozoa signal depends on an LBA artefact, we predict support for Nephrozoa would decrease in favour of Xenambulacraria when fast evolving members of Xenacoelomorpha are removed. The Acoelomorpha have clearly evolved more rapidly than *Xenoturbella* (Figure 1A) and this difference seems to be mirrored in the more derived gene content of acoelomorph genomes [15, 16].

The validity of this approach requires the Xenacoelomorpha to be monophyletic. In our jackknife tree, and in previous phylogenomic analyses, the Xenoturbellida is strongly supported as the sister group of Acoelomorpha. This conclusion is further supported by a *Xenoturbella*/Acoelomorpha specific rare genomic change involving their Caudal/CDX ortholog (Figure S4E). If we therefore accept Xenacoelomorphs as monophyletic, it is

legitimate to use the slowly evolving member of the clade (*Xenoturbella*) as a representative of the Xenacoelomorpha, so reducing the effects of rapid evolution in the Acoelomorpha. When we removed the long branched Acoelomorpha but included the slower evolving *Xenoturbella* and repeated the jackknifing of the complete data set, the support for Xenambulacraria increased to 81% (Figure 1C). This result is consistent with the support for Xenacoelomorpha being reduced in part due to LBA caused by the fast evolving Acoelomorpha.

Stratifying genes according to phylogenetic accuracy: genes with difficult to extract phylogenetic signal support Nephrozoa

A given gene is expected to vary in its ability to reconstruct the phylogeny of interest according to the method being used. More accurate genes ('better' genes with respect to the phylogenetic method used) will have more appropriate or more even rates of substitution or, more generally, some genes may fit the assumptions of the models used more closely than others; equally, some alignments may contain non-orthologous - e.g. contaminant - sequences. We reason that the genes that perform best at reconstructing known clades with a given method should be the most reliable when solving a related phylogenetic problem. To stratify the genes in our concatenated alignment according to their ability to reconstruct an accurate tree, we measured the capacity of each gene to reconstruct uncontroversial monophyletic groups of animals using two different methods that gave virtually identical results. After stratifying our genes, we concatenated them in order from best to worst and took the genes covering the first 25% of genes (best) and those covering the last 25% of genes (worst). The proportions of missing data and constant positions were similar for the two sub-datasets, but the worst genes evolved faster and were more saturated (Table 1); CATGTR is the best fitting model in each case, and improvement over GTR seems to be more important for the worst genes (Table 1). Posterior predictive checks show that the best genes violate the models much less than the worst genes (Table 1), but that even the best fitting CATGTR model does not explain the data well. We performed gene jackknife analysis with CATGTR

using 50 samples of ~30,000 positions. The best performing genes according to our criterion supported Xenambulacraria (including the long branched acoelomorphs) with 94% jackknife support (Figure 1D). The worst genes supported Nephrozoa with a weak 48% jackknife support and we observed lower support for other clades across the tree in agreement with the expected difficulty in extracting phylogenetic signal from these genes. The best genes also support Xenambulacraria (JP= 63%) when the short branched *Xenoturbella* is removed leaving just the fast evolving Acoelomorpha (Figure 2S). Since the genes with the better phylogenetic to non-phylogenetic signal ratio consistently support Xenambulacraria, the likely explanation is that support for Nephrozoa is an artefact caused by the limitations of reconstruction methods when applied to problematic data.

Better fitting models support Xenambulacraria, worse models support Xenambulacraria if long branch acoelomorphs are removed

Consistent with previous studies [5, 17, 18], the site heterogeneous CATGTR model we used has a better fit to our data set than the site homogeneous LG and GTR models predominantly used by Cannon et al. [2] and Rouse et al. [1] (cross-validation score of 3034 ± 152 and 2001 ± 155, respectively). While we have shown the best genes analysed with CATGTR support Xenambulacraria even with long branch Acoelomorpha included, analysing this data set with less well-fitting site homogeneous GTR models supports Nephrozoa (100% bootstrap support). When reanalysing the best data after removing the long branched Acoelomorpha, however, even the less well fitting GTR model supports Xenambulacraria (92% bootstrap support, Figure 2). For the worst performing genes, all analyses (CATGTR, and GTR with or without Acoelomorpha) supported Nephrozoa (Figure 2). Data and analyses that are better by specified, measurable, objective criteria consistently result in increased support for Xenambulacraria.

Addressing the effects of compositional bias reduces support for Nephrozoa

After LBA, probably the best-known source of systematic error is compositional bias, in which a systematic tendency of substitutions towards certain amino acids in subsets of taxa affects tree reconstruction [19]. Considering the possibility that compositional biases in the proportions of amino acids found in different species were inadequately accounted for by the models used, we looked for evidence of the existence of compositional bias by using posterior predictive checks in PhyloBayes to compare real amino acid frequencies of the 59 species in our data with their mean values under the null distribution predicted by the best fitting CATGTR model. A strong compositional bias was observed in our data although not specifically in Xenacoelomorpha. Interestingly, part of the superiority of the 'better' genes discussed previously may be explained by the lower compositional bias we observe in the best 25% of data compared to the worst 25% (mean squared heterogeneity - best genes = ~100; worst genes = ~190). If compositional bias is contributing to the support for Nephrozoa, then reducing the effects of this bias would be predicted to lower support for Nephrozoa. To minimise the effects of species specific compositional bias we recoded the amino acids in our alignment using a reduced alphabet that gathers similar (and frequently substituted) amino acids into the following 6 'Dayhoff' groups (A,G,P,S,T) (D,E,N,Q) (H,K,R) (F,Y,W) (I,L,M,V) (C). Recoding also tends to reduce model violations and saturation as frequently substituting amino acids are consolidated into a single character state [19]. We reran the jackknife analyses of the complete data set using the recoded data in PhyloBayes [12]. Using all species and all genes, jackknife support for Xenambulacraria increased from 61% to 90% suggesting that compositional bias affects tree reconstruction and specifically reduces support for Xenambulacraria (Figure 3). We repeated this analysis using a bootstrapping approach instead of jackknifing and the support for Xenambulacraria was found to be 98%. This increase is in line with other evidence indicating the relatively conservative nature of jackknife support values.

The effects of model and data testing are not data set specific

One possible criticism of our findings is that they depend on the particular subset of genes and taxa used. We repeated our analyses using the data sets of Cannon et al. [2] and Rouse et al. [1]. For each test (removing long branched taxa, stratifying genes according to phylogenetic accuracy and recoding to reduce compositional bias) we observed the same direction of change as we observe in our data, albeit with lower support values, especially for the taxon-poor Rouse et al. data [1] (see Figures S4). While Cannon et al. [2] analysed their data with long branched Acoelomorpha omitted, they used the site homogenous LG model and recovered the Nephrozoa tree. Using CATGTR on the same data we recovered the Xenambulacraria tree (Figure S2). With the same results coming from three, large, independently assembled data sets it is reasonable to conclude that the support for Xenambulacraria cannot be explained by the choices made during data set assembly.

Discussion

Determining the correct phylogenetic position of the Xenacoelomorpha has significant implications for our understanding of their evolution and that of the Metazoa. If Xenacoelomorpha diverged prior to other bilaterian animals, then this could explain their relative morphological simplicity and lack, for example, of several bilaterian Hox genes and microRNAs [20-22]. Under the assumption of such an 'early-diverging' scenario, xenacoelomorphs were naturally considered to be of particular interest, as a branch intermediate between non-bilaterians (such as Cnidaria) and Nephrozoa [23, 24]. If, on the other hand, xenacoelomorphs are the sister group of the Ambulacraria, their simplicity, both morphological and genetic, must have been derived from a more complex ancestor by a process of character loss. If we accept that the Xenambulacraria clade is real, we should expect additional evidence for this relationship to remain in the embryology, morphology and genomes of these animals and such evidence would be a valuable corroboration of our results. Although it seems that the branch separating the Xenambulacraria from other Bilateria is short, it would still be predicted that certain characters uniting these taxa exist. Accordingly, the

278 occurrence of neuropeptides in xenacoelomorphs related to echinoderm SALMFamides [25]
279 has been reported previously based on immunohistochemical evidence [26, 27] to add to other
280 known shared molecular characters [5, 28, 29].
281
282 One surprising result from our work is the lack of support for a monophyletic clade of
283 deuterostomes when using site heterogenous models - the relationships between chordates,
284 Xenambulacraria and protostomes are essentially unresolved. While the majority of our
285 analyses recover a monophyletic group of chordates plus protostomes the support values are
286 very low meaning there is no solid evidence to refute the traditional protostome/deuterostome
287 dichotomy. All possible relationships between chordates, protostomes and Xenambulacraria
288 are observed in different analyses (see extended info). This observation nevertheless implies
289 an extremely short branch between the bilaterian common ancestor (Urbilateria) and the
290 deuterostomes. If the deuterostomes are ultimately shown to be monophyletic then the short
291 branch leading to the deuterostome common ancestor, Urdeuterostomia, suggests it should
292 have much in common with Urbilateria. If the deuterostomes do prove to be paraphyletic then
293 Urbilateria and Urdeuterostomia must be considered synonymous and this result has
294 significant implications for our understanding of the characteristics of the common ancestor of
295 Bilateria. Given that the internal branches separating the Xenambulacraria, Chordata and
296 Protostomia are short, larger datasets and more refined methodologies (e.g. [30]) are required
297 to adequately test the deuterostome monophyly.
298

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Declaration of interests.

The authors declare no competing interests.

Figure 1. Support for Xenambulacraria is strengthened in experiments designed to reduce systematic errors. A. Full data set using all 1,173 genes and 350,088 positions shows limited support (60% of Jackknife replicates highlighted in red) for a sister group relationship between Xenacoelomorpha and Ambulacraria (Xenambulacraria). B. Summary figure of result in 1A. C. Full data set with long branched Acoelomorpha removed, results in increased support for Xenambulacraria (81% jackknife support). D. Data set of all species and the best 25% of genes (as measured by their ability to reconstruct known monophyletic groups) results in increased support for Xenambulacraria (94% jackknife support). Monophyletic deuterostome clade is not supported though the position of the Chordata is not reliably resolved in any analysis. All analyses used 50 Jackknife replicates (support values shown to right of nodes) analysed with PhyloBayes using the CATGTR+Gamma model. Major clades are indicated with corresponding colours. Jackknife proportions = 100% unless shown. The outgroups are shown in green. See also Figures S1-4 and Table S1.

Figure 2. Best genes and best fitting model support Xenambulacraria hypothesis under different conditions (green box). Worst genes and less well-fitting model support the Nephrozoa hypothesis (red box). Summary trees with jackknife support values shown for relationships between key clades for different methods of analysis. Best genes were selected by their ability to reconstruct known monophyletic groups. Top row analysed with better fitting site heterogeneous CATGTR+Gamma model. Bottom row analysed with less well-fitting site homogenous GTR+Gamma model. 'Dayhoff6' used Dayhoff recoding to reduce compositional bias. 'No Acoel' excluded long branched Acoelomorpha. 'All' included all species with no data recoding. Chords = Chordata, Proto = Protostomia, Ambula = Ambulacraria, Xenacoels = Xenacoelomorpha. JP= Jackknife Proportion. BP = Bootstrap proportion.

Figure 3. Dayhoff recoding to reduce compositional bias and saturation increases support for Xenambulacraria. PhyloBayes jackknife and bootstrap analyses of all genes and all taxa using CATGTR and Dayhoff recoding. The jackknife topology is shown though the

bootstrap topology was identical and branch lengths were almost identical. Jackknifing used 50 replicates of 30,000 amino acids. Jackknife proportions (first number) and bootstrap proportions (second number) for nodes with less than 100% support for either measure are shown to the right of node supported. Bootstrap proportions are consistently higher, suggesting jackknifing provides a conservative measure of support. Xenambulacraria support is highlighted in red.

Table 1. Comparisons of characteristics of best and worst quarters of genes from the three data sets. For the data from this study, from Cannon et al [2] and from Rouse et al [1] we compare several aspects of the best and worst quarter of genes as ranked using our monophyly score. The first five rows show posterior predictive tests of diversity and heterogeneity of best and worst quarters of genes from the three data sets using site homogenous (GTR) and heterogenous (CATGTR) models of site evolution. For all three data sets and for all three tests the CATGTR model provides a closer fit to the observed statistic than the site homogenous GTR model as estimated by the z-score shown here. There is a slightly better fit of model to data for the best genes compared to the worst genes. The three tests are computed with the readpb_mpi programme of the PhyloBayes_mpi suite: diversity (site-specific amino acid preferences), max heterogeneity (maximal compositional heterogeneity observed across the taxa), and mean heterogeneity (mean squared heterogeneity across taxa). The remaining rows show comparisons of best and worst genes made using the CATGTR model: Congruence score measures average monophyly score per gene and % recovered clades measures percentage of clans present in the super matrix LG+F+G tree recovered by single genes using the same model, in all cases the best quarter are better. # positions, % missing data and number of constant positions have similar values between best and worst genes. Cross validation scores show how much better the CATGTR model fits the data compared to the GTR model. For all data sets and partitions, trees based on the best genes are consistently longer and slightly more saturated (saturation estimated as in [7]

382 from the a0 parameter, using the CATGTR patristic distances) than those based on the worst
383 genes.

STAR methods

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Max Telford (m.telford@ucl.ac.uk).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Xenoturbella bocki were collected from mud dredged at approx. 60 metres depth in Gullmarsfjord, Sweden.

Symsagittifera roscoffensis were collected from intertidal regions of beaches in region of Roscoff, France.

Meara stichopi were collected by dissection from the pharynx of the sea cucumber *Stichopus sp*. The sea cucumbers were collected in the sea close to Bergen, Norway.

Pseudaphanostoma variabilis were found in sediment collected close to the island of Hållö close to Smögen, West coast Sweden.

Praesagittifera naikaiensis were collected in sediment dredged from the sea bed close to Onomichi, Hiroshima, Japan

Paratomella rubra were collected from intertidal sands of Filey bay, Yorkshire, United Kingdom.

Isodiametra pulchra came from a lab strain from the University of Innsbruck, Austria.

METHOD DETAILS

Xenoturbella bocki genome

Independent Illumina fragment libraries were made from two single animals, which had been starved for at least 3 months in the presence of Penicillin, Streptomycin and Gentamycin antibiotics to minimize environmental bacterial contaminations. The fragment libraries had insert sizes of ~200bp and ~150 bp and were sequenced as single paired reads with read

411 length of 36-100bp. Overlapping paired reads were joined using flash [31]. The majority of
412 sequences were obtained from these two libraries of which 10 lanes were sequenced.
413
414 Mate pair libraries were constructed from DNA isolated from several animals with insert
415 sizes of 700, 1,000, 1,500 and 2,000 bp. After standard Illumina filtering all sequences
416 shorter than 31bp were discarded. All reads were subsequently filtered for adaptor
417 sequences, PCR duplicates and quality with SOAPfilter_v2.0
418 (https://github.com/tanghaibao/jcvi-bin/blob/master/SOAP/SOAPfilter_v2.0) using standard
419 settings except setting the insert sizes and the appropriate asci quality shifts. A total of
420 731,057,046 reads were assembled simultaneously using SOAPdenovo (v2) [32] using
421 settings –K 31 -M3 –F –U -g200. A total of 108,063,238 bp were assembled in a total of
422 21,594 scaffolds. The average scaffold length was 5004 bp, the longest scaffold had a size
423 of 317,597 bp. Including contigs not merged into scaffolds the total sequence size was
424 119,097,168 bp with an average length of 1210 bp an N50 of 22,208 and an N90 of 443bp.
425 Additional gaps were filled using SOAP Gapcloser v1.12
426 (http://soap.genomics.org.cn/soapdenovo.html).
427
428 Using the human matrix, Genescan [33] was used to generate predictions of coding regions
429 resulting in 23 Mb of protein coding sequence (N50: 1872 bp) in 21,769 predicted protein or
430 peptide sequences, which were subsequently used for phylogenomic analyses.
431
432 ***Symsagittifera roscoffensis genome***
433 A standard fragment Illumina library was made from a pool of symbiont free hatchlings,
434 which were raised in artificial sea water in the presence of antibiotics. Reads were
435 processed as described for *Xenoturbella* above. 526,232,442 reads were assembled using
436 SOAPdenovo2 (-M3, -R, –d1, -K31) and the Celera assembler using the settings for large
437 and heterozygous genomes. Single gene analyses indicated that the two assemblers had
438 different qualities in different regions of the genome. Hence the entire Soap assembly and

the Celera assembly using its contigs and degenerate contigs larger than 500 bp were jointly assembled using minimus2 [34]. Although the total assembled genome size of about 1 Gbp from the SOAPdenovo assembly was reduced to about 450 Mb of assembled sequence many single gene analyses and PCR amplifications indicated that many more genes are represented in the joint assembly in significantly longer gene models. The joint assembly had an N50 of 2,905bp and a N90 of 587bp. Analysis of missing sequences indicated that most of the removed part is composed of repetitive sequence. The total number of predictions for coding sequences is 113,993 and comprising a total of 52Mb. A transcriptome was also sequenced from *S. roscoffensis* mixed stage embryos using standard methods.

Amplifying genomes of small acoels

Due to their small sizes one whole animal each of *Meara stichopi*, *Nemertoderma westbladi*, and *Pseudaphanostoma variabilis* were used without prior DNA extraction to directly amplify genomic DNA using the illustra Genomphi V2 DNA amplification Kit (GE Healthcare Nr.: 25-6600-30). Amplified DNA was cleaned by Isopropanol precipitation and shared to 1.5-3 kb fragments using speed code SC6 on the Hydroshear DNA Shearing Device (Thermo Fisher Scientific). After additional cleaning and quantification 1 micrograms DNA from each animal was used to generate standard illumina fragment libraries and these were sequenced as paired end with sequence length 100 bp. Sequence data have been submitted to the European Nucleotide Archive (ENA) under accession number PRJEB25577.

Nemertoderma westbladi was collected from mud at the site "Telekabeln" in the Gullmarsfjord in July 2009. For *Nemertoderma westbladi*, 800,863,374 reads equalling ~80 Gb of sequence were used for the genome assembly using SOAPdenovo2. The best results were obtained using the settings -K39 -d0 –M 3 –map 45. The assembly comprised about 205 Mb with an N50 of about 380 bp. 80,966 gene predications resulted in 38Mb of coding sequence.

For *Meara stichopi* 1,167,743,394 reads (~110 Gb) were read. An assembly was generated using standard settings and –K –M 3. The assembly had a total size of about 1.4 Gbp and an N50 of 1.1 Kb. A total of 130,115 protein or peptide fragments were predicted comprising 37Mb of coding sequence.

Pseudaphanostoma variabilis was collected from shell gravel near the island Hållö close to Smögen in July 2009. The *Pseudaphanostoma variabilis* genome was assembled from 672,950,533 reads with the SOAPdenovo2 settings –K 31 –d 0 –M 3 –map 36 and resulted in an assembly size of about 413 Mb. 115,245 gene predictions comprised 45 Mb of coding sequence.

The *Praesagittifera naikaiensis* genome was sequenced and assembled at the Okinawa Institute of Science and Technology. 1,148,317 sequences with a total size of about 1.2 Gb and an N50 of 4,452 bp resulted in 400,106 gene predictions comprising 233Mb of coding sequence.

***Paratomella rubra* transcriptome**

Specimens of the acoel *Paratomella rubra* were collected from intertidal sand in Filey Bay, Yorkshire, UK. RNA was prepared and sequenced, the transcriptome was assembled and cross-contaminants were removed and proteins predicted as described in (Egger et al 2015 [18]). Data available in the NCBI Short Read archive: SRX3470480.

***Isodiametra pulchra* transcriptome**

Specimens of the acoel *Isodiametra pulchra* were harvested from a lab stock provided by B Egger, Innsbruck. RNA was prepared and sequenced, the transcriptome was assembled and cross-contaminants were removed and proteins predicted as described in ref [18]. Data available in the NCBI Short Read archive SRX3469680.

Initial contaminant cleaning

All sequences were scanned for contaminating bacterial sequences using the PhymmBL program [35]. Sequences were additionally clustered based on tetranucleotide frequencies using an emergent self-organizing map (ESOM).

Removing redundancy

We translated gene predictions from genomes and transcriptomes into protein sequence and, when both present from a given species, we joined both predictions and clustered using CD-HIT with a 97% identity threshold [36], resulting in non-redundant proteomes for each species. We obtained 32,456 complete gene predictions in *Symsagittifera roscoffensis,* 35,867 complete gene predictions in *Meara stichopi,* 23,233 complete gene predictions in *Nemertoderma westbladi,* 27,378 complete gene predictions in *Pseudophanostoma variabilis,* 24,329 complete gene predictions in *Paratomella rubra,* 19,206 complete gene predictions in *Xenoturbella bocki*.

Initial ortholog predictions using OMA

Non-redundant peptide datasets from 67 species including 9 Xenacoelomorpha species, 8 Chordata, 15 Ambulacraria, and 13 Protostomia and 22 non-Bilateria organisms were processed by the OMA standalone software version 0.99w [37], using default settings. This identified 245,524 Orthologous Groups (OGs)—sets of genes in which all members are orthologous to all other members. From these, we selected the 3,683 OGs which had a minimum of 34 species represented (at least 50% of all species), and further filtered 1,665 OGs containing at least one member of Xenoturbellida and Nemetodermatida and Acoela.

Reducing missing data, adding species and initial cleaning using 42 software

Transcriptomic data from 77 species were then incorporated into the 1,665 previously assembled core orthologous clusters using a multiple Best Reciprocal Hit approach

implemented in the newly designed Forty-Two software (https://bitbucket.org/dbaurain/42/downloads). First, we removed the most divergent sequences, which are the most likely to be paralogs or contaminants. More precisely for each species having multiple sequences, each sequence was BLASTed against the rest of the alignment and the best hit identified; a sequence was removed if it overlapped with the best hit sequence by ≥ 95% and if its BLAST score was below the best hit score by a given threshold. Using a threshold of 10%, 17,480 sequences were removed. The resulting clusters were cleaned using HmmCleaner version 1.8 [38] and the same process was repeated, this time removing 4,267 additional sequences. Most of these sequences were sequencing variants of the same transcripts (due to sequencing errors or to *in vivo* transcript degradation).

Removing potential contaminants

As in Simion et al. [9], alignments of ribosomal proteins containing a large eukaryotic taxonomic diversity were used to detect contaminations. We used BLASTP against several custom databases to detect and remove the contaminants. An additional screening was done using BLASTN to remove the few remaining contaminants from *Homo sapiens* and *Danio rerio*. The case of homoscleromorph and calcareous sponges was analysed differently, because of the absence of clean complete genomes that can serve as a reference for decontamination. For each alignment, we BLASTed each poriferan sequence against the other sequences and removed the 2,434 sequences that had a BLAST bit score to the 'wrong' clade that was 5% higher than to the expected clade (i.e., Calcarea, Demospongiae, or Homoscleromorpha).

To discard genes for which orthology/paralogy relationships are difficult to infer, we made alignments using Mafft [39] (mafft --quiet --localpair --maxiterate 5000 —reorder), cleaned alignments with HmmCleaner and constructed RAxML trees [40] using the LG+Gamma+F model. We then computed the number of taxonomic groups (among the 14 clades displaying

551 a long basal branch: Acoela, Anthozoa, Calcarea, Chordata, Demospongiae, Ecdysozoa,
552 Echinodermata, Hemichordata, Homoscleromorpha, Lophotrochozoa, Medusozoa,
553 Nemertodermatida, Rotifera and Xenoturbellida) displaying paralogous copies (see [9]) and
554 eliminated the 157 genes with >= 5 cases of paralogy.
555
556 To reduce the amount of missing data and the computational burden, we removed 21
557 species (highly incomplete, taxonomically redundant or fast-evolving) and then the 137
558 genes in which more than one of the following 8 groups (Acoela, Nemertodermatida,
559 Xenoturbellida, Echinodermata, Hemichordata, Chordata, Protostomia and outgroup) is
560 missing. We had three criteria for choosing which taxa to retain: 1. Taxonomic diversity with
561 the aim of picking a member of each of the major groups of a given clade (i.e. not all
562 arthropods for Ecdysozoa). 2. Avoiding taxa with known issues such as extreme branch
563 lengths or compositional biases (e.g. picking a shorter branch nematode rather than the
564 familiar but rapidly evolving *Caenorhabditis elegans*). 3. choosing a species with fewest
565 missing data.
566
567 Our last quality check was based on the rationale that non-orthologous sequences (being
568 either a contaminant or a paralog and thus misplaced) typically display very long branches
569 when constrained on the species tree. First, alignments were cleaned with HmmCleaner
570 version 1.8 [38] and BMGE [41], and concatenated using SCaFoS [42]. The phylogeny
571 inferred using RAxML [40] from the supermatrix under the LG+Gamma$_4$+F model was
572 considered as a proxy of the species tree (note that xenacoelomorphs were sister to all other
573 bilaterians in this tree). Then, for each alignment, the reference topology was pruned of the
574 species missing in that alignment, and branch lengths on this constrained topology were
575 estimated using RAxML (LG+Gamma$_4$+F model). This allowed us to compare terminal
576 branch lengths observed in the single-gene tree to those observed in the pruned supermatrix
577 tree, and to remove sequences for which the branch-length ratio was > 5, hence eliminating
578 642 questionable sequences.

579 Finally, we only kept the 1173 alignments in which at most 16 species were missing. We
580 used SCaFoS to assemble the supermatrix, build chimeras of closely-related species
581 (*Oscarella carmela*/*Oscarella* SN2011, *Saccoglossus kowalevskii*/*Saccoglossus*
582 *mereschkowskii* and *Cephalodiscus gracilis*/*Cephalodiscus hodgsoni*) and retained only the
583 slowest-evolving sequence when multiple copies were available for a given species (using
584 Tree-Puzzle and the WAG+F model to compute distances). This produced a supermatrix
585 containing 350,088 amino acid positions for 59 species, with an overall amount of 23.5%
586 missing data.

587 **Dataset quality**

588 To compare of our dataset with those of Cannon and Rouse [1, 2], for each gene separately
589 we computed a phylogeny using RAxML (LG+Gamma$_4$+F model) [40]. We then computed
590 the number of tree bipartitions observed in the supermatrix tree (constructed with the same
591 model) that are recovered by each gene. We assume that the majority of partitions in the
592 supermatrix tree are likely to be correct and the percent of recovered bipartitions in the
593 single gene trees is thus an estimation of dataset quality. Dataset quantity was measured as
594 total amino acids.
595

596 **Phylogenetic inference**

597 The supermatrix was analysed with the site-heterogeneous CATGTR model [43] using
598 PhyloBayes-MPI version 1.8 [44] after the removal of constant positions ('-dc' option) and
599 with the site-homogeneous GTR model using raxml version 8.2.8 [40]. The use of LG or
600 LG4X models gave virtually the same results as GTR. The robustness of phylogeny was
601 inferred with 100 rapid bootstraps in the case of the GTR model and with 100 gene
602 jackknifes in the case of the CATGTR model.
603

604 **Stratifying genes according to support for known monophyletic groups**

605 To select the genes from all three data sets (this study, Rouse et al. [1] and the larger 881
606 genes data set of Cannon et al. [2]) most likely to contain easy to extract phylogenetic
607 signal, we used two different approaches. First, each gene was analysed separately to find
608 their individual level of support for known monophyletic groups. All Xenacoelomorph
609 sequences were removed such that the monophyly measure was independent of the
610 presence of this clade. For each aligned and trimmed gene, a tree was reconstructed using
611 phyml [45] (settings -d aa -o tlr -a e -c 5). Each resulting tree was analysed using a custom
612 perl script that measured the support for the following uncontroversial monophyletic groups:
613 Cnidaria, Ambulacraria, Hemichordata, Echinodermata, Chordata, Ecdysozoa,
614 Lophotrochozoa, Porifera, Ctenophora (where present) Protostomia and Bilateria The
615 monophyly score for each clade was calculated as the size of the largest clade on the tree
616 containing species from the monophyletic group in question divided by the total number of
617 species from that monophyletic group in the dataset. For example, if there were five
618 chordates in the data set and the largest chordate-only grouping on the tree contained four
619 of them, the monophyly score for chordates would be ⅘ = 0.8. The total score for the tree
620 was calculated as the monophyly score averaged over all clades. Clades with fewer than
621 two species in the tree were ignored. The data sets were then ranked by monophyly score
622 and concatenated (with Xenacoelomorphs now included) in order from best (highest
623 monophyly score) to worst.
624
625 For each of the three stratified data sets (ours, Cannon et al. [2] and Rouse et al. [1]) we
626 took the genes representing the first 25% of positions (best) and the last 25% positions
627 (worst)
628 and performed jackknife resampling to produce 50 jackknife replicates each containing
629 ~30,000 positions. Each jackknife replicate data set was analysed using PhyloBayes-MPI
630 and a CATGTR+Gamma model with a single run and stopping after 1500 cycles. The
631 jackknife summary tree was produced using a bpcomp analysis using all 50 replicates with a
632 burnin discarding the first 1000 cycles. We also inferred Maximum LIkelihood trees using the

GTR+Gamma model with RAxML [40] based on the concatenations of the best and worst 25% of genes.

In a second closely related approach, we sorted the genes according to the percentage of bipartitions observed in the supermatrix tree that are recovered by each gene and took the 25% genes with the highest (lowest) values as the best (worst) genes this time including all species. These approaches gave congruent results and we present only those from the first approach.

Dayhoff recoding

This was performed using the "-recode Dayhoff6" command in PhyloBayes-MPI.

Posterior Predictive Analyses (ppred)

These were conducted using PhyloBayes ppred command as described in ref [19].

Carbon footprint calculations

The carbon footprint for travel was computed only for flights for the three meetings specifically organised for this project, so constitute a small underestimate. We used the calculator of the International Civil Aviation Organization (https://www.icao.int/environmental-protection/Carbonoffset/Pages/default.aspx), which did not include radiative forcing, so seriously underestimating the impact on global warming (Table S2).

The carbon footprint for computation was more difficult to compute since analyses were done in multiple labs, using various computers. More importantly, we did not archive all computations done for this work (e.g. preliminary analyses). We used the reasonable hypothesis that the jackknife analyses with the CATGTR model are by far the largest contributor and compute their footprint only. This certainly leads to an underestimation

(ignoring for example assembly of genomes/transcriptomes, dataset building, dataset curation, RAxML analyses and Dayhoff analyses were ignored). For simplicity we also assumed that all the computations were done on a single computer, mp2 of ComputeCanada (https://wiki.calculquebec.ca/w/Accueil).

For 3 taxon sampling experiments, the 100 jackknife replicates of ~90,000 positions were performed on 6 nodes of 24 cores. The average CPU time for a single replicate was 520.5 hours, giving a total of 936,900 hours (=520.5*6*100*3). The 50 jackknife replicates of ~30,000 positions were performed on 2 nodes of 24 cores, for 3 datasets (Our data, Cannon and Rouse), 2 taxon samples, 2 data samples (best/worse) and 2 methods. The average time for a single replicate is 188.8 hours, so a total of 453,120 hours of a single node (=188.8*2*50*3*2*2*2*2). Total time for all jackknife experiments assuming a single node is 1,390,020 hours.

A node of mp2 consumes 300 W, to which we add cooling (22,75%) and other components (~5%) (Suzanne Talon, personal communication), so one hour of computation corresponds to ~0.38 kWh (=0.3*1.2775). Total electric energy consumption for our CATGTR jackknife replicates was 531,683 kWh (=1,390,020*0.38). To convert this into CO_2 emissions, we used the world average carbon intensity of power generation in 2017 (https://www.iea.org/tcep/power/), 491 gCO2/kWh, which leads to an estimate of 261 tonnes of CO_2 (=531,683*0.000491).

QUANTIFICATION AND STATISTICAL ANALYSIS

Jackknife procedure and tests for reliability.

A jackknife replicate was generated by randomly sampling single-gene alignments without replacement until >90,000 positions (~390 genes per replicate for most) or >30,000 positions (~130 genes per replicate for the analyses of best and worst genes) depending on analysis were selected. For PhyloBayes-MPI analysis of jackknife replicates, 3000 cycles were performed and consensus tree and jackknife support were obtained as in Simion et al. [9].

To see whether the number of cycles gives an accurate measure, we experimented by extended our chains. Increasing the number of cycles did not alter jackknife proportions (Table S1.).

Similarly, running two chains of each jackknife replicate until convergence also strengthens our results. We performed an experiment where we ran two chains for each of 100 jackknife samples of 30k positions for the 'best' quarter of positions of our data with all taxa. Of these, 51 pairs of chains converged (maxdiff <0.3) and 49 pairs did not (maxdiff > 0.3) - we compared the results from converged and imperfectly converged sets (Table S1.).

50 of 59 nodes received 100% support (Jackknife Proportion JP = 100%) in both converged and non-converged datasets and all but 4 received >90% support in both converged and non-converged pairs of chains. For all nodes that did not receive maximum support, the level of support is very similar for the converged and the imperfectly converged set. Interestingly, for 7 out of 9 nodes, the level of support in the converged set of runs was higher. Xenambulacraria support increased from 0.91 to 0.96. Chordata + Protostomia from 0.45 to 0.58. Only support for monophyly of Acoelomorpha and sister-group of *Ircinia* and *Chondrilla* was lower in the converged data (0.5 and 0.98) than in non-converged (0.65 and 1).

We also compared the results from Jackknifing to those from Bootstrapping (which uses full sized data sets as opposed to jackknifing which uses a smaller subsample). Bootstrapping can be applied in some of the less CPU intensive analyses (reduced alphabet analyses which are significantly quicker). When we do this (100 replicates) for our full data set with all species, the supports were very similar to those of the jackknife based on 90K positions, and, as expected, slightly higher (see below). Interestingly, the support value for monophyletic Xenambulacraria increases from 90% jackknife to 98% bootstrap support (Table S1.). This supports our contention that jackknifing provides a conservative estimate of support.

Due to the relatively small size of the main Cannon et al. [2] data set (~45k positions) we managed to run a full PhyloBayes analysis to convergence on a complete data set. We used the CATGTR site heterogeneous model on a data set from which the long branched Acoelomorpha had been removed. We found *Xenoturbella* + Ambulacraria supported with a value of 1.0 posterior probability showing that our jackknife analysis of the same was conservative (Figure S3B).

Model fit

To assess the fit of different models, we performed 10-fold model cross-validations. Model fit tests were done using training data sets of 10,000 amino acids and test data sets of 2,000 amino acids we used PhyloBayes version 4.1 [12] to perform cross-validation for the following models: LG+Γ, GTR+Γ, CAT+Γ and CAT-GTR+Γ. PhyloBayes was run for 1100 (LG and GTR) or 3100 (CAT and CATGTR) cycles and we kept the last 1000 cycles for following likelihood computations. Cross validation was run for full data sets as well as for the best and worst genes from the gene stratification experiments. The model cross-validations in all cases clearly favoured CAT-GTR+Γ > CAT+Γ > GTR+Γ > LG+Γ (for our principal, complete data set likelihood scores with respect to LG are 3034±152, 2270±151 and 268±40).

DATA AND SOFTWARE AVAILABILITY

The sequence alignments, phylogenetic trees that support the findings of this study, as well as the script for measuring monophyletic groups, are available on GitHub (https://github.com/MaxTelford/Xenacoelomorpha2019). Genome and transcriptome assemblies are available at https://figshare.com/search project number PRJNA517079. Raw data for novel sequences are available at the Sequence Read Archive BioProject PRJNA517079.

Table S1. Experiments to show that a Jackknifing approach gives conservative estimates of clade support. Related to Figure 1. A. Adding more cycles makes minor differences to clade support suggesting our estimates are accurate. B. Running two chains for each replicate to convergence makes minor difference (generally slightly strengthening support for less well supported clades) suggesting our clade support estimates are conservative. B. Comparison of bootstrapping and jackknifing shows the latter is gives more conservative estimates of clade support than bootstrapping. All clades not shown in the table have a support value of JP/BP = 1.

Table S2. Calculations of CO_2 produced by authors travelling to meetings related to this work. Related to STAR methods. For each of three meetings the origins, destinations and number of flights are shown with the approximate CO2 produced in tonnes.

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880

881

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Bacterial and Virus Strains		
Biological Samples		
Xenoturbella bocki	Gullmarsfjord, Sweden	NCBI:txid242395
Symsagittifera roscoffensis	Beaches off Roscoff, France	NCBI:txid84072
Meara stichopi	From pharynx of *Stichopus*, off Bergen Norway	NCBI:txid84115
Nemertoderma westbladi	Gullmarsfjord, West coast Sweden	NCBI:txid172109
Pseudaphanostoma variabilis	Hållö close to Smögen, West coast Sweden	NCBI:txid2510493
Praesagittifera naikaiensis	Onomichi, Hiroshima, Japan	N/A
Paratomella rubra	Sand from Filey bay, Yorkshire, UK	NCBI:txid90914
Isodiametra pulchra	Lab strain from Innsbruck, Austria	NCBI:txid504439
Chemicals, Peptides, and Recombinant Proteins		
Critical Commercial Assays		
Deposited Data		
Alignments, software and trees	GitHub	https://github.com/MaxTelford/Xenacoelomorpha2019
Genome and transcriptome assemblies	https://figshare.com/ search project number	PRJNA517079
Raw data for novel sequences.	Sequence Read Archive BioProject	PRJNA517079
Experimental Models: Cell Lines		
Experimental Models: Organisms/Strains		
Oligonucleotides		
Recombinant DNA		
Software and Algorithms		
PhyloBayes	[44]	*www.atgc-montpellier.fr/phylobayes*
Flash	[31]	*http://ccb.jhu.edu/software/FLASH/index.shtml*
SOAPfilter_v2.0	[32]	*https://github.com/tanghaibao/jcvi-bin/blob/master/SOAP/SOAPfilter_v2.0*
SOAPGapcloser v1.12	[32]	*http://soap.genomics.org.cn/soapdenovo.html*

Genescan	[33]	*http://genes.mit.edu/GENSCAN.html*
Soapdenovo2	[32]	*https://github.com/aquaskyline/SOAPdenovo2*
minimus2	[34]	*https://github.com/sanger-pathogens/circlator/wiki/Minimus2-circularization-pipeline*
PhymmBL	[35]	*https://ccb.jhu.edu/software/phymmbl/index.shtml*
CD-Hit	[36]	*http://weizhongli-lab.org/cd-hit/*
42		*https://bitbucket.org/dbaurain/42/downloads*
HmmCleaner version 1.8	[38]	*https://metacpan.org/pod/HmmCleaner.pl*
Mafft	[39]	*https://mafft.cbrc.jp/alignment/software/*
OMA	[37]	*https://omabrowser.org*
RAxML	[40]	https://cme.h-its.org/exelixis/software.html
BMGE	[41]	*ftp://ftp.pasteur.fr/pub/gensoft/projects/BMGE/*
SCaFoS	[42]	*http://megasun.bch.umontreal.ca/Software/scafos/scafos.html*
Other		

Figure 1

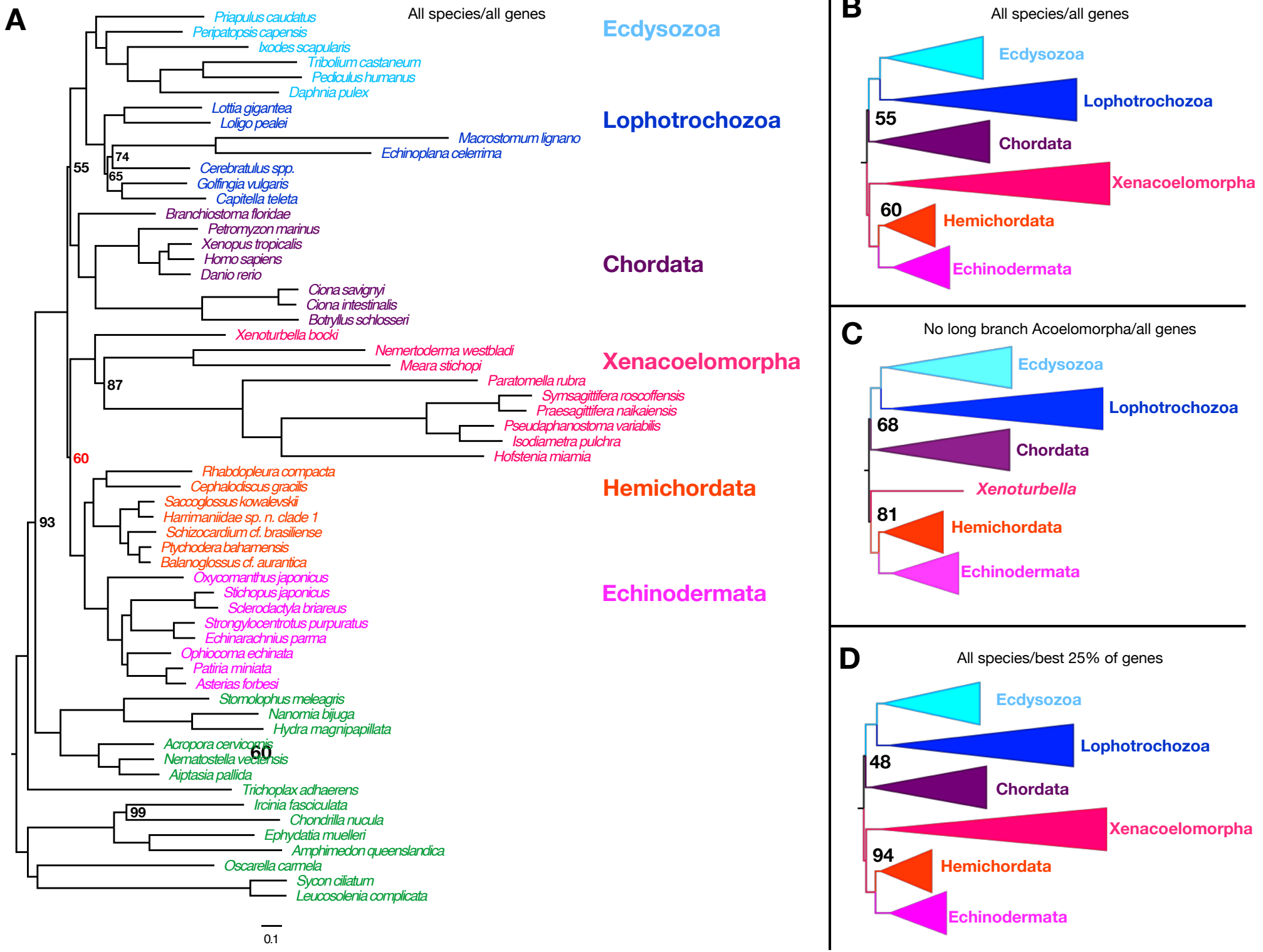

A
All species/all genes
Priapulus caudatus
Peripatopsis capensis
Ixodes scapularis
Tribolium castaneum
Pediculus humanus
Daphnia pulex
Lottia gigantea
Loligo pealei
Macrostomum lignano
Echinoplana celerrima
Cerebratulus spp.
Golfingia vulgaris
Capitella teleta
Branchiostoma floridae
Petromyzon marinus
Xenopus tropicalis
Homo sapiens
Danio rerio
Ciona savignyi
Ciona intestinalis
Botryllus schlosseri
Xenoturbella bocki
Nemertoderma westbladi
Meara stichopi
Paratomella rubra
Symsagittifera roscoffensis
Praesagittifera naikaiensis
Pseudaphanostoma variabilis
Isodiametra pulchra
Hofstenia miamia
Rhabdopleura compacta
Cephalodiscus gracilis
Saccoglossus kowalevskii
Harrimaniidae sp. n. clade 1
Schizocardium cf. brasiliense
Ptychodera bahamensis
Balanoglossus cf. aurantica
Oxycomanthus japonicus
Stichopus japonicus
Sclerodactyla briareus
Strongylocentrotus purpuratus
Echinarachnius parma
Ophiocoma echinata
Patiria miniata
Asterias forbesi
Stomolophus meleagris
Nanomia bijuga
Hydra magnipapillata
Acropora cervicornis
Nematostella vectensis
Aiptasia pallida
Trichoplax adhaerens
Ircinia fasciculata
Chondrilla nucula
Ephydatia muelleri
Amphimedon queenslandica
Oscarella carmela
Sycon ciliatum
Leucosolenia complicata
55
74
65
87
60
93
60
99
0.1
Ecdysozoa
Lophotrochozoa
Chordata
Xenacoelomorpha
Hemichordata
Echinodermata
B
All species/all genes
Ecdysozoa
Lophotrochozoa
55
Chordata
Xenacoelomorpha
60
Hemichordata
Echinodermata
C
No long branch Acoelomorpha/all genes
Ecdysozoa
Lophotrochozoa
68
Chordata
Xenoturbella
81
Hemichordata
Echinodermata
D
All species/best 25% of genes
Ecdysozoa
Lophotrochozoa
48
Chordata
Xenacoelomorpha
94
Hemichordata
Echinodermata

Figure 2

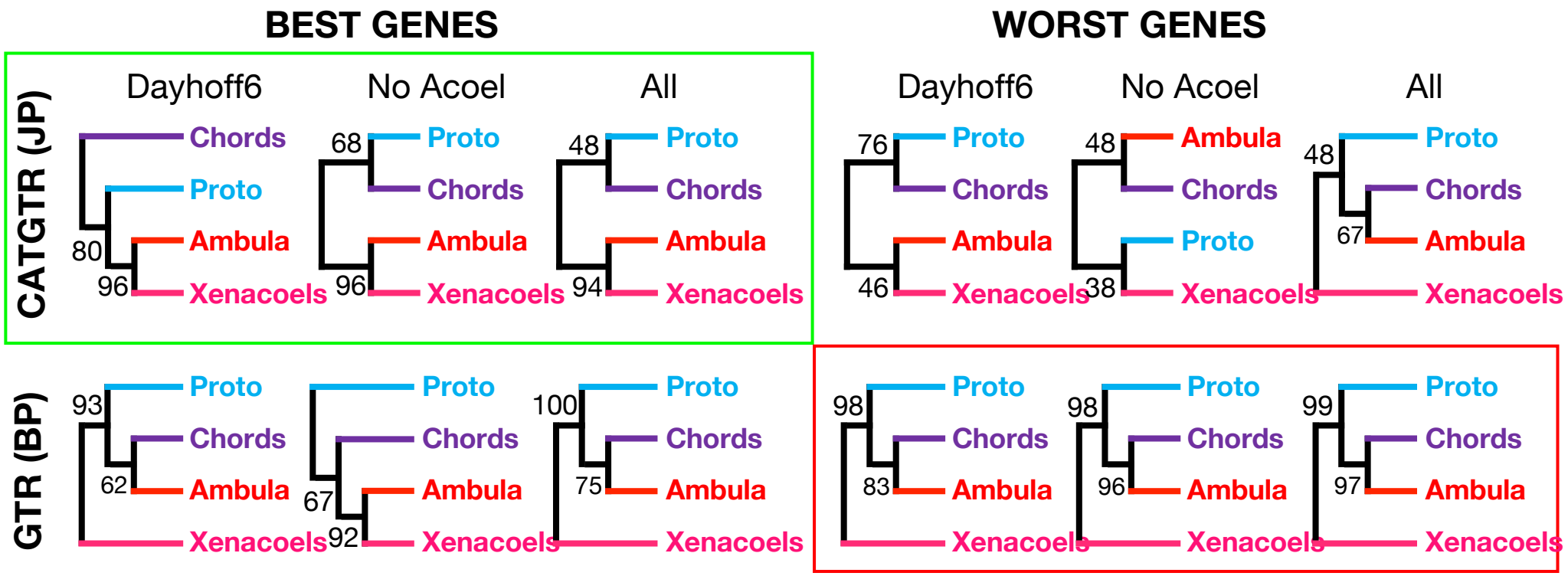

BEST GENES
WORST GENES
CATGTR (JP)
GTR (BP)
Dayhoff6
No Acoel
All
Chords
Proto
Ambula
Xenacoels
80
96
68
96
48
94
76
46
48
38
48
67
93
62
67
92
100
75
98
83
98
96
99
97

Figure 3

All species/all genes/no recoding/bootstrap

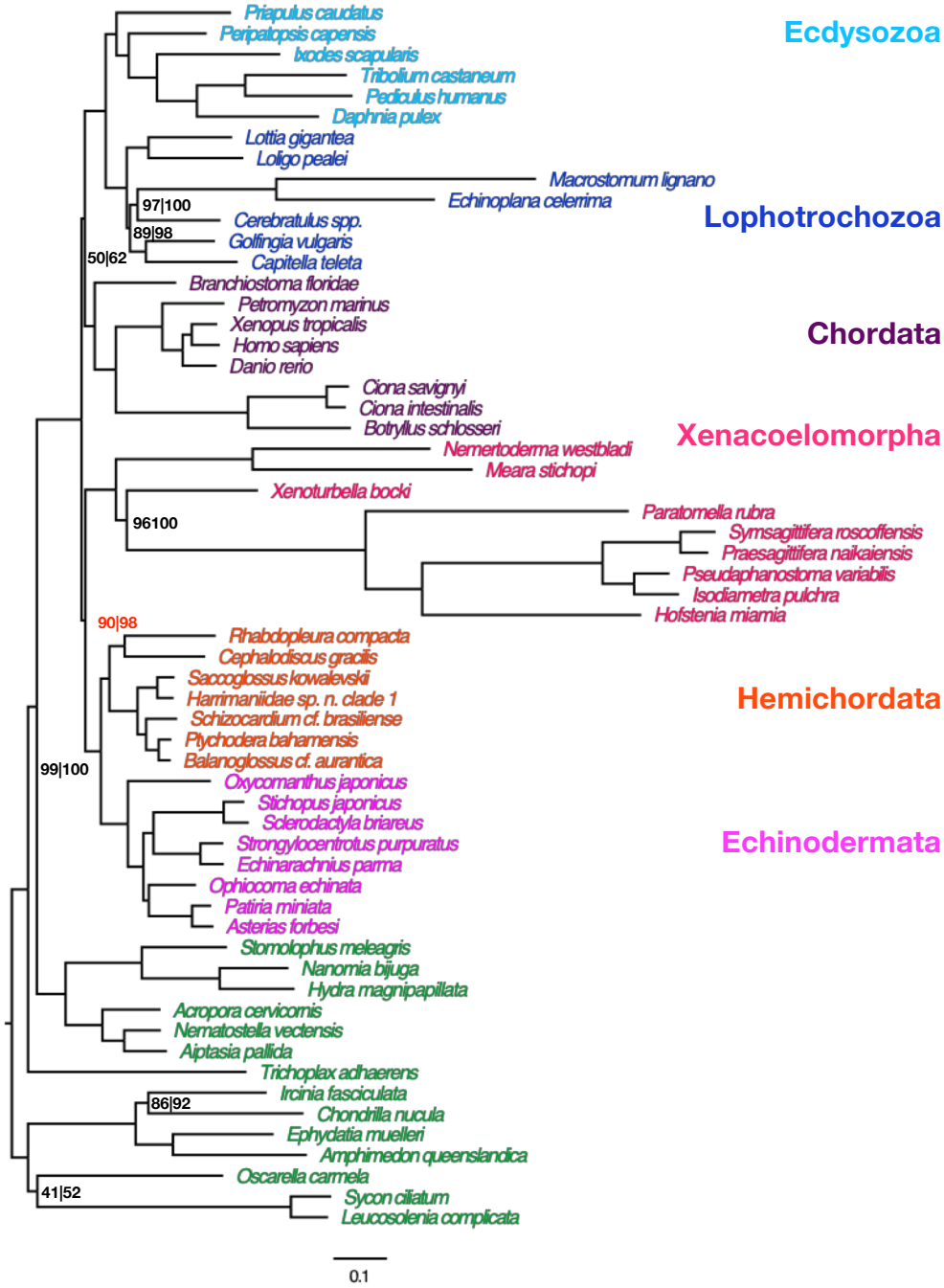


Table 1

	This study				[2]				[1]			
	Best Genes		Worst Genes		Best Genes		Worst Genes		Best Genes		Worst Genes	
model	CATGTR	GTR	CATGTR	GTR	CATGTR	GTR	CATGTR	GTR	CATGTR	GTR	CATGTR	GTR
diversity (Zscore)	5.7	122.0	7.0	139.7	7.8	132.4	10.3	199.5	3.1	69.7	3.7	84.7
max heterogeneity (Zscore)	17.1	37.2	88.5	197.3	9.3	12.1	43.4	106.6	1.4	1.8	2.5	4.7
mean heterogeneity (Zscore)	120.0	152.7	208.2	325.7	50.5	68.6	169.0	276.8	6.9	7.9	29.6	39.4
topology supported	X+A (94%)	X+PCA (100%)	X+PCA (48%)	X+PCA (99%)	X+A (42%)	X+PCA (100%)	X+P (76%)	X+PCA (100%)	X+A (50%)	X+PCA (93%)	X+PCA (50%)	X+PCA (87%)
Congruence score	0.87		0.53		0.80		0.44		0.8		0.44	
%recovered clades	72.58		37.38		60.45		25.17		47.40		3.47	
#positions	87791		87562		84276		84462		98630		98579	
%missing data	24.75		22.74		39.89		36.39		43.86		40.80	
%constant positions	20.44		24.35		14.66		14.04		20.75		24.05	
Cross validation	2078 ± 82		3539 ± 147		2914 ± 113		4960 ± 175		701 ± 62		997 ± 54	
Tree length	28.2		35.1		50.4		63.1		27.9		31.4	
Saturation	0.23		0.19		0.21		0.19		0.17		0.14	

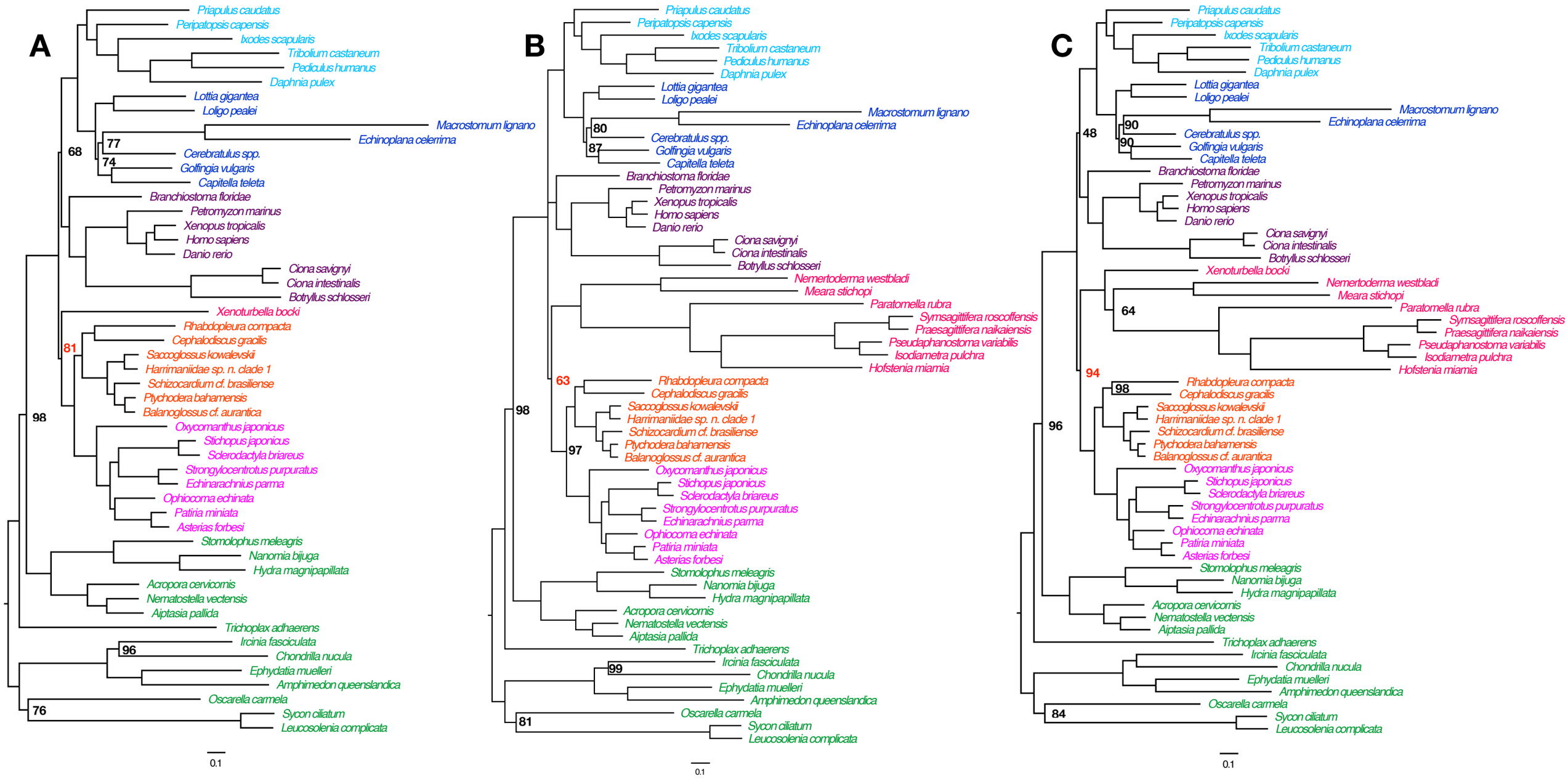


Figure S1. Phylobayes analyses of the data produced in this study. Related to Figure 1.

A. Phylobayes jackknife analysis. This study, All genes, No Acoelomorphs, CATGTR, 50 x 30,000 amino acids Jackknife. Jackknife proportions less than 100% shown to right of node supported. Xenambulacraria support highlighted in red.

B. Phylobayes jackknife analysis. This study, Best quarter of genes, No *Xenoturbella*, CATGTR, 50 x 30,000 amino acids Jackknife. Jackknife proportions less than 100% shown to right of node supported. Xenambulacraria support highlighted in red.

C. Phylobayes jackknife analysis. This study, Best genes, All taxa, CATGTR, 50 x 30,000 amino acids. Jackknife proportions less than 100% shown to right of node supported. Xenambulacraria support highlighted in red.

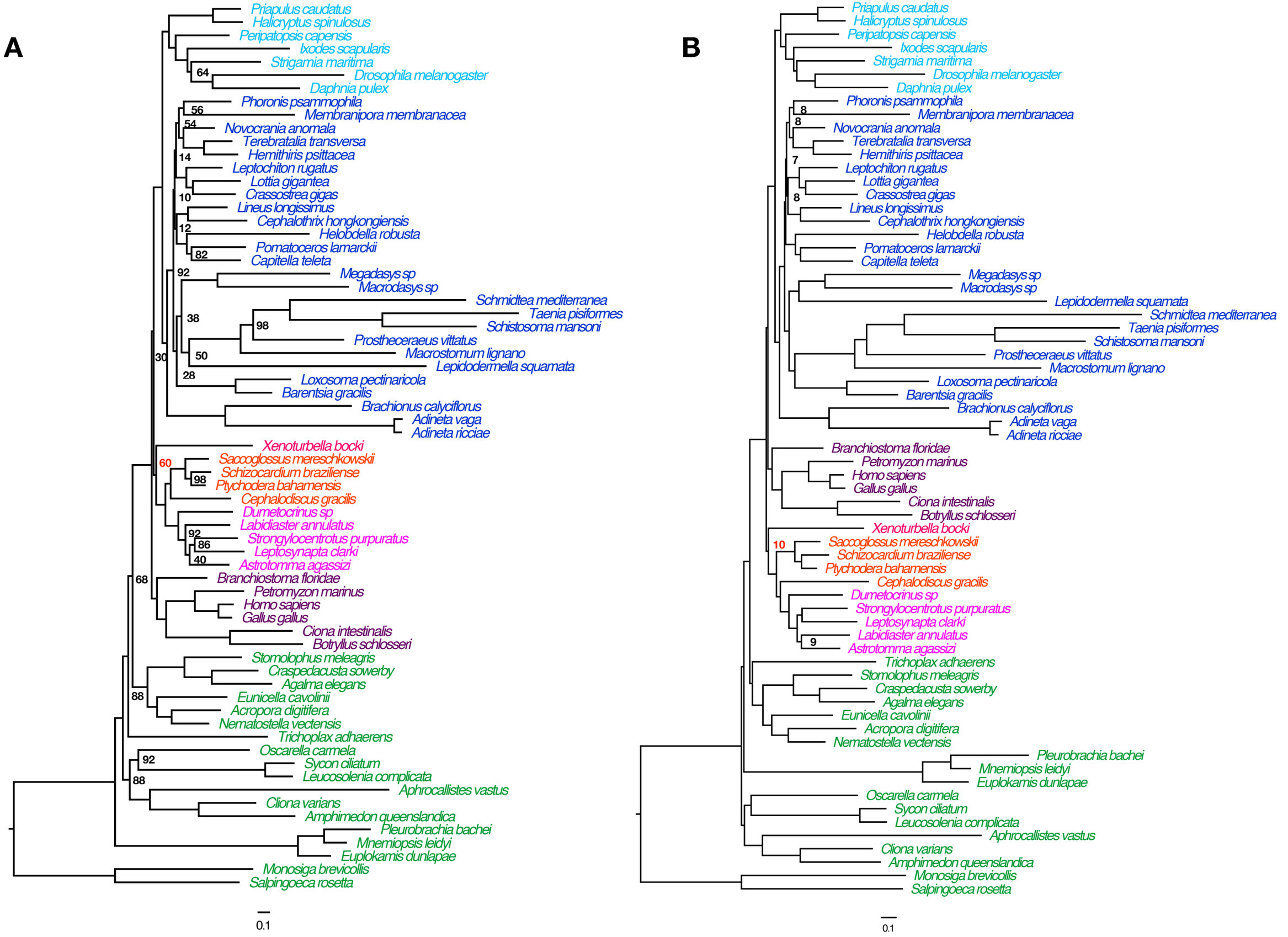


Figure S2. Reanalyses of data from Cannon *et al.* [S1] using Phylobayes. Related to Figure 1.

A. Phylobayes jackknife analysis. Cannon *et al.* [S1] data, All genes, No Acoelomorphs, CATGTR, 50 x 30,000 amino acids. Jackknife proportions less than 100% shown to right of node supported. Xenambulacraria support highlighted in red.

B. Phylobayes full dataset analysis. Cannon *et al.* [S1] data, All 212 genes, No Acoelomorphs, CATGTR. Posterior probabilities proportions less than 100% shown to right of node supported. Xenambulacraria support highlighted in red.

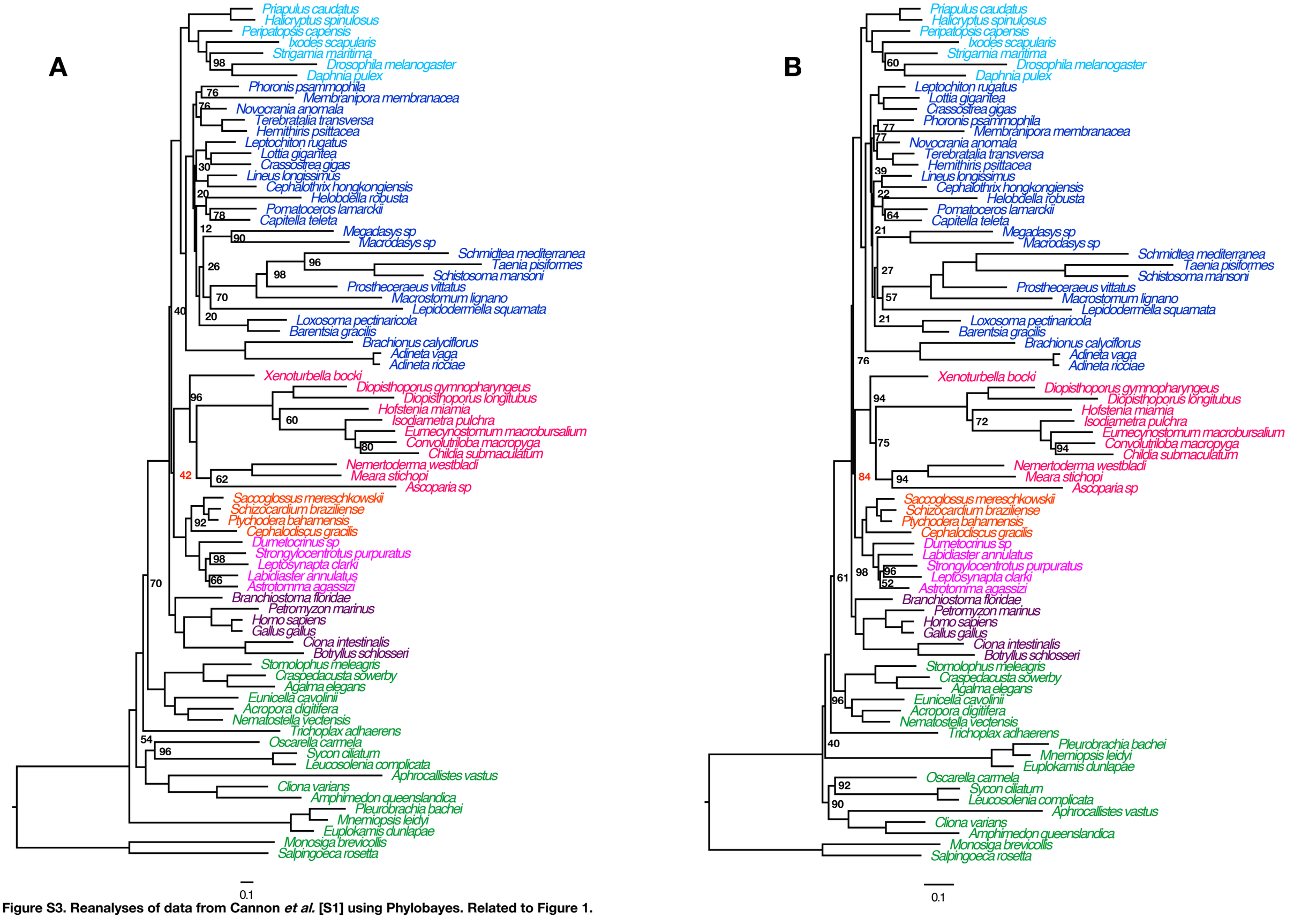
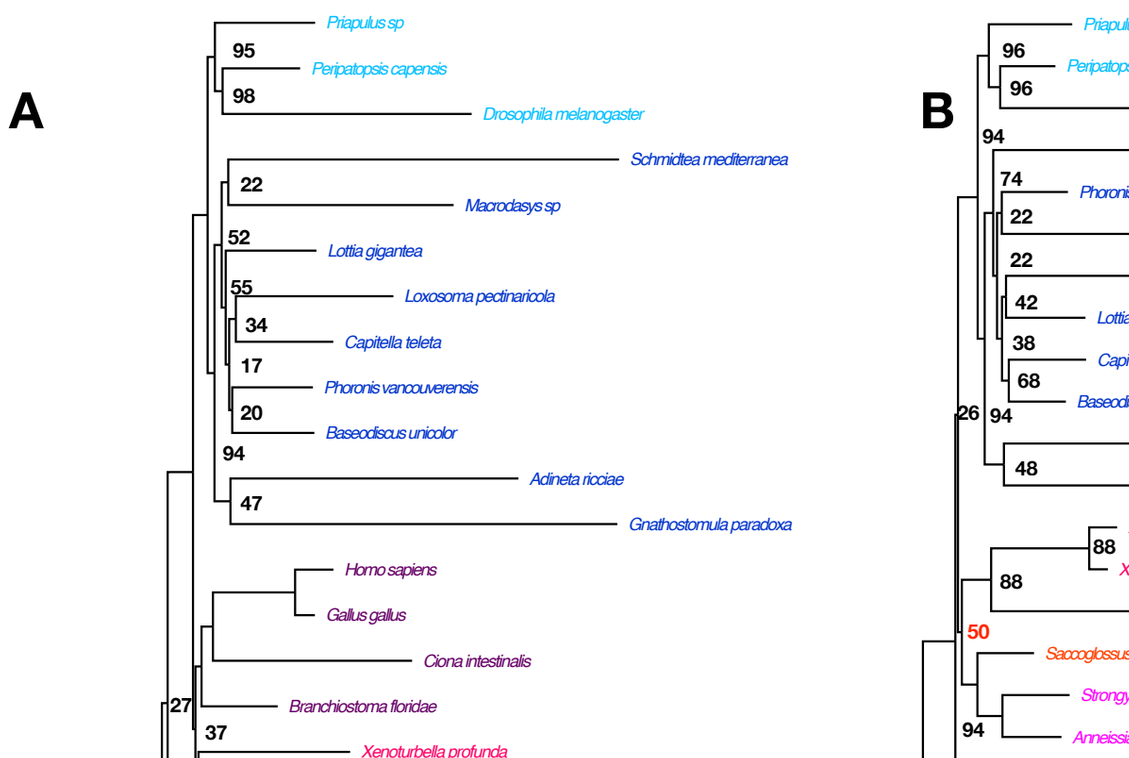
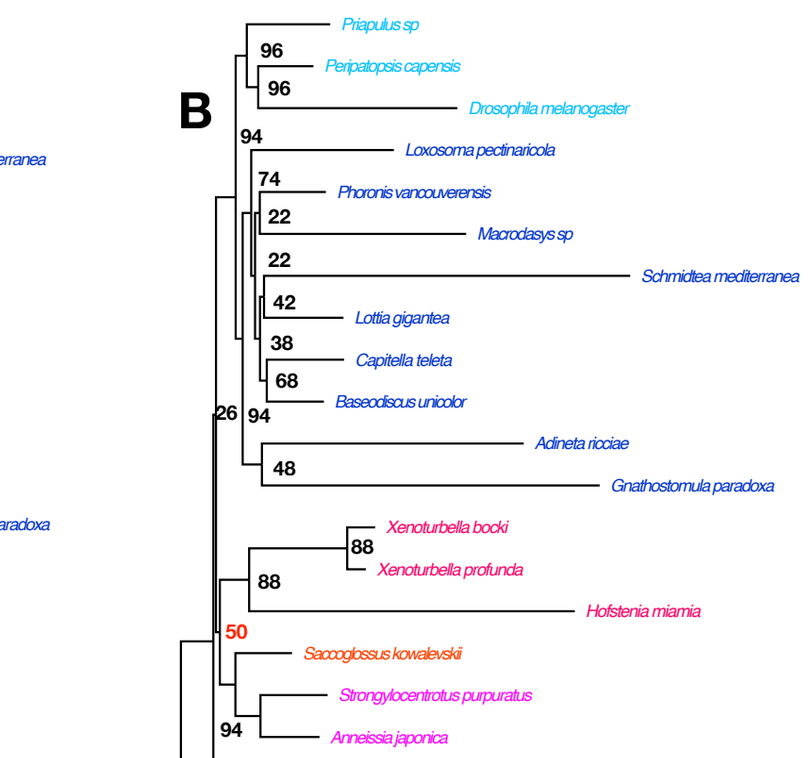


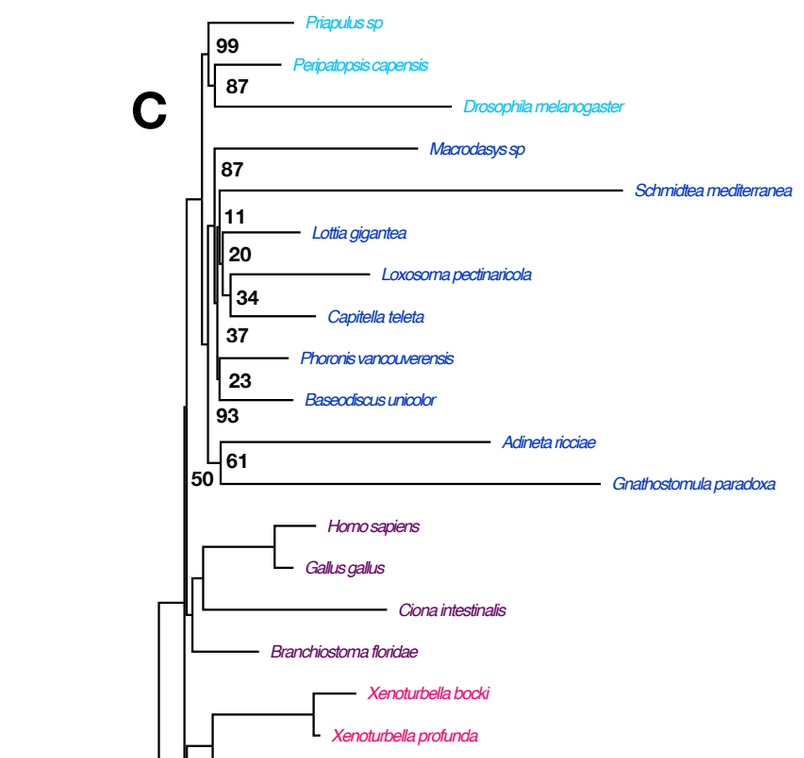
Figure S3. Reanalyses of data from Cannon *et al.* [S1] using Phylobayes. Related to Figure 1.

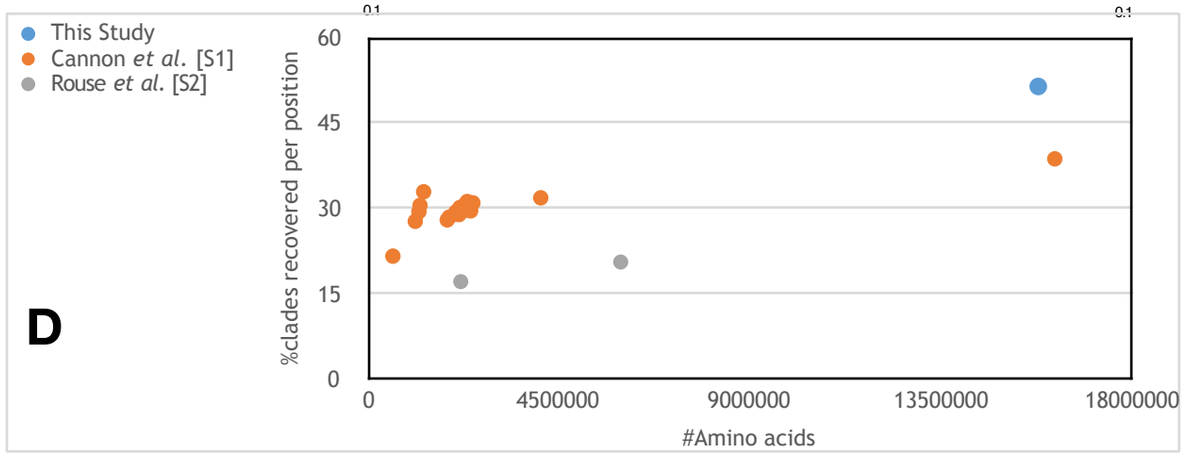
A. Phylobayes jackknife analysis. Cannon *et al.* [S1] data, Best genes, All taxa, CATGTR, 50 x 30,000 amino acids. Jackknife proportions less than 100% shown to right of node supported. Xenambulacraria support highlighted in red.

B. Phylobayes jackknife analysis. Cannon *et al.* [S1] data, All genes, All taxa, Dayhoff Recoded, CATGTR, 50 x 30,000 amino acids. Jackknife proportions less than 100% shown to right of node supported. Xenambulacraria support highlighted in red.









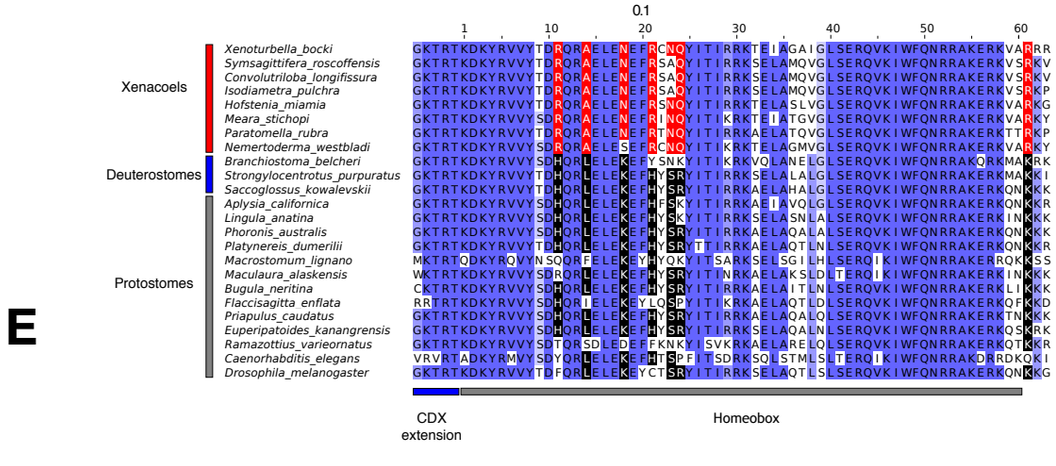


Figure S4. Reanalyses of data from Rouse *et al.* [S2] using Phylobayes, comparison of three data sets and sequences of CDX genes supporting monophyly of Xenacoleomorpha. Related to Figure 1.

A. Phylobayes jackknife analysis. Rouse *et al.* [S2] data, All genes, No Acoelomorphs, CATGTR, 50 x 30,000 amino acids. Jackknife proportions <100% shown to right of node supported. Xenambulacraria support highlighted in red.

B. Phylobayes jackknife analysis. Rouse *et al.* [S2] data, Best genes, All taxa, CATGTR, 50 x 30,000 amino acids Jackknife. Jackknife proportions <100% shown to right of node supported. Xenambulacraria support highlighted in red.

C. Phylobayes jackknife analysis. Rouse *et al.* [S2] data, All genes, All taxa, Dayhoff Recoded, CATGTR, 50 x 30,000 amino acids. Jackknife proportions <100% shown to right of node supported. Xenambulacraria support highlighted in red.

D. Comparison of size and ability to reconstruct clades of different recent data sets used to reconstruct position of xenacoelomorphs. X axis: total number of amino acids in alignment. Y axis: % of clades that are present in the tree reconstructed from the total data set that are recovered by individual genes - score is the average % across genes. Cannon *et al.* [S1] and Rouse *et al.* [S2] presented several different data sets as shown.

E. Alignment of homeobox region of the CDX (Caudal) gene from bilaterians. Amino acids unique to, and supporting monophyly of Xenacoelomorpha are indicated in red.

Supplemental References

S1. Cannon, J.T., Vellutini, B.C., Smith III, J., Ronsquist, F., Jondelius, U., and Hejnol, A. (2016). Xenacoelomorpha is the sister group to Nephrozoa. Nature *530*, 89–93.

S2. Rouse, G.W., Wilson, N.G., Caravajal, J., I, and Vrijenhoek, R.C. (2016). New deep-sea species of *Xenoturbella* and the position of Xenacoelomorpha. Nature *530*, 94–97.

Supplemental Table 1

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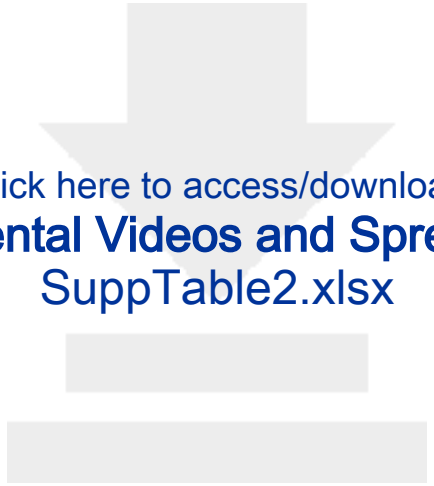
Supplemental Videos and Spreadsheets

SuppTable1.xlsx

Supplemental Table 2



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SuppTable2.xlsx