LETTER

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New deep-sea species of *Xenoturbella* and the position of Xenacoelomorpha

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The discovery of four new *Xenoturbella* species from deep waters of the eastern Pacific Ocean is reported here. The genus and two nominal species were described from the west coast of Sweden[1,2], but their taxonomic placement remains unstable[3,4]. Limited evidence placed *Xenoturbella* with molluscs[5,6], but the tissues can be contaminated with prey[7,8]. They were then considered deuterostomes[9–13]. Further taxon sampling and analysis have grouped *Xenoturbella* with acoelomorphs (=Xenacoelomorpha) as sister to all other Bilateria (=Nephrozoa)[14,15], or placed Xenacoelomorpha inside Deuterostomia with Ambulacraria (Hemichordata + Echinodermata)[16]. Here we describe four new species of *Xenoturbella* and reassess those hypotheses. A large species (>20 cm long) was found at cold-water hydrocarbon seeps at 2,890 m depth in Monterey Canyon and at 1,722 m in the Gulf of California (Mexico). A second large species (~10 cm long) also occurred at 1,722 m in the Gulf of California. The third large species (~15 cm long) was found at ~3,700 m depth near a newly discovered carbonate-hosted hydrothermal vent in the Gulf of California. Finally, a small species (~2.5 cm long), found near a whale carcass at 631 m depth in Monterey Submarine Canyon (California), resembles the two nominal species from Sweden. Analysis of whole mitochondrial genomes places the three larger species as a sister clade to the smaller Atlantic and Pacific species. Phylogenomic analyses of transcriptomic sequences support placement of Xenacoelomorpha as sister to Nephrozoa or Protostomia.

Xenoturbellida Bourlat *et al.*, 2006

Genus *Xenoturbella* Westblad, 1949

Xenoturbella monstrosa sp. nov.

Etymology. Latin for extraordinary size.

Material examined. Holotype, Scripps Institution of Oceanography Benthic Invertebrate Collection SIO-BIC BI1037, sex unknown (Extended Data Fig. 1c). Paratype SIO-BIC BI1038, from type locality, sex unknown. Paratype SIO-BIC BI1039, female, from Mexican locality (Fig. 1b and Extended Data Fig. 1d–h).

Locality. Monterey Submarine Canyon, California, 36° 36.8′ N, 122° 26.0′ W, ~2,890 m depth, vesicomyid clam field (Extended Data Fig. 1a, b). Collected via slurp system, research vessel (R/V) *Western Flyer*/remote operated vehicle (ROV) *Tiburon* dive 610, 25 October 2004. Also from Guaymas Transform Fault, Gulf of California, Mexico, 27° 34.659′ N, 111° 27.036′ W, 1,722 m depth, near a cold-water methane seep, 'Pinkies Vent'. Collected via slurp, R/V *Western Flyer*/ROV *Doc Ricketts* dive 385, 13 April 2012.

Description. Body ~20 cm long in life, purple or pale pink. Dorsal body wall with two deep, longitudinal furrows. Ring furrow and side furrow found. Body in front of ring furrow rounded; posterior tapers gradually. Mouth oval-shaped (when relaxed), lying ventrally midway between ring furrow and anterior end. Epidermal network over two-thirds of ventral surface. Body wall with gametes dorsally and ventrally. Exogenous DNA present in tissue from co-occurring vesicomyid bivalves *Archivesica diagonalis* (in holotype) or *Calyptogena pacifica* (in Mexican paratype).

Xenoturbella churro sp. nov.

Etymology. Noun used in apposition. Resembles fried-dough pastry called churro.

Material examined. Holotype (SIO-BIC BI1040), female (Fig. 1b and Extended Data Fig. 2a–e).

Locality. Guaymas Transform Fault, Gulf of California, Mexico, 27° 34.659′ N, 111° 27.036′ W, 1,722 m depth, near 'Pinkies Vent'. Collected via slurp, R/V *Western Flyer*/ROV *Doc Ricketts* dive 385, 13 April 2012.

Description. Body 10 cm long in life, uniformly orange/pink. Dorsal body wall with four deep, longitudinal furrows. Ring furrow and side furrow present. Anterior end rounded, posterior tapers sharply. Mouth oval (when relaxed), lying ventrally midway between ring furrow and anterior end. Epidermal network over two-thirds of ventral surface. Body wall with gametes dorsally and ventrally. Exogenous DNA from co-occurring vesicomyid bivalve *C. pacifica* present in tissue.

Xenoturbella profunda sp. nov.

Etymology. Latin for deep.

Material examined. Holotype SIO-BIC BI1041, male (Extended Data Fig. 3b, c, g, h). Paratypes (SIO-BIC BI1042-46, UNAM ICML-EMU-11010), a female and five males or juveniles (Fig. 1c and Extended Data Fig. 3a, d–f), from type locality.

Locality. Pescadero Basin, Mexico, 23° 57.23′ N, 108° 51.73′ W, 3,700 m depth on sediment near hydrothermal vent. Collected via slurp, R/V *Western Flyer*/ROV *Doc Ricketts* over several dives in April 2015.

Description. Body to 15 cm in life, uniformly pale pink, with epidermis, circular and longitudinal muscles, parenchyma and gastrodermis. Dorsal body with a pair of deep, longitudinal furrows. Ring furrow and side furrow found. Anterior end rounded, posterior tapering gradually. Mouth oval (when relaxed), ventral, anterior to ring furrow. Epidermal network over two-thirds of ventral surface. Gametes dorsally and ventrally. Oocytes reach 450 μm in diameter, sperm with spherical heads. Exogenous DNA of vesicomyid bivalve *A. gigas* present in tissue.

Xenoturbella hollandorum sp. nov.

Etymology. Named for Linda and Nicholas Holland for their contributions to biology.

Material examined. Holotype SIO-BIC BI1036, sex unknown (Fig. 1d and Extended Data Fig. 4a–d).

Locality. Monterey Submarine Canyon, California, 36° 48.132′ N, 121° 59.647′ W; ~631 m depth, on sediment adjacent to bones of a grey whale. Collected via push core, R/V *Western Flyer*/ROV *Tiburon* dive 1,160, 18 December 2007.

Description. Body short (2.5 cm) and uniformly bright pink. Dorsal body with a pair of longitudinal furrows. Ring furrow and side furrow found. Ventral mouth present, diamond-shaped (when relaxed), just anterior to ring furrow. Epidermal network over ventral surface anterior to ring furrow. Exogenous cytochrome-*c*-oxidase subunit I (*COI*) sequences of bivalves not detected.

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Figure 1 | New species of *Xenoturbella*. a, *X. monstrosa* (arrows) in a field of *A. diagonalis* (*Ad*) and *Ectenagena elongata* (*Ee*) clams at 2,890 m depth in Monterey Bay, California. Scale estimated from the average size of *A. diagonalis*[27]. **b**, *X. monstrosa* (arrow at upper left) and *X. churro* (asterisk at lower right) at 1,722 m depth near a methane seep in the Guaymas Basin, Mexico. Red spots (lasers) are 29 cm apart. Shell fragments in sediment, but no living clams observed in vicinity. Both specimens contained *C. pacifica* DNA in their tissues. **c**, *X. profunda* female in an *A. gigas* (*Ag*) clam field near a carbonate-hosted hydrothermal vent at ~3,700 m depth in Pescadero Basin, Mexico. **d**. *X. hollandorum* on sediment adjacent to whale bones (b). Abbreviations: a, anterior; rf, ring furrow; h, hemichordate; o, bone-eating *Osedax* worms; p, polynoid scaleworm; s, shrimp.

While we have markedly increased the diversity of the group, the morphological similarities of the new species leads us to keep them in *Xenoturbella*[1]. Live specimens of all four new species exhibited an epidermal branching network ventrally that has not been previously recognized. This network was inconspicuous in *X. hollandorum* and consequently may have been overlooked previously in *X. bocki*. *X. profunda* is gonochoric, as opposed to the hermaphroditism reported for *X. bocki*, which also has much smaller eggs[1,4]. *X. monstrosa* was seen in abundance once (Fig. 1a, Extended Data Fig. 1a, b and Supplementary Video 1) in a vesicomyid clam field (mostly *Ectenagena extenta*, some *A. diagonalis*) in Monterey Canyon, but repeated visits to this site failed to reveal more specimens. The *X. monstrosa* collected from Monterey were in a patch of mainly *A. diagonalis* that were dying or spawning, producing large amounts of mucus (Supplementary Video 1). The Gulf of California specimen extends the range of *X. monstrosa* to >2,500 km and depth from ~1,700 to ~3,000 m. The single specimen of *X. churro* was found only 30 cm from the Mexican specimen of *X. monstrosa* (Fig. 1b and Supplementary Video 2). *Xenoturbella profunda* lives deepest of the known species, and all seven collected specimens were observed on sediments (showing bacterial mats and various fauna, including vesicomyids) near a hydrothermal vent (Supplementary Video 3).

Mitochondrial *COI* was sequenced for all specimens examined and compared with other *Xenoturbella*. The nominal species from Sweden, *X. bocki* and *X. westbladi*, comprised a single haplotype network with several shared sequences (Extended Data Fig. 5a), which strongly suggests it is a single species, although this has previously been dismissed[17]. Here we treat *X. westbladi* as a junior synonym of *X. bocki*. The two specimens of *X. monstrosa* from California differed by one base pair (bp) and from the Mexican specimen by up to 7 bp (Extended Data Fig. 5b). Seven specimens of *X. profunda* exhibited four haplotypes, differing by one to three bp (Extended Data Fig. 5c).

Whole mitochondrial genomes of the four new species (Extended Data Fig. 6) exhibited the same gene order as *X. bocki*[9,18]. Phylogenetic analysis of these genomes placed the smaller, shallower-dwelling species, *X. hollandorum* and *X. bocki*, as sister taxa differing at ≥6.4% of the nucleotide sites and 59 amino acids. The distinction of *X. hollandorum* as a new species is also supported by nuclear sequence data (Supplementary Table 1). Among all other *Xenoturbella* species, mitochondrial genomes differed by 10–20% (Supplementary Data Table 1), with the deep-water species forming a clade with respect to *X. hollandorum* and *X. bocki* (Fig. 2). Phylogenetic comparison of mitochondrial proteins placed Xenacoelomorpha with deuterostomes, as previously reported[9,16,18] (Extended Data Fig. 7), although poorly supported. Notably, the PhyloBayes[19] analysis recovered Xenacoelomorpha as sister group to Chordata, also with poor support, and we question the utility of such data at deep phylogenetic scales.

Phylogenomic analyses of *Xenoturbella* compared our transcriptomic data from *X. profunda* with limited transcriptomic (expressed sequence tag) data from *X. bocki*[11], and genomic and transcriptomic data from other animals. The main data set included 26 terminals and 1,178 genes with 70% average gene occupancy (Supplementary Table 5). Maximum likelihood analyses using RAxML[20] with either the WAG, LG or GTR amino-acid substitution models and gamma (Γ) correction for rate heterogeneity produced identical tree topologies and similar support values, grouping *Xenoturbella* with the acoel *Hofstenia* (=Xenacoelomorpha) as sister to Nephrozoa

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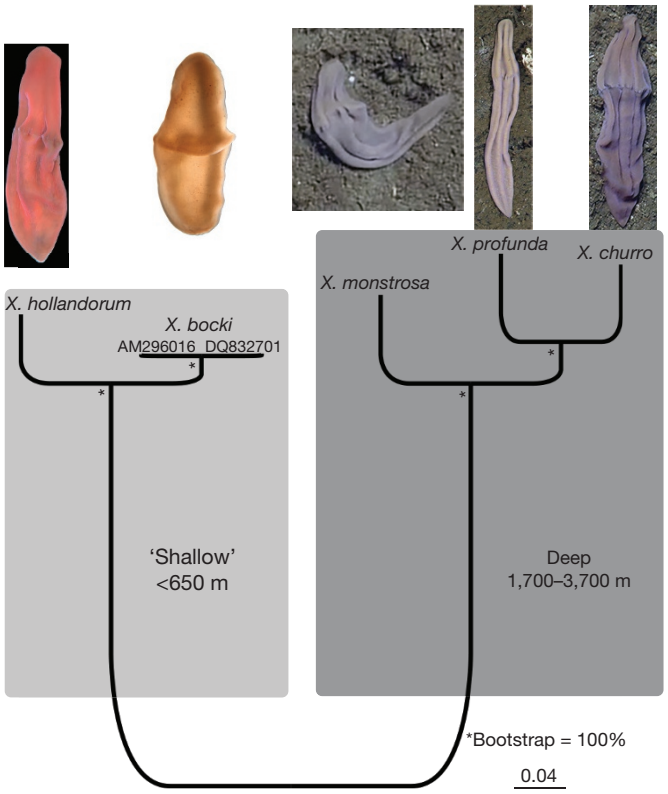

Figure 2 | Phylogeny of *Xenoturbella* based on mitochondrial genomic DNA sequences (15,532 base-pair alignment) using RAxML and GTR + Γ. Support assessed via 100 pseudo-replicates of bootstrapping. Scale bar, substitutions per nucleotide position. All nodes were highly supported as indicated by bootstrap support of 100% (asterisk). Rooting based on the topology recovered from the analysis of mitogenomes of Metazoa (Extended Data Fig. 7). Scale bar, substitutions per nucleotide position.

(deuterostomes + protostomes; Fig. 3). All key nodes were well supported. Alternative hypotheses assessed using SOWH[21] constraining Xenacoelomorpha as sister group to Ambulacraria ($n = 100$, Δ-likelihood = 1,313.9, $P = 0.027$), or to Deuterostomia ($n = 100$, Δ-likelihood = 1,042.07, $P = 0.031$), were rejected as significantly worse. A species tree[22] generated from the consensus of 393 individual gene-trees (average occupancy = 80%) (Extended Data Fig. 8) placed Xenacoelomorpha as sister to Nephrozoa, although there was poor support for the latter clade. We also used PhyloBayes under CAT + GTR + Γ, as recommended in a previous assessment of the position of *Xenoturbella*[16]. The result placed Xenacoelomorpha as sister to protostomes with moderate support, a placement not previously recovered (Extended Data Fig. 9). To explore if elevated substitution rates in some loci could be influencing our analyses[23], we removed the ribosomal proteins (51 loci) from the 1,178-locus data set and then split this into slowest- and fastest-evolving matrices. Maximum likelihood analyses revealed no major differences between the two data sets or incongruence with the complete data set (Extended Data Fig. 10).

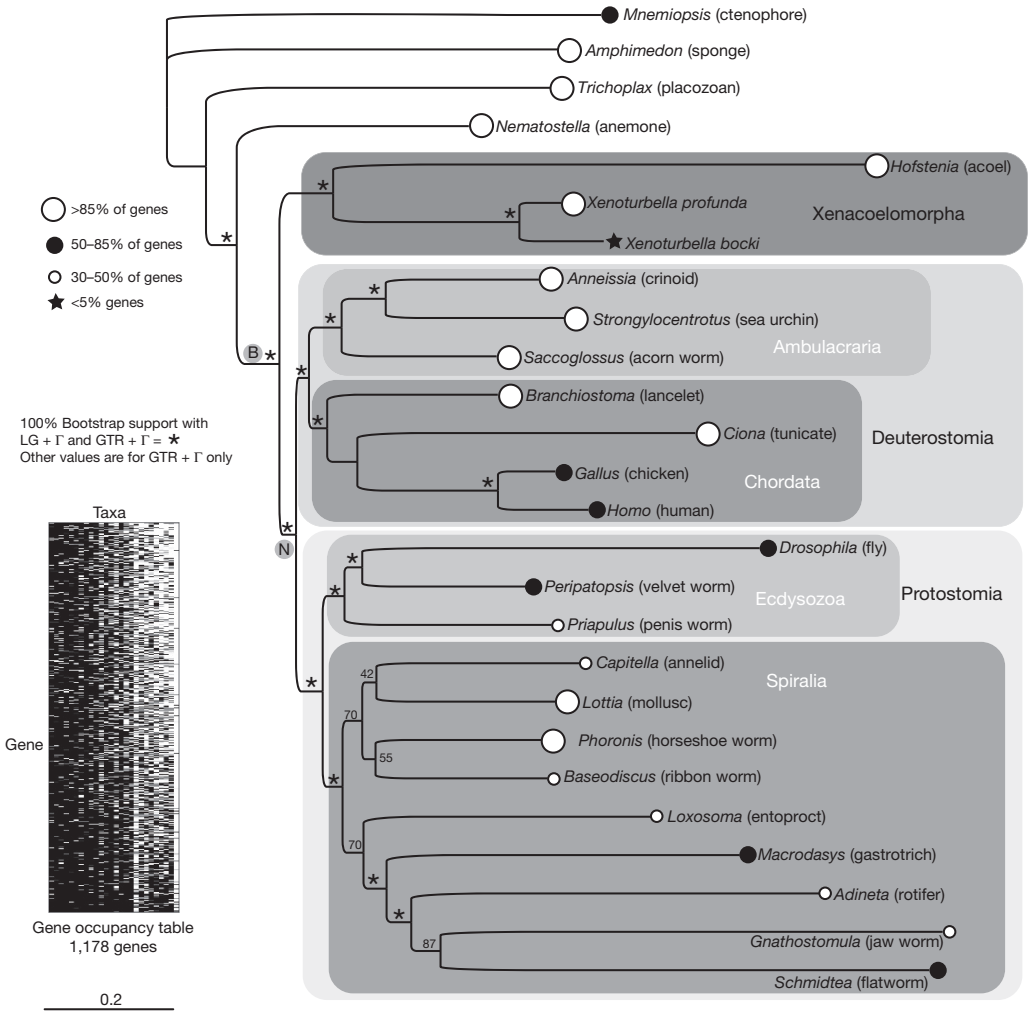

Figure 3 | Maximum likelihood phylogeny of animals based on 1,178 genes (394,818 amino acids) with 70% average gene occupancy. Within Bilateria (B), Xenacoelomorpha is sister group to Nephrozoa (N), which includes Protostomia and Deuterostomia. Key nodes were highly supported, as indicated by bootstrap support of 100% (asterisks). Data were analysed using RAxML with GTR, LG and WAG models, + Γ. Support values of less than 100 are shown for GTR + Γ; the same nodes had higher support with LG + Γ and WAG + Γ. Scale bar, substitutions per amino-acid position.

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We sampled four new *Xenoturbella* species from cold-water hydrocarbon seeps, a hydrothermal vent, and from the vicinity of a whale-fall, all environments that host dense communities of animals. These discoveries highlight the possibility of further *Xenoturbella* discoveries in deep-sea environments that sustain their likely food source, bivalve molluscs. Although the placement of *Xenoturbella* has been a challenge in metazoan phylogenetics, the present application of transcriptomic data resulted in much larger sampling of genes, which has helped to resolve deep phylogenetic relationships[24]. Our analyses showed no support for the hypothesis that Xenacoelomorpha lies inside deuterostomes[7,9,16], a result also corroborated by another phylogenomic study on the position of Xenacoelomorpha[25]. The placement of Xenacoelomorpha as sister group to Nephrozoa (Fig. 3 and Extended Data Figs 8 and 10), or to Protostomia (Extended Data Fig. 9), suggests the suite of features previously proposed to be character losses in *Xenoturbella*[13] appear instead to be plesiomorphic absences.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions G.W.R., N.G.W. and R.C.V. collected the specimens. N.G.W. and J.I.C. generated and assembled mitochondrial data for the new *Xenoturbella* species. J.I.C. generated the *Xenoturbella* Illumina transcriptome and assembled the data. G.W.R. and J.I.C. performed phylogenetic analyses of mitochondrial genomes and transcriptome data. G.W.R. analysed the morphology of *Xenoturbella* spp. for the taxonomic descriptions. G.W.R. drafted the paper with J.I.C., N.G.W. and R.C.V. All authors commented on the manuscript.

Author Information Sequence data have been deposited in GenBank; accession numbers can be found in Supplementary Tables 3–5. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to G.W.R. (grouse@ucsd.edu).

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METHODS

No statistical methods were used to predetermine sample size.

Collecting details. Once on board the ship, specimens were maintained in chilled seawater in a 4 °C cold-room. *Xenoturbella* was photographed under a Leica MZ8 stereomicroscope. Larger animals were photographed with a macro lens. Animals were then relaxed in 7% MgCl in freshwater before further photography. Pieces of tissue were then taken for molecular sequencing and frozen or placed into RNAlater or 95% ethanol. Other pieces of tissue were fixed in 4% paraformaldehyde in 0.2 M sodium phosphate buffer overnight before rinsing and being placed in buffer with sodium azide or processed further and kept in 70% ethanol. One specimen of *X. monstrosa* was fixed and preserved in 95% ethanol and the other in 10% formalin in seawater. All other specimens were fixed in 4% paraformaldehyde in buffer and preserved in 70% ethanol. All specimens are lodged at the Scripps Institution of Oceanography Benthic Invertebrate Collection (SIO-BIC), La Jolla, California, except for a paratype of *X. profunda*, which is lodged at the Instituto de Ciencias del Mar y Limnologia (UNAM), Mazatlán, Mexico, and catalogued as ICML-EMU11010. Taxonomic acts are registered on Zoobank (http://zoobank.org/) as urn:lsid:zoobank.org:pub:135BCEF9-A1E4-4926-B346-3711A6485690.

Amplification and sequencing of mitochondrial and nuclear genes and mitochondrial genomes. We extracted DNA from *Xenoturbella* individuals using a DNeasy Blood & Tissue Kit following the manufacturer's specifications (Qiagen). *COI* for all *Xenoturbella* individuals and their bivalve prey was amplified with the primers listed in Supplementary Table 2. Histone H3 (HH3) was also amplified with the primers listed in Supplementary Table 2. NCBI accession numbers are listed in Supplementary Table 3. To acquire *Xenoturbella* mitochondrial genomes, we initially amplified portions of three genes: 16S rRNA (*16S*), cytochrome B (*CytB*) and cytochrome-*c*-oxidase subunit III (*COIII*). Primer sequences and thermocycling conditions are provided in Supplementary Table 2. All amplifications were done using illustra PuReTaq Ready-To-Go PCR Beads (GE Life Sciences) following the manufacturer's protocol. PCR products were cleaned using USB ExoSAP-IT, sequenced by Eurofins MWG Operon. Geneious R7 (ref. 28) was used to inspect and trim sequences.

We then used the *16S*, *CytB* and *COIII* sequences and the Primer3 (ref. 29) algorithm in Geneious to design *Xenoturbella*-specific primers for long PCR amplifications. The mitochondrial genomes of *X. monstrosa*, *X. hollandorum* and *X. churro* were amplified in two overlapping fragments that were each 8,000 bp long using primers listed in Supplementary Table 2. All long PCR products were amplified using Platinum Taq DNA Polymerase High Fidelity (Invitrogen) following the manufacturer's specifications. The PCR products were visualized on 0.9% agarose gels run at 80 V for 90 min. PCR products were cleaned using USB ExoSAP-IT or GelElute Extraction kit (5 Prime). The *X. hollandorum* mitochondrial genome was outsourced to Engencore (Selah Genetics) for sequencing and assembly with the Roche 454 platform and Newbler version 2.3. The *X. monstrosa* and *X. churro* mitochondrial genomes were sequenced by Macrogen using Illumina HiSeq2000. The reads were assembled *de novo* after low-quality reads and adaptor sequences were removed using Geneious. The *X. profunda* mitochondrial genome was obtained from the transcriptome data. *X. profunda* and *X. churro* had fragments of the mitochondrial genome that were not sequenced. These missing 'fill-in' fragments were recovered via direct sequencing using primers listed in Supplementary Table 2 (refs 12, 30–32).

RNA extraction and transcriptome sequencing. *X. profunda* tissue was finely chopped and placed in RNAlater (Ambion) for 24 h at 4 °C and subsequently at −80 °C for long-term storage. RNA was extracted using Direct-zol RNA Kits (Zymo Research) following the manufacturer's protocol. The optional DNase I step was performed during the RNA extraction to remove residual genomic DNA. RNA extractions were then purified using RNA Clean & Concentrator kits (Zymo Research) following the manufacturer's protocol. Libraries were prepared from the purified mRNA using KAPA Stranded mRNA-Seq Kits (Kapa Biosystems) following the manufacturer's protocol. Libraries were sequenced (150 bp paired-end) using a 300 cycle Miseq kit (Illumina) by the IGM Genomics Center (University of California, San Diego). 9,941,202 read pairs were generated from this run, which were then assembled using Trinity into 31,374 transcripts after low-quality, adaptor-contaminated or ribosomal rRNA reads were removed.

Mitochondrial data analysis. We used TCS[33] to construct *COI* haplotype networks for the *Xenoturbella* species with multiple sequences. The mitochondrial phylogenetic analysis was based on the taxon sampling used previously[16]. All 13 protein coding regions of the *Xenoturbella* mitochondrial genomes were extracted and translated into amino-acid sequences in Geneious using translation table 5 (invertebrate mitochondrial). Translated amino-acid sequences from the other taxa were downloaded directly from GenBank (Supplementary Table 4). The complete mitochondrial genomes of the four new *Xenoturbella* species were uploaded to the MITOS web server for annotation using translation table 5 (http://mitos.bioinf.uni-leipzig.de).

The translated amino-acid sequences of each protein-coding gene were aligned independently using MAFFT[34] and the FFT-NS-i ×1000 algorithm. Each of these 13 alignments was then run on the GBLOCKS server with the least stringent settings to remove poorly aligned regions (http://molevol.cmima.csic.es/castresana/Gblocks_server.html)[35]. We used Geneious to concatenate the trimmed sequences into a single alignment of 2,519 positions.

The amino-acid data were analysed using RAxML 8.1.22 (ref. 20) with the data partitioned by gene (13 partitions) and with the GTR + Γ model. Clade support was assessed using 100 bootstrap pseudo-replicates. A PhyloBayes analysis of the data was also run using CAT + GTR + Γ, as done previously with a similar data set[16], although the files available from that study contained only 12 of the 13 protein coding genes (*COIII* was missing) and was also missing a further 361 bp from cytochrome *b* for *Amphipholis*.

For assessing the relationships among the five *Xenoturbella* species, the nucleotide data were analysed using RAxML with GTR + Γ. Clade support was assessed using 100 bootstrap pseudo-replicates. Rooting was based on the topology found in the analysis of the metazoan mitochondrial genomes (Extended Data Fig. 7).

Phylogenomic analyses. Taxa included in the transcriptome analysis are listed in Supplementary Table 5. Agalma[36] is an analysis pipeline (https://bitbucket.org/caseywdunn/agalma) for phylogenetics that was used to construct alignments of orthologous gene sequences from raw sequence data.

Adaptor sequences, low quality reads and ribosomal RNA sequences were removed from the *X. profunda* raw Illumina and NCBI SRA data. The transcriptomes were assembled using Trinity (version r20140413p1)[37] from the cleaned reads. The UCSC genome browser was used to download transcribed DNA sequences from the mrna.fa.gz file of the taxa with sequenced genomes. The assemblies and genome data were translated into amino-acid sequences by longest open reading frame. All of the translated transcriptomes were loaded into the Agalma database and then sent through the 'post-assemble' and 'phylogeny' pipelines. An all-by-all blast was used to find homologous sequences across the species based on sequence similarity. These blast results were aligned and run through RAxML 8.0 to make gene trees. These gene trees were used to identify orthologues; non-homologous sequences were removed. Two supermatrices were constructed using the homologous protein alignments. The complete matrix had 7,878 genes and 37% average gene occupancy. The matrix used in the phylogenetic analysis was trimmed down to 1,178 genes with 70% average gene occupancy (Supplementary Table 5).

The 70% matrix was used in all phylogenetic analysis. RAxML was used to calculate maximum likelihood trees. The -m PROTGAMMAAUTO option was called to automatically test for the best-fitting amino-acid substitution model. This selected the LG model. However, the more complex GTR model was not included in this test. For this reason, we ran rapid bootstrap analyses (100 replicates) and searches for the best scoring ML tree using both the LG and GTR amino-acid substitution models and the Γ model of rate heterogeneity (PROTLGGTR or PROTGAMMAGTR). We also ran the suboptimal model WAG + Γ, as used in previous studies involving *Xenoturbella*[11].

The Swofford–Olsen–Waddell–Hillis (SOWH) test[38] evaluates statistical support for incongruent phylogenetic topologies. We used SOWHAT (https://github.com/josephryan/sowhat)[21] to perform SOWH tests by comparing the following best constrained trees: (1) with Xenacoelomorpha as sister to Deuterostomia (((*Ciona*, *Gallus*, *Homo*, *Branchiostoma*, *Anneissia*, *Saccoglossus*, *Strongylocentrotus*), (*Hofstenia*, *X_bocki*, *X_profunda*)), *Peripatopsis*, *Capitella*, *Macrodasys*, *Baseodiscus*, *Priapulus*, *Lottia*, *Phoronis*, *Loxosoma*, *Gnathostomula*, *Adineta*, *Drosophila*, *Mnemiopsis*, *Trichoplax*, *Amphimedon*, *Nematostella*, *Schmidtea*); or (2) with Xenacoelomorpha as sister to Ambulacraria within Deuterostomia ((((*Saccoglossus*, *Strongylocentrotus*, *Anneissia*), (*Hofstenia*, *X_profunda*, *X_bocki*)), *Branchiostoma*, *Homo*, *Gallus*, *Ciona*), *Peripatopsis*, *Schmidtea*, *Capitella*, *Macrodasys*, *Baseodiscus*, *Priapulus*, *Lottia*, *Phoronis*, *Loxosoma*, *Gnathostomula*, *Adineta*, *Drosophila*, *Mnemiopsis*, *Trichoplax*, *Amphimedon*, *Nematostella*); against the maximum likelihood tree topology recovered using GTR + Γ. SOWHAT was run with RaxML for 130 replicates using WAG + Γ. WAG was used instead of LG or GTR because of computational capacity restrictions. Astral II[22] was used to estimate a species tree given a set of unrooted gene trees. A total of 393 gene alignments with an average occupancy of 80% were input into Astral II (https://github.com/smirarab/ASTRAL/). The Astral II species tree was generated from the 393 gene trees with 100 replicates of bootstrapping. PhyloBayes[19] MPI was run on CIPRES (https://www.phylo.org/) for 4,460 generations using the CAT + GTR + Γ4 options shown previously to be optimal[16]. The first 2,200 trees were discarded as burn-in and the posterior consensus was computed on the remaining 2,260 trees. All PhyloBayes analyses were conducted using the CIPRES Science Gateway[26] and all other phylogenetic analysis were conducted on Amazon Web services C4 Instances (AWS EC2).

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To assess if the phylogenetic signal differed between slow- and fast-evolving proteins, submatrices were constructed from the 70% average gene occupancy matrix. First, ribosomal proteins were screened from the original 70% matrix by blasting to the Uniprot ribosomal protein database (http://www.uniprot.org/docs/ribosomp). This filtered data set consisted of 1,127 genes (51 ribosomal proteins were removed). A gene tree was made for each gene and ranked by evolutionary rate[23] using a custom python script from (https://github.com/NathanWhelan/Order-genes-by-evolutioanry-rate). The ranked genes were divided into the fastest-evolving 50% and the slowest-evolving 50%. The alignments were concatenated and then used to construct a maximum likelihood tree using the PROTGAMMAGTR amino-acid substitution model in RAxML. Support was assessed with 100 bootstrap pseudo-replicates.

All alignments, SOWHAT constraint trees and partition information are available on the Dryad repository (http://datadryad.org/resource/doi:10.5061/dryad.79dq1).

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Extended Data Figure 1 | *X. monstrosa* sp. nov. a, Photograph taken by ROV of a vesicomyid clam field at ~3,000 m depth, Monterey Bay, California. Most clams are *E. extenta*. Two specimens of *Xenoturbella* are visible (arrows). Scale bar estimated from size of *E. extenta* (5 cm average width). **b**, Supplementary Video 1 frame grab showing same clam field as **a** with *A. diagonalis* (*Ad*) and *E. extenta* clams. Numerous *Xenoturbella* (arrows) were observed, including two specimens sampled for this study. Scale bar estimated from size of *E. extenta* (5 cm average width). **c**, Ventral view of the holotype SIO-BIC BI1037. Although highly contracted and incomplete (the posterior end was removed and frozen and the ventral area removed for histology), the specimen is still over 10 cm long. The mouth (m), ring furrow (rf) and side furrow (sf) are visible. **d**, Frame grab of paratype SIO-BIC BI1039, a female *in situ*. **e**, Dorsal view of paratype SIO-BIC BI1039 (relaxed) showing ring furrow (rf), side furrow (sf), oocytes in body wall (oo) and part of the ventral surface (v). **f**, Ventral view of paratype SIO-BIC BI1039 showing the mouth (m), ring furrow (rf), oocytes in body wall (oo) and part of the epidermal ventral glandular network (vgn). **g**, Close-up of the ventral posterior of paratype SIO-BIC BI1039 showing the trailing off of the ventral glandular network (vgn) and oocytes clearly visible in the body wall. **h**, Close-up of the ventral anterior of paratype SIO-BIC BI1039 showing the mouth (m), ring furrow (rf) and the beginning of the ventral glandular network (vgn) near the anterior tip of the animal.

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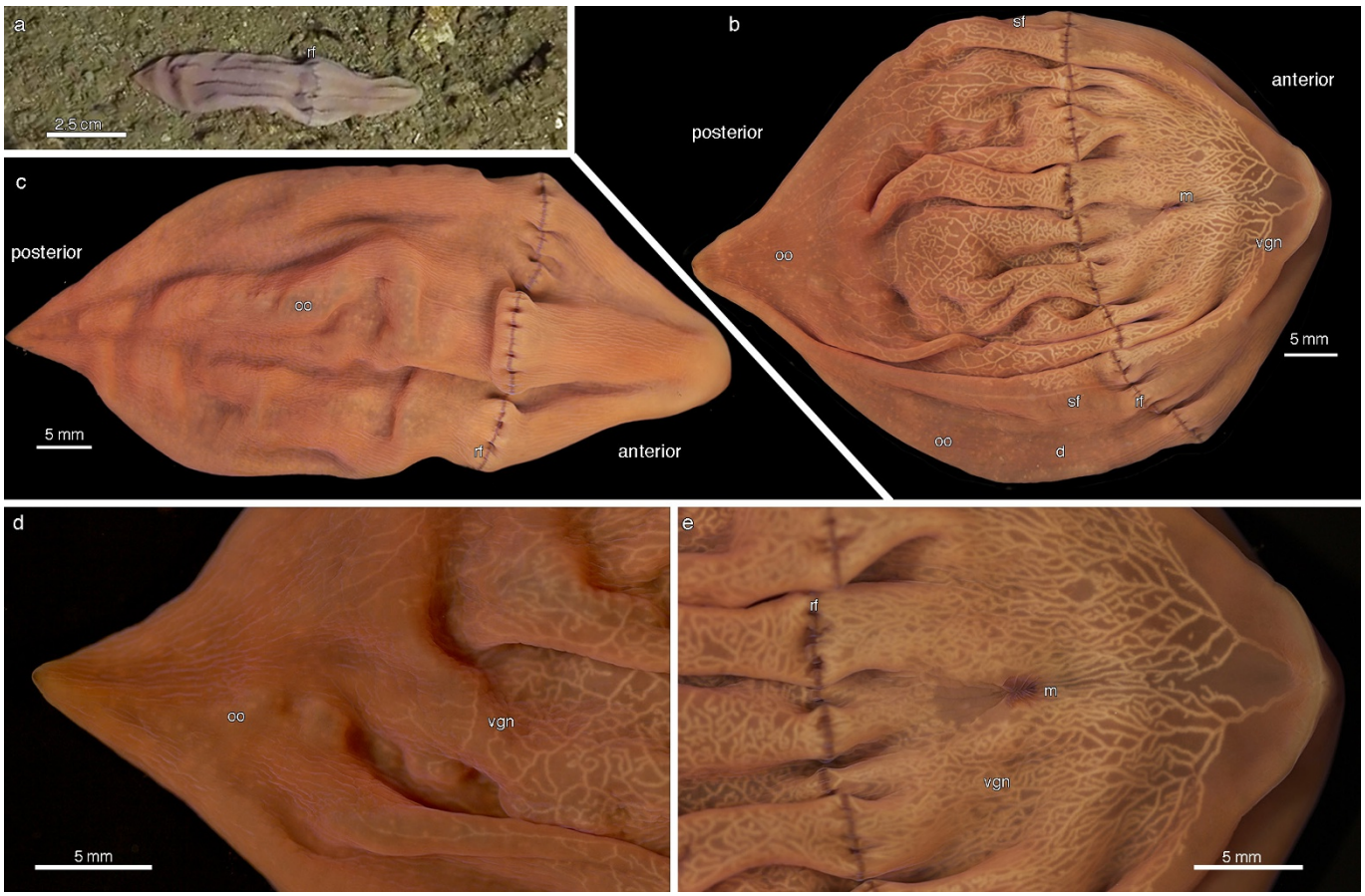

Extended Data Figure 2 | *X. churro* sp. nov. a, Frame grab of holotype SIO-BIC BI1040, a female, from the Guaymas Transform Fault, Gulf of California, Mexico, at ~1,700 m depth *in situ*. The ring furrow (rf) is visible towards the anterior end. **b**, Ventral view of the holotype SIO-BIC BI1040, (relaxed) showing mouth (m), ring furrow (rf), oocytes (oo), side furrow (sf) and epidermal ventral glandular network (vgn). Part of the dorsal side (d) is visible. **c**, Dorsal view of paratype SIO-BIC BI1039 showing ring furrow (rf) and oocytes (oo). **d**, Close-up of the ventral posterior of the holotype, showing the trailing off of the ventral glandular network (vgn), oocytes and the distinctively tapering posterior tip. **e**, Close-up of the anterior end of the holotype, showing the mouth (m), ring furrow (rf) and the beginning of the ventral glandular network (vgn) near the anterior tip of the animal.

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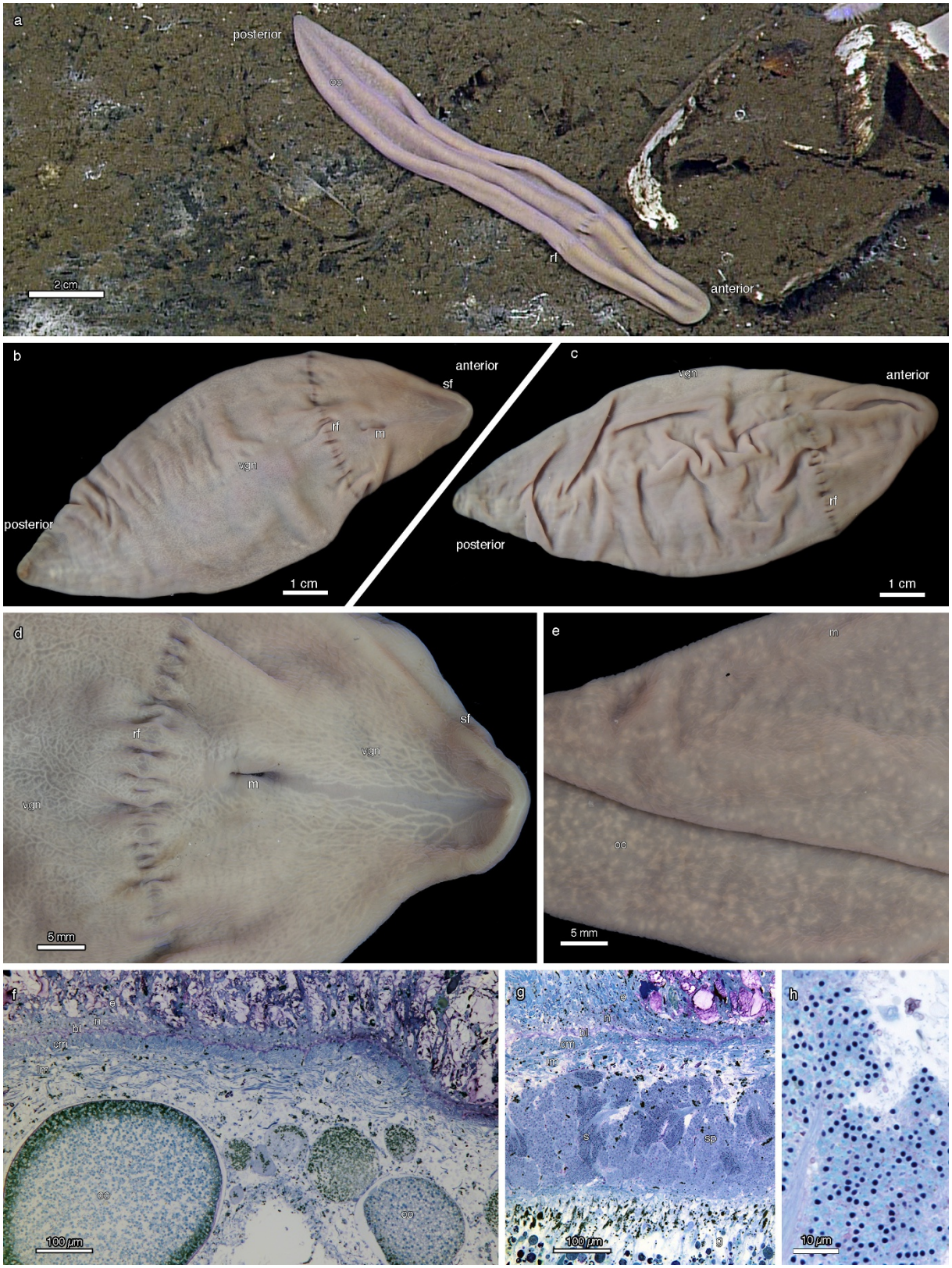

Extended Data Figure 3 | See next page for figure caption.

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Extended Data Figure 3 | *X. profunda* sp. nov. a, Frame grab of paratype SIO-BIC BI1044, a female, from the Pescadero Basin, Gulf of California, Mexico, at ~3,700 m depth *in situ*. The ring furrow (rf) is visible towards the anterior end. **b**, Ventral view of the holotype SIO-BIC BI1041, a male (relaxed) showing mouth (m), ring furrow (rf), side furrow (sf) and epidermal ventral glandular network (vgn). **c**, Dorsal view of the holotype showing the ring furrow (rf). The ventral glandular network (vgn) is visible where part of the ventral side is exposed. **d**, Close-up of the ventral anterior end of female paratype SIO-BIC BI1044, showing mouth (m), ring furrow (rf), side furrow (sf) and beginning of ventral glandular network (vgn) near the anterior tip of the animal. **e**, Close-up of the dorsal posterior of female paratype SIO-BIC BI1044, showing oocytes of different sizes distributed in the parenchyma. **f**, Parasagittal section (1 μm, stained with toluidine blue) through dorsal body of female paratype SIO-BIC BI1044 showing thick epidermis (e), subepidermal nerve net (n), basal lamina (bl), circular muscle layer (cm), longitudinal muscle layer (lm) and parenchyma with oocytes of various sizes (oo). The gastrodermis is not shown here. **g**, Parasagittal section (1 μm, stained with toluidine blue) through dorsal body wall of the male holotype SIO-BIC BI1041. Tissue layers as in the female, but a layer of developing (sp.) and mature sperm (s) instead of oocytes is present. The gastrodermis (g) lies beneath the parenchyma layer. **h**, Parasagittal section (1 μm, stained with toluidine blue) showing parenchyma layer with late spermatids or mature sperm with spherical heads and free flagella.

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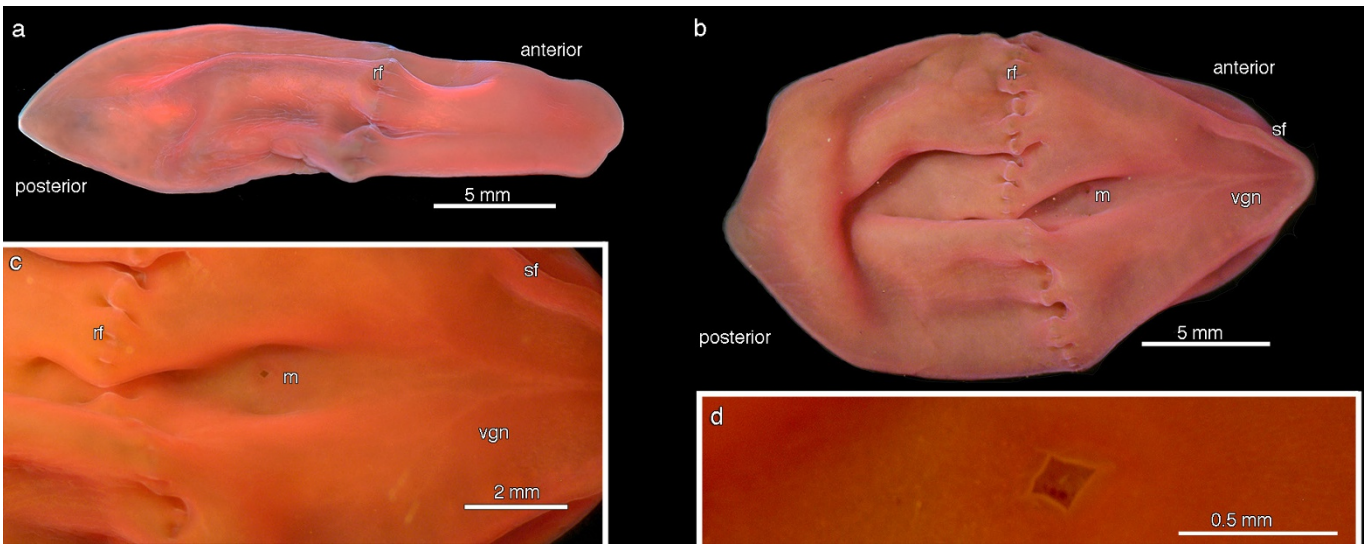

Extended Data Figure 4 | *X. hollandorum* sp. nov. a, Dorsal view of the live unrelaxed holotype SIO-BIC BI1036, showing ring furrow (rf). **b**, Ventral view of the holotype (relaxed) showing mouth (m), ring furrow (rf) side furrow (sf) and epidermal ventral glandular network (vgn). **c**, Close-up of the anterior end of the holotype, showing mouth (m), ring furrow (rf), side furrow (sf) and the ventral glandular network (vgn) near the anterior tip of the animal. **d**, Close-up of the diamond-shaped mouth.

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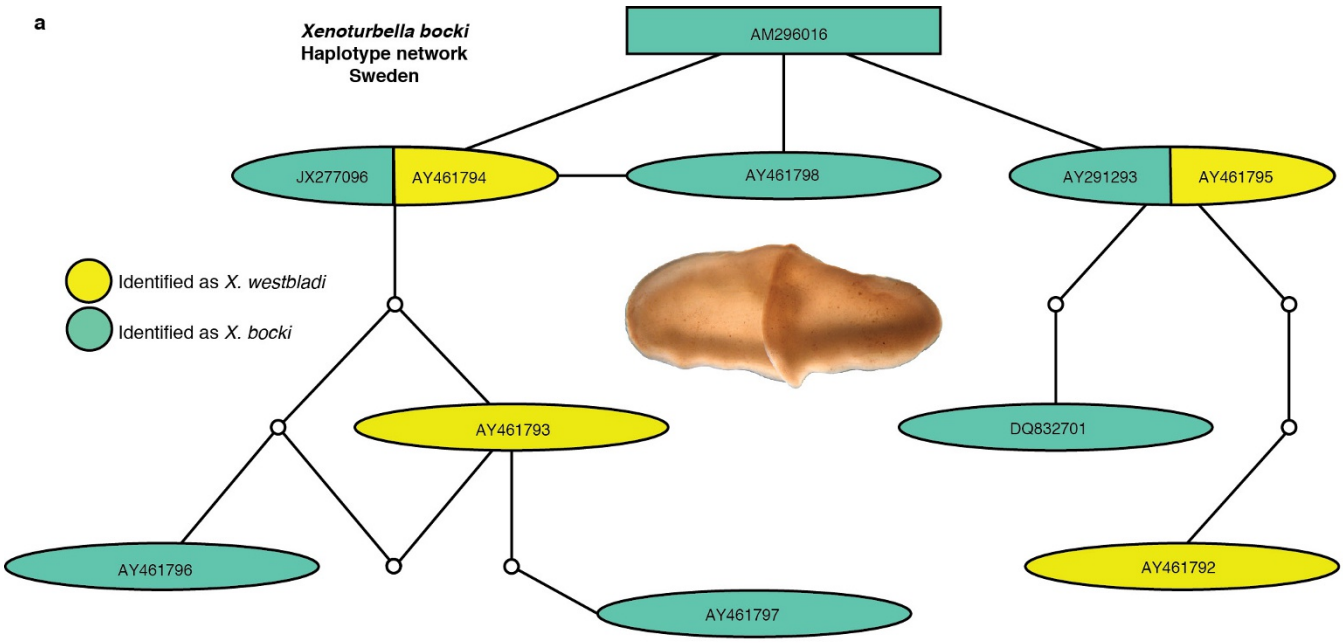

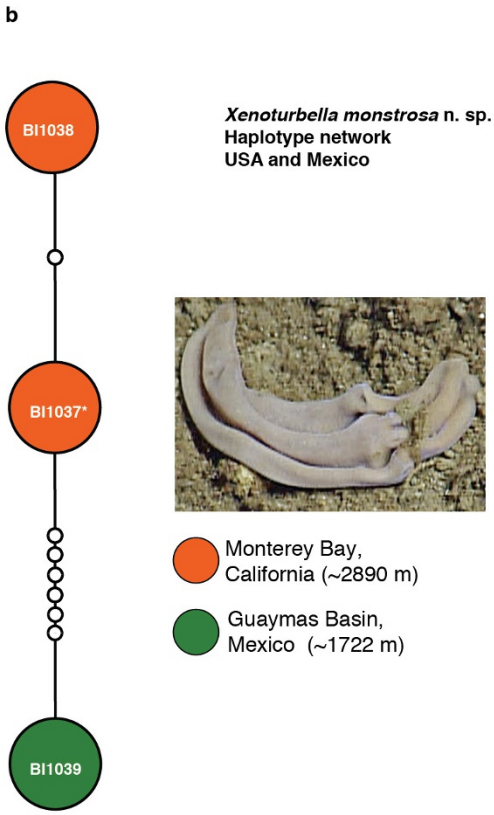

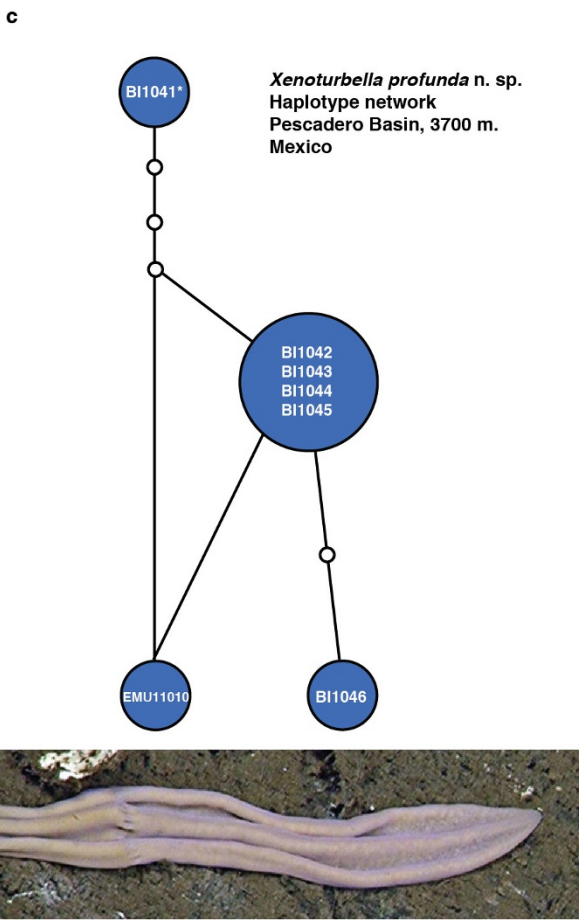

Extended Data Figure 5 | Mitochondrial *COI* haplotype networks. **a**, *X. bocki* and *X. westbladi* haplotypes (GenBank accession numbers indicated). Sequences from the two nominal species are completely interconnected. This network suggests that there is only one species in Swedish waters, which should be recognized under the older name, *X. bocki*. **b**, *X. monstrosa* from Monterey Bay (California) and Gulf of California (Mexico) (SIO-BIC accession numbers indicated). **c**, *X. profunda* from the vicinity of a hydrothermal vent at 3,700 m in the Pescadero Basin, Mexico (SIO-BIC and UNAM specimens indicated). Holotypes are designated with asterisks in the figure. Networks generated with TCS[33].

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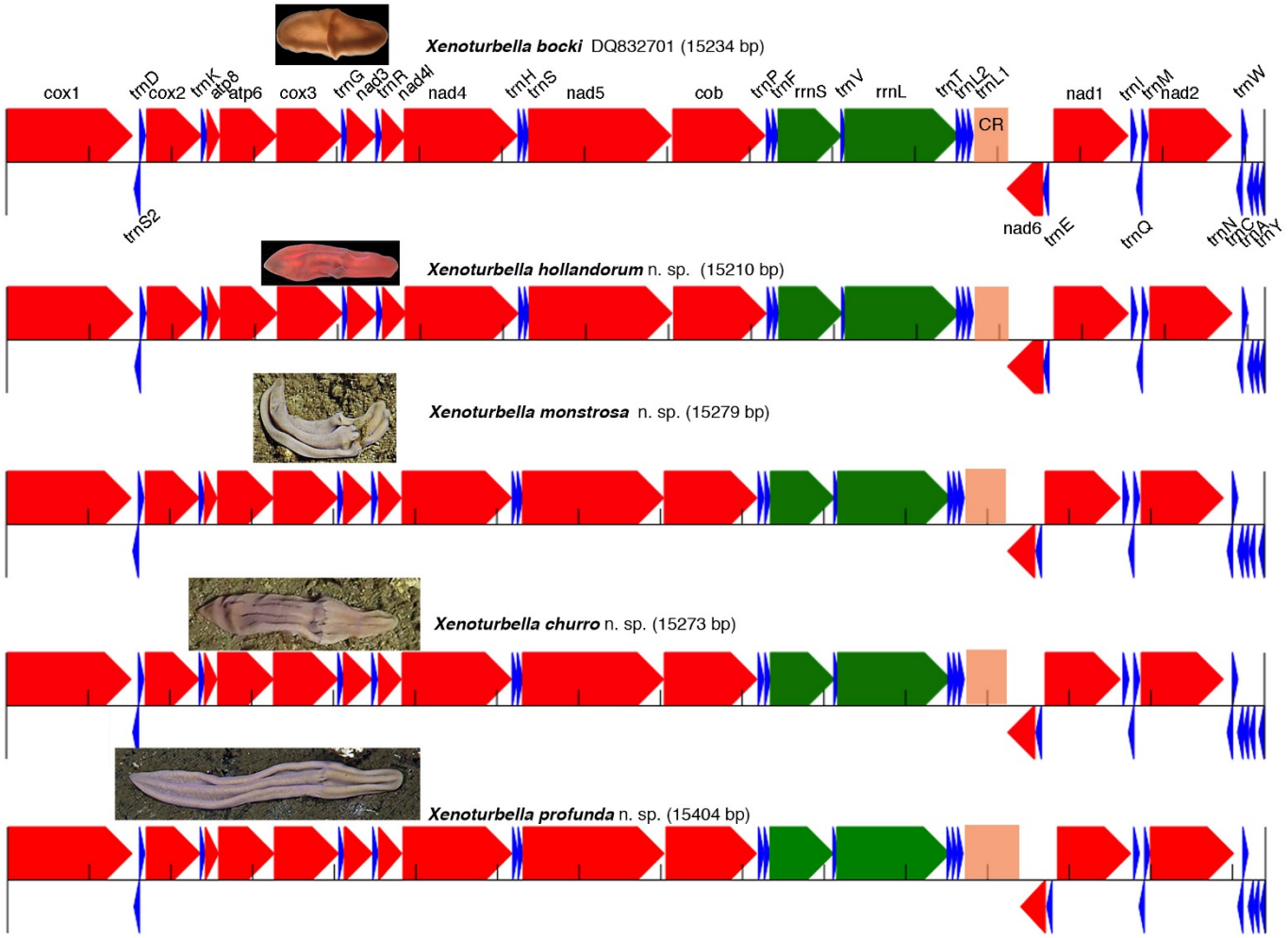

Extended Data Figure 6 | Mitochondrial genomics of *Xenoturbella*. a, Gene order for the four new species compared with that of *X. bocki*[9,18]. The gene order was consistent across all species with only minor variation in amino acids and length of the control region (also see Supplementary Table 1).

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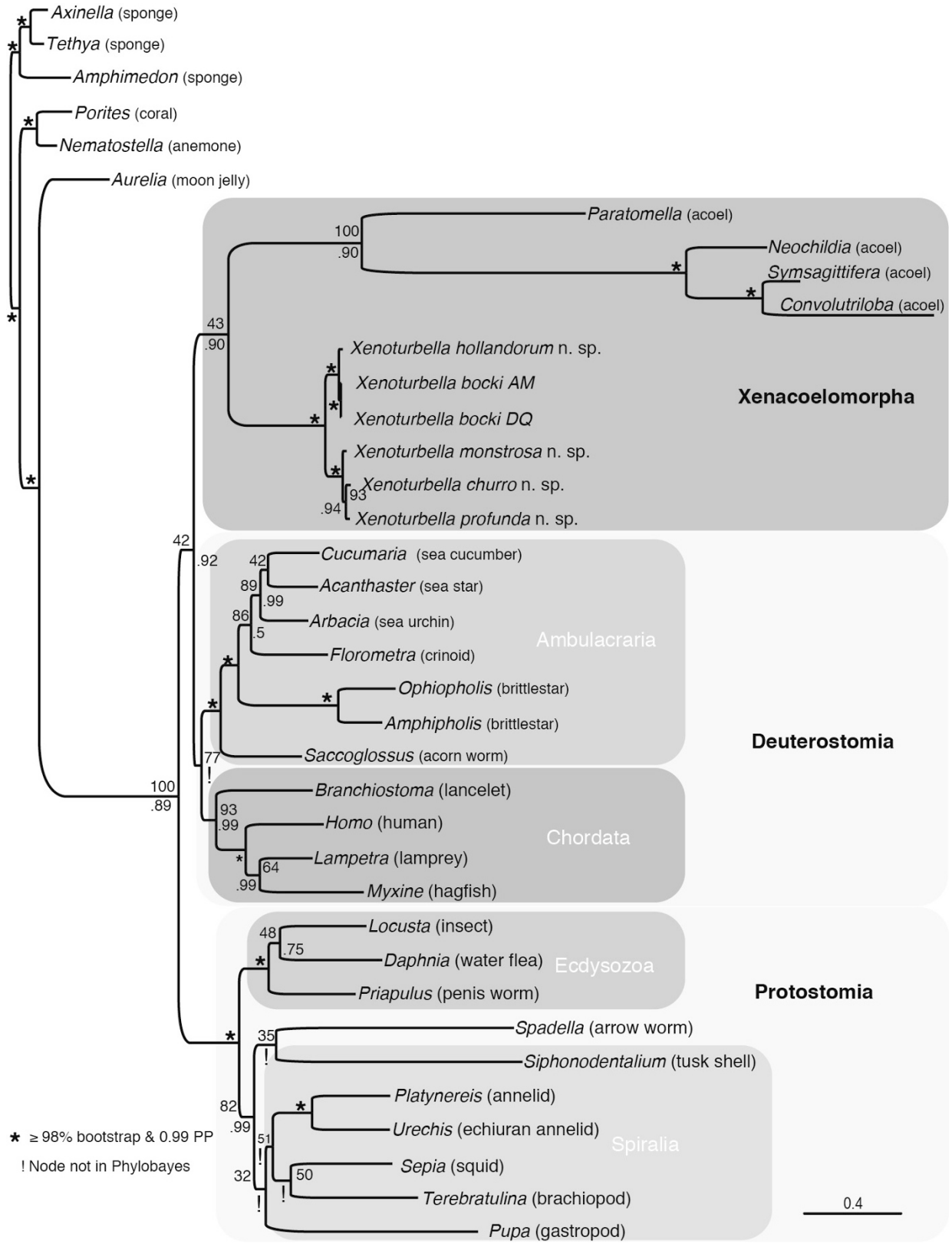

Extended Data Figure 7 | Maximum likelihood phylogeny based on all 13 mitochondrial proteins. Five *Xenoturbella* species formed a weakly supported sister clade to Acoela = Xenacoelomorpha. Xenacoelomorpha was sister to deuterostomes with weak support. Data were partitioned and analysed using RAxML and GTR + Γ. Analysis using PhyloBayes under CAT + GTR + Γ placed Xenacoelomorpha inside deuterostomes as a weakly supported sister group to Chordata (not shown), a result not recovered previously[16] with the same program, model and terminals (except for *X. profunda*). Scale bar, substitutions per amino-acid position. The lower support for Xenacoelomorpha in its placement with deuterostomes, or its placement with Chordata, compared with previously shown, may be due to additional *Xenoturbella* data and/or the fact that all 13 mitochondrial proteins were used here.

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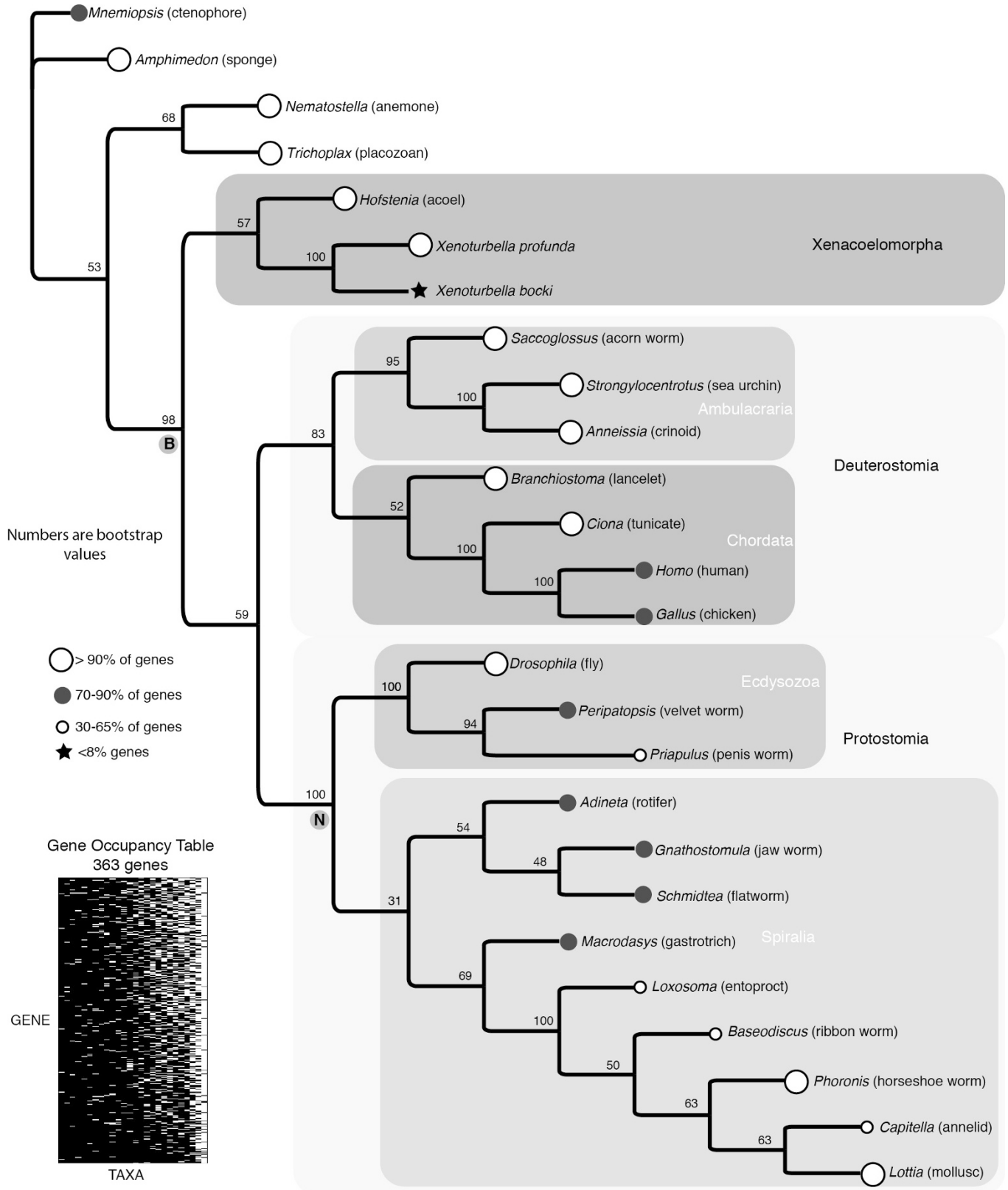

Extended Data Figure 8 | Species tree based on 393 gene trees using Astral II[22]. Nodal support assessed via 100 pseudo-replicates of bootstrapping for each gene, combining both information from each gene tree and its respective bootstrap replicates. B, Bilateria; N, Nephrozoa.

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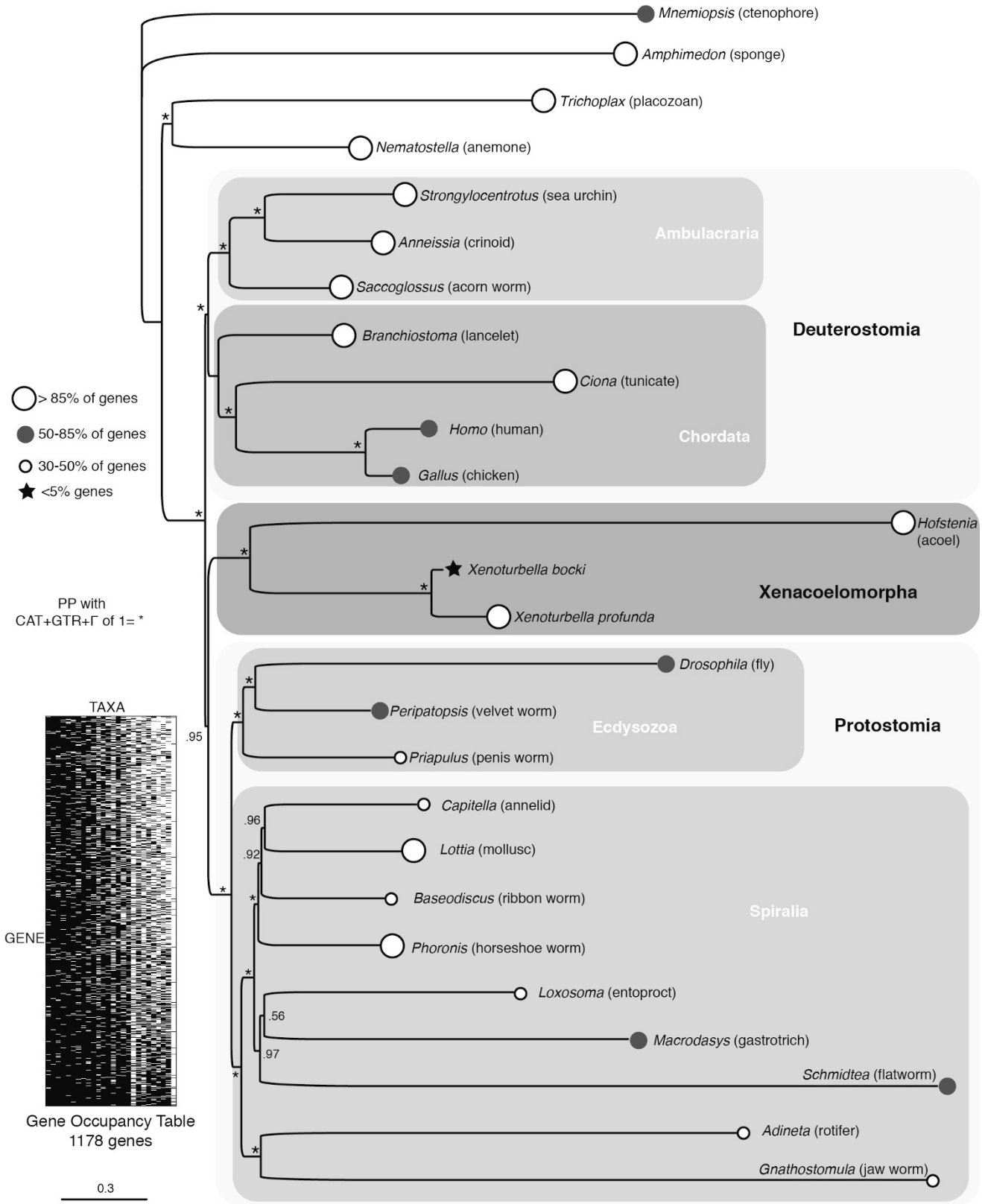

Extended Data Figure 9 | PhyloBayes analysis of 1,178 genes with 70% average gene occupancy. CAT + GTR + Γ was used and the first 2,000 out of 4,460 generations for two chains were discarded as burn-in and the remainder used to generate a consensus tree. Scale bar, substitutions per amino-acid position. The largest discrepancy observed between chains was 0.164609 and the mean was 0.000989. Xenacoelomorpha grouped with protostomes. B, Bilateria.

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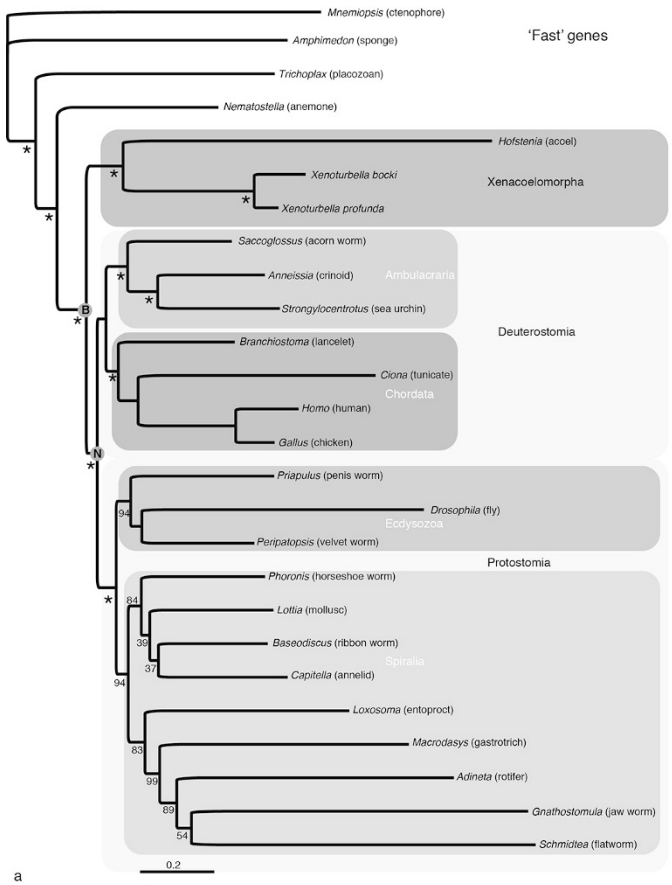

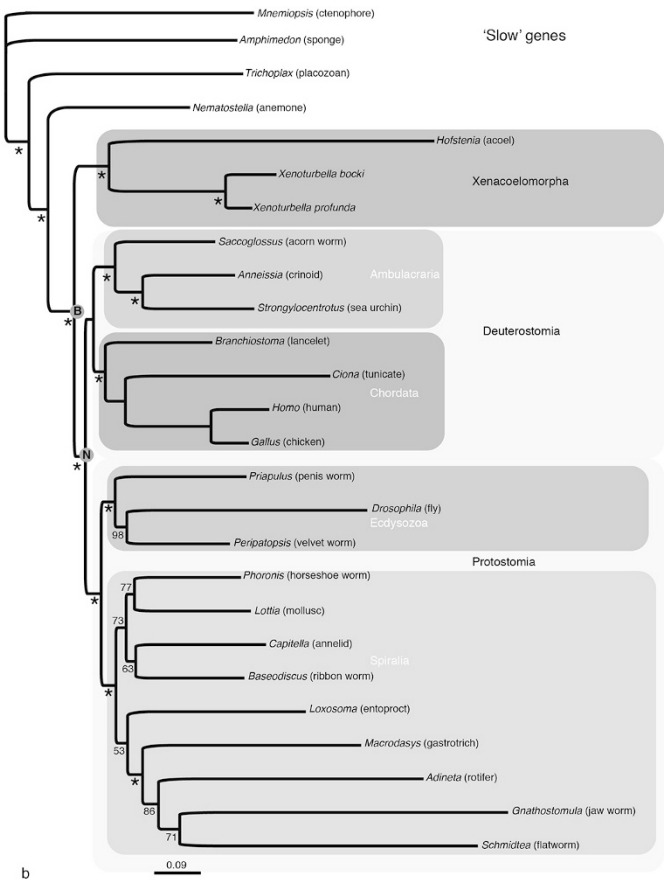

Extended Data Figure 10 | Maximum likelihood analyses of 'fast'- and 'slow'-evolving data sets. The 70% occupancy data set was reduced to 1,127 genes (51 ribosomal proteins removed) and then divided into the fastest-evolving 50% and the slowest-evolving 50%. These were analysed using the PROTGAMMAGTR amino-acid substitution model in RAxML. **a**, Phylogeny based on the 'fast-evolving' data set with 216,066 amino acids. **b**, Phylogeny based on the 'slow-evolving' data set with 168,571 amino acids. Nodal support assessed via 100 pseudo-replicates of bootstrapping. Asterisks indicate 100% bootstrap support. B, Bilateria; N, Nephrozoa.

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