A screen for gene paralogies delineating evolutionary branching order of early Metazoa

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The evolutionary diversification of animals is one of nature's greatest mysteries. In addition, animals evolved wildly divergent multicellular life strategies featuring ciliated sensory epithelia. In many lineages epithelial sensoria became coupled to increasingly complex nervous systems. Currently, different phylogenetic analyses of single-copy genes support mutually-exclusive possibilities that either Porifera or Ctenophora is sister to all other animals. Resolving this dilemma would advance the ecological and evolutionary understanding of the first animals. Here we computationally identify and analyze gene families with ancient duplications that could be informative. In the TMC family of mechano-transducing transmembrane channels, we find that eumetazoans are composed of Placozoa, Cnidaria, and Bilateria, excluding Ctenophora. Likewise, in the MLX/MLXIP family of bHLH-ZIP regulators of metabolism, we find that members of a proposed clade of "Benthozoa" (Porifera + Eumetazoa) share a duplication, absent in Ctenophora. These results suggest a new avenue for deducing deep phylogeny by choosing rather than avoiding ancient gene paralogies.

The branching order of the major metazoan lineages has received much attention due to its importance in understanding how animals and their sensory and nervous systems evolved (JEKELY *et al.* 2015). To date, most phylogenetic analyses have used single-copy orthologs, with different genes and approaches finding support for either Ctenophora (RYAN *et al.* 2013; MOROZ *et al.* 2014; BOROWIEC *et al.* 2015; WHELAN *et al.* 2015; SHEN *et al.* 2017) or Porifera (PISANI *et al.* 2015; FEUDA *et al.* 2017; SIMION *et al.* 2017) as outgroup animal lineage. In contrast, comparative analysis of single-cell transcriptomes from different lineages and cell types is consistent with an independent origin of neuron-like cells in ctenophores (SEBE-PEDROS *et al.* 2018). Another recent study that does not even rely on sequence analysis directly provides evidence that the sponge choanocyte does not correspond to the sponge cell type most similar transcriptomically to the choanoflagellate cell type (SOGABE *et al.* 2019). In summary, few studies (SEBE-PEDROS *et al.* 2018; SOGABE *et al.* 2019) have addressed the question of early animal branching without using single-copy genes for phylogenetic analysis.

Single-copy genes are preferred for phylogenetic inference of lineage branching order for several reasons (FITCH AND MARGOLIASH 1967; WOESE AND FOX 1977). First, single-copy genes more closely approximate clock-like divergence (ZUCKERKANDL AND PAULING 1965; FITCH AND MARGOLIASH 1967; WOESE AND FOX 1977; PETT *et al.* 2019) compared to duplicated genes, which frequently experience neofunctionalization and/or uneven subfunctionalization and rate asymmetries (compare left and right-hand trees in Fig. 1A) (WALSH 1995; LYNCH *et al.* 2001; HOLLAND *et al.* 2017). An organismal tree of animals (*e.g.*, left cladogram in Fig. 1A) can be constructed from a single-copy gene, or from a set of concatenated single-copy genes, provided orthologous outgroup genes are included in the analysis as an aid for rooting. The specific tree in Fig. 1A depicts the "benthozoic hypothesis", which is premised on the latest common ancestor (LCA) of Choanozoa (Choanoflagellatea + Metazoa) being holopelagic, and the LCA of "Benthozoa" (a name that we propose here for a clade uniting Porifera and Eumetazoa) having evolved a biphasic pelagic larva and a benthic adult form. Other alternative life cycle scenarios have been proposed that correspond to different branching patterns (NIELSEN 2008; NIELSEN 2013; JEKELY *et al.* 2015) some of which are based on fossil interpretation (ZHAO *et al.* 2019).

Second, single-copy genes (strict orthologs across all lineages) offer a practical advantage in eliminating the ambiguity associated with gene duplications (paralogs). Some paralogs represent recent lineage-specific duplications while others stem from deeper duplications contributing to a larger gene super-family. Nonetheless, a gene tree of a pair of genes produced by a duplication in the stem-metazoan lineage can depict lineage-branching order doubly so, once in each paralog's subtree (right phylogram in Fig. 1A). Furthermore, a gene tree of paralogs offers a unique advantage unavailable in single-copy gene trees: a tree of paralogs captures the duplication itself and unites lineages sharing the duplication relative to outgroup lineages with a single gene.

A majority of gene orthologs are part of larger super-families (OHNO 1970; TAYLOR AND RAES 2004) as has been well documented for the Hox gene family (HOLLAND *et al.* 2017). Thus,

many “single-copy” genes are only ostensibly so because they can be evaluated separately from their ancient paralogs; and because *in principle* single-copy genes diverged lineally from a single ancestral gene present in the latest common ancestor (LCA) of a taxonomic clade. However, the choice of homologs is a poorly examined aspect of modern phylogenetic analysis even though various ortholog-calling errors associated with ancient paralogy have been noted (NOUTAHI *et al.* 2016).

Here we identify candidate gene paralogies established prior to the evolution of Eumetazoa (Bilateria + Cnidaria + Placozoa) for the purpose of determining early animal branching order. The majority of these paralogies predate the metazoan LCA and/or experienced apparent gene losses in either candidate first animal sister lineage (Porifera or Ctenophora) and are not informative (see Table S1). Nonetheless, we find at least three analyzed gene families that possibly support the proposed clade of Benthozoa (Porifera + Eumetazoa) while we have found none that support the traditional grouping of Ctenophora with Eumetazoa. Among the most intriguing of our findings are the recently identified family of multimeric transmembrane mechanosensitive channel proteins, the TMC proteins (KERESZTES *et al.* 2003; BALLESTEROS *et al.* 2018), in which family we find definitive independent duplications in Eumetazoa (*Tmc48756* → *Tmc487* + *Tmc56*), Ctenophora (*Tmc48756* → *Tmc-α*, *Tmc-β*, *Tmc-γ*, and *Tmc-δ*), and possibly Benthozoa (*Tmc12348756* → *Tmc48756* + a neofunctionalized *Tmc123* clade). In summary, our identification and analysis of genes duplicating and diversifying in the stem-benthozoic lineage will help to outline the extent of a shared biology for Benthozoa and the nature of independent evolutionary neuralization in Eumetazoa and Ctenophora.

Material & Methods

Comparative genomic orthology counting and screening. For the data depicted in Fig. 1D, we used BioMart query tool (DURINCK *et al.* 2005; HAIDER *et al.* 2009; SMEDLEY *et al.* 2009) and the EnsemblCompara orthology calls (VILELLA *et al.* 2009) for MetazoaEnsembl Genomes Release 41. We also used these same tools to identify 2146 unique protein-coding genes in the placozoan *Trichoplax adhaerens*, which share the following properties: (1) these genes all can be grouped into a smaller number of paralogy groups; (2) these genes all have homologs in the cnidarian *Nematostella vectensis*; (3) the cnidarian homologs can also all be grouped into a smaller number of paralogy groups; (4) these genes all have homologs in the molluscan genome of *Lottia gigantea*, representing Lophotrochozoa, as well as in *Drosophila melanogaster*, representing Ecdysozoa; and (5) these genes all have homologs in the sponge *Amphimedon queenslandica* (a sponge in class Demospongiae). To ensure that our results would not be skewed by errors in gene annotation and curation, we focused on genes for which we could identify homologs in other cnidarians (the anthozoan *Stylophora pistillata* and the hydrozoan *Hydra vulgaris*), another sponge (the homoscleromorphan sponge *Oscarella carmela*), and throughout Bilateria. We then constructed phylogenetic trees for several different gene families from this list.

Sequence alignment. Sequences were obtained from a variety of sources including NCBI BLAST with taxonomic specification and ComparaEnsembl orthology calls (VILELLA *et al.* 2009). Care was taken to get sequences from representative taxa with whole-genome sequence assemblies. Several *TMC* genes identified in this manner are the result of computational annotation that were then hand curated for this study. These are indicated in the tree figure (Fig. 1) and in the FASTA headers (Supplementary files) by the accession numbers with “CUR” appended. Alignment of protein-coding sequence was conducted using MUSCLE alignment option in MEGA7 (KUMAR *et al.* 2016). The multiple sequence alignment (MSA) was conducted

using default parameters that were adjusted as follows. The gap existence parameter was changed to -1.6 and the gap extension parameter was changed to -0.01. Excessively long protein sequences were trimmed at the N- and C- termini so that they began and ended on either side of the ten transmembrane domains. Lengthy, fast-evolving, loop segments and/or repetitive amino acid sequences occurring in between transmembrane domains were trimmed. Supplementary Files for curated data sets are provided as explained under “Data availability”.

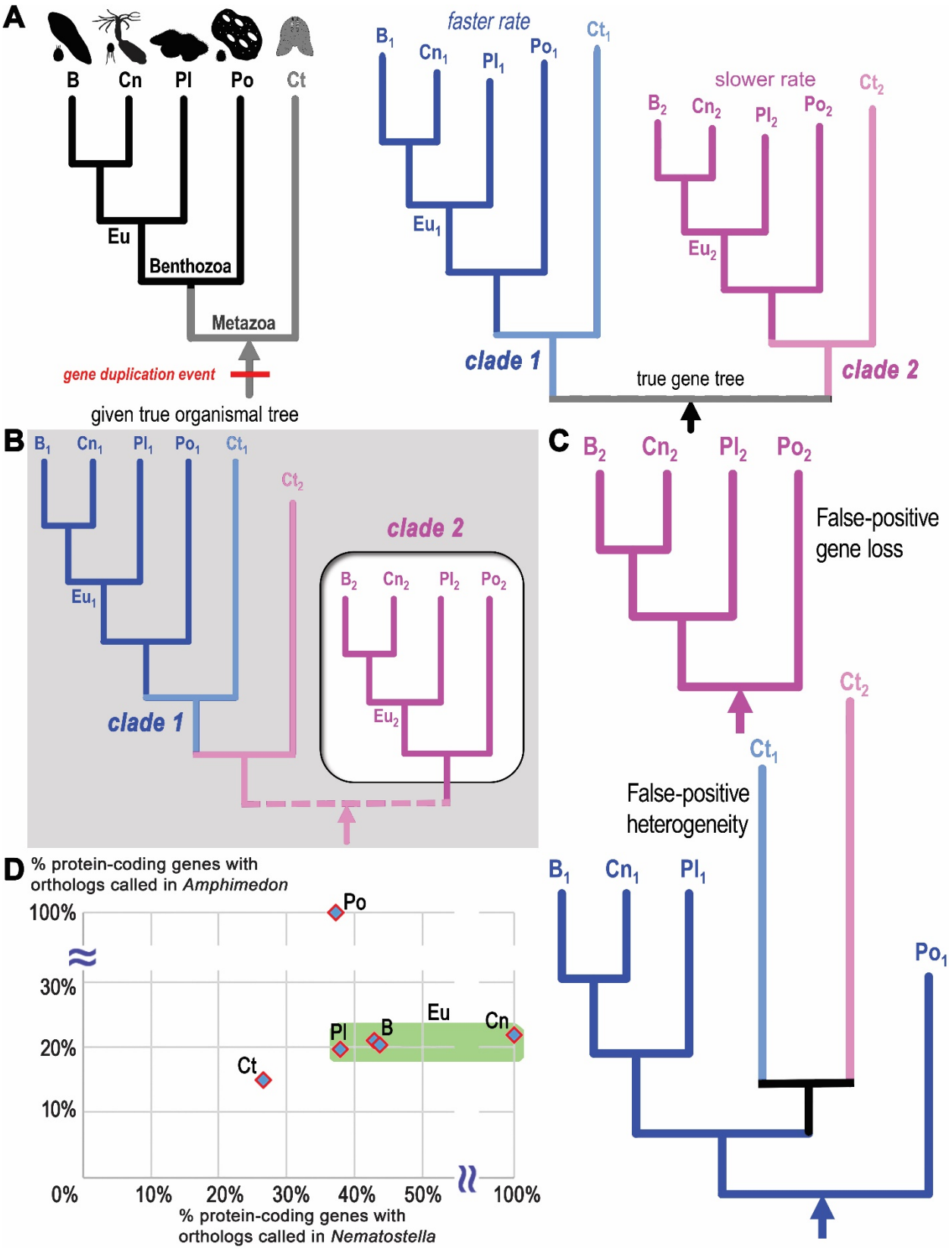
Bayesian Inference. To conduct metropolis-coupled-MCMC Bayesian phylogenetic we used the MrBayes (version 3.2) software (HUELSENBECK AND RONQUIST 2001; RONQUIST AND HUELSENBECK 2003; RONQUIST *et al.* 2012). All runs used two heated chains (“nchains = 2”, “temperature = 0.08”) that were run for 1.2 M generations with a burn-in fraction of 20%. Initial runs for all gene families sampled all substitution models, but we always found 100% percent posterior probability assigned to the WAG substitution model (WHELAN AND GOLDMAN 2001). Subsequently all finishing runs used the WAG model with invariant-gamma rates modeling. Double precision computing was enabled using BEAGLE toolkit (AYRES *et al.* 2012; RONQUIST *et al.* 2012). All trees were computed multiple times during the process of sequence curation and annotation. The final *cornichon*/*cornichon-related* gene family tree of Supporting Figure 2 (ver. 21) finished with 0.009 average standard deviation of split frequencies from two heated chains. The final *Tmc* gene family tree of Figure 3 (ver. 69) finished with 0.005 average standard deviation of split frequencies from the two heated chains. The final *MLX*/*MLXIP* gene family tree of Figure 4 (ver. 20) finished with 0.007 average standard deviation of split frequencies from the two heated chains. Tree graphics were rendered with FigTree version 1.4.4 and annotated with Adobe Photoshop tools.

Data availability. All data files, including sequence files (*.fas), multiple sequence alignment files (*.masx and *.nexus), and curation documents (Curated_*_Genes.docx), are provided with

the Supporting Information as a zipped archive. Four files are provided for each of the three gene families shown for a total of 12 files.

Figure 1. Dangers and promise of ancient paralogy when deducing the deep phylogeny of animals. (A) A hypothetical true organismal tree (left tree) is shown depicting branching order of the principal metazoan lineages: Ctenophora (Ct), Porifera (Po), Placozoa (Pl), Cnidaria (Cn), and Bilateria (B). Eu = Eumetazoa. This tree depicts the benthozoic hypothesis, in which Ctenophora is sister to all other animals (here referred to as Benthozoa). On the right is a corresponding tree for a gene duplicated in the stem-metazoan lineage resulting in two sub-clades for paralog one (blue) and paralog two (magenta). Asymmetric rates (faster or slower) are depicted in the cladogram, typical with neo-functionalized paralogs. **(B)** The gene tree in panel 1a is redrawn here with a different rooting procedure in order to isolate paralog clade two (magenta clade) such that the sponge (Po_2) lineage appears as the outgroup lineage for that subclade. The dotted line anticipates the analyses of each subclade as separate "single-copy" gene clades **(C)** Shown are trees constructed when the isolated gene clades are forced into the false rooting scheme conflicting with the hypothetical true organismal tree shown in a. Focusing on the more slowly evolving gene tree (right hand tree in magenta in 1a), the first re-rooting flips the ctenophore Ct_2 gene so that it is swept into the faster evolving clade (left hand side tree in blue), which has a higher propensity for long branch attraction. Thus, the erroneous rooting of each sub-clade results in false-positive gene loss (top tree) and false-positive compositional and rate heterogeneity (bottom tree). **(D)** Consistent with the predicted false-positive errors, comparative genomic orthology data sets that are based on the premise that sponge genomes are the outgroup predict a small number of orthologs called in a ctenophore genome relative to other metazoan genomes. Shown are the percentages of each organism's protein-coding gene set that have orthologs called in a cnidarian (*Nematostella*, *x*-axis) or a sponge (*Amphimedon*, *y*-axis) as inferred from the Metazoa ComparaEnsembl orthology calls (see Methods).

Fig. 1


A
B
Cn
Pl
Po
Ct
Eu
Benthozoa
Metazoa
gene duplication event
given true organismal tree
faster rate
B_1
Cn_1
Pl_1
Po_1
Ct_1
Eu_1
clade 1
slower rate
B_2
Cn_2
Pl_2
Po_2
Ct_2
Eu_2
clade 2
true gene tree
B
clade 2
clade 1
C
False-positive gene loss
False-positive heterogeneity
D
% protein-coding genes with orthologs called in *Amphimedon*
100%
30%
20%
10%
0%
10%
20%
30%
40%
50%
100%
% protein-coding genes with orthologs called in *Nematostella*
Po
Pl
B
Eu
Cn
Ct

Gene duplications from the early metazoan radiation

Ever since the elucidation of the first non-bilaterian animal genomes, mainly those from Cnidaria (PUTNAM *et al.* 2007), Placozoa (SRIVASTAVA *et al.* 2008), Porifera (SRIVASTAVA *et al.* 2010), and Ctenophora (RYAN *et al.* 2013; MOROZ *et al.* 2014), we have attempted to identify gene duplications that could definitively order early metazoan phylogeny. Given the preponderance and constancy of gene duplication (and gene loss) throughout evolution, one should in principle be able to find a candidate duplication shared by all major animal lineages except the one true sister animal lineage. As a possible explanation for our repeated failures to find such a signature gene family, we hypothesized the existence of a methodological bias associated with the various ortholog-calling pipelines on which we were relying. This hypothesis of inherent bias related to ancient gene paralogy motivated the comparative genomic screen that we present here.

To illustrate possible problems that arise by not accounting for ancient paralogy, we consider the "benthozoic" hypothesis in which Ctenophora is the sister group to Benthozoa (Porifera + Eumetazoa) (RYAN *et al.* 2013; MOROZ *et al.* 2014; BOROWIEC *et al.* 2015; JEKELY *et al.* 2015; WHELAN *et al.* 2015; SHEN *et al.* 2017). By choosing the most closely-related homologs to one of a pair of duplicated genes (the more slowly evolving magenta subclade two of Fig. 1), or by internally re-rooting the true gene tree so that the subclade in question fits the standard model in which Porifera is sister to all other animals (Fig. 1A right gene tree re-rooted to Fig. 1B tree), we end up with an isolated subclade with a false-positive gene loss (top phylogram in Fig. 1C). Furthermore, the ctenophoran true outgroup sequence ("Ct_2") is easily collected into the more divergent sub-clade (blue subclade one). When the fast evolving gene sub-clade is rooted separately with sponge as outgroup ("Po_1"), the inherent topology unites both ctenophore paralogs in an apparent ctenophore-specific duplication (bottom phylogram in Fig. 1C). Due to this topology this second fast-evolving subclade would feature false-positive rate heterogeneity and compositional heterogeneity. False-positive compositional heterogeneity is consistent with

recent attributions of (true) evolutionary sequence bias in ctenophores (FEUDA *et al.* 2017). Last, as predicted by the benthozoic hypothesis and deep paralogy-induced miscalling of orthologs, we find that using Porifera as the first sister animal lineage results in the automated annotation of fewer orthologs being called in the ctenophore *Mnemiopsis leidyi* relative to sponge (*Amphimedon queenslandica*), placozoan (*Trichoplax adhaerens*), cnidarian (*Nematostella vectensis*), and bilaterian (the lophotrochozoan *Lottia gigantea* and *Capitella teleta*) genomes (Fig. 1D).

Based on the above observations and rationale, we devised a comparative genomic screen in which orthology calls to the ctenophore *Mnemiopsis* and the sponge *Amphimedon* are not considered during the initial candidate paralogy group screen. We begin with the 16,590 protein-coding genes from the beetle *Tribolium castaneum* (Tcas5.2 genes), which we chose as a model bilaterian that has not experienced extensive gene loss as in nematodes (ERIVES 2015), nor extensive gene duplications as in vertebrates. The *Tribolium* gene set becomes 8,733 genes if we only consider those that exist in paralogous relationship(s) with other *Tribolium* genes. Of these only 2,617 have orthologs called in the mosquito *Aedes aegypti*, the lophotrochozoan *Capitella teleta*, and the placozoan *Trichoplax adhaerens*. Then we discard those paralogous *Tribolium* genes which are related by a common ancestor in more recent taxonomic group such as Cucujiformia (an infraorder of beetles), Holometabola, Hexapoda, Mandibulata, Arthropoda, Pancrustacea, Protostomia, Bilateria, and Eumetazoa. This narrows the list to 2,322 candidate paralogous genes. However, many of these genes exist in impractical paralogy groups that are too large to attempt careful curation from multiple genomes. We thus removed 1,133 genes from the 153 largest paralogy groups, which contained 4 to 41 genes each. We were then left with about <500 small paralogy groups containing 2 to 3 genes each (Supporting Table S1). We sampled from the candidate small paralogy groups to find sets that had at least one ortholog called in *Amphimedon queenslandica* and at least one in *Mnemiopsis leidyi*. We note that we cannot rule out the possibility that metazoan-relevant paralogies were

excluded because true orthologs failed to be computationally called in sponges and ctenophores. Many of the candidate paralogy groups have paralogs called in sponges and ctenophores, indicating that the duplications occurred prior to the latest common ancestor (LCA) of Metazoa.

We then subjected candidate paralogy groups that passed our first test to a draft phylogeny by Maximum Parsimony, and if warranted by Bayesian Inference. Extensive gene curation of unannotated exons was then conducted to build the best possible (most informative) trees (see Methods). We report on the first three gene families for which we could construct a clear picture (well-supported nodes) based on gene duplications (*cornichon*, *TMC*, and *MLX/MLXIP* families). All trees shown were computed with metropolis-coupled-MCMC Bayesian phylogenetic inference to construct multiple gene trees (see Methods).

Duplication of the *cornichon*-family of transmembrane chaperones

We find that the *cornichon* (*cni*/*CNIH1-CNIH3*) and *cornichon-related* (*cnir*/*CNIH4*) paralogy groups, which encode eukaryotic chaperones of transmembrane receptors (HERRING *et al.* 2013), was either duplicated in the stem-choanozoan lineage with subsequent gene loss only in choanoflagellates and ctenophores, or else duplicated in the stem-benthozoan lineage with subsequent neofunctionalization. We find that both *cni* and *cnir* clades exist only in Porifera, Placozoa, Cnidaria, and Bilateria but not Ctenophora and Choanoflagellatea (Fig. 2). This tree is based on a protein that is only ~150 amino acids long and suggests that the ancestral *cni*/*cnir* gene was duplicated in the stem-choanozoan lineage with two subsequent independent losses in the stem-ctenophore lineage and the stem-choanoflagellate lineage (Fig. 2). However, an alternative interpretation is that the progenitor gene was duplicated in the stem-benthozoic lineage with the *cnir* clade undergoing neofunctionalization to an extent promoting artifactual basal branching. In this alternative interpretation there is no need to invoke independent gene

losses for Ctenophora and Choanoflagellatea. In either case the progenitor gene was present as a single copy gene in the LCA of Holozoa as shown by the basal branching of the single gene from the filozoan *Capsaspora owczarzaki*, which branches before the duplication (Fig. 2).

Fig. 2

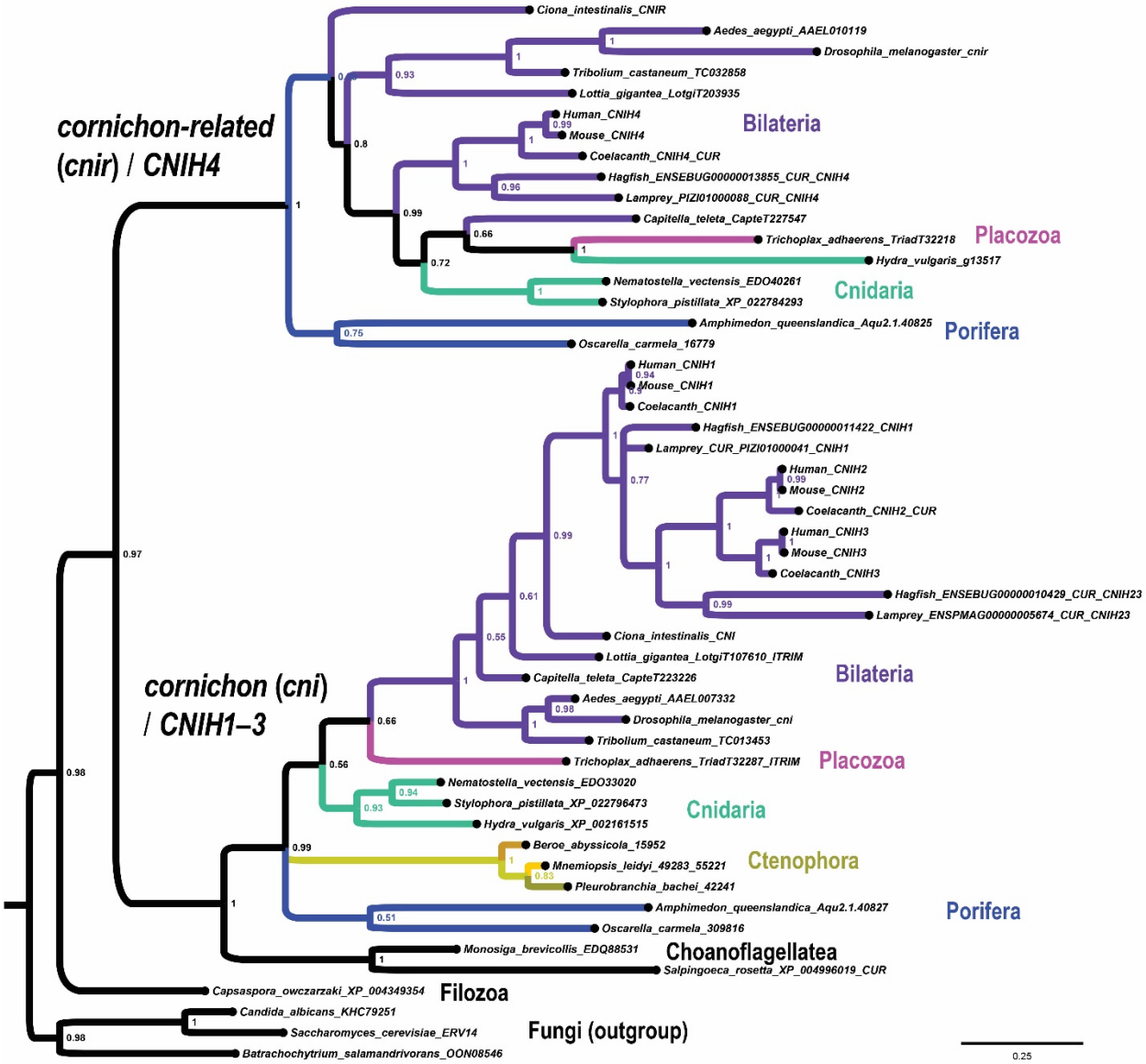


Figure 2. A stem-choanozoan duplication: *cornichon* and *cornichon-related*. Shown is the gene tree for a eukaryotic transmembrane receptor chaperone (see text for details). Tree was generated from a multiple sequence alignment composed of 167 alignment columns and is rooted with Holomycota (Fungi) as outgroup.

A TMC duplication defines Eumetazoa and excludes Ctenophora

We find that a phylogenetic analysis of gene duplications of the transmembrane channels (TMCs), a large family of ion leak channels with roles in mechanotransduction (DELMAS AND COSTE 2013; PAN *et al.* 2013; BALLESTEROS *et al.* 2018; PAN *et al.* 2018; QIU AND MULLER 2018), excludes ctenophores from a eumetazoan super-clade composed of Bilateria, Cnidaria, and Placozoa (Fig. 3). We describe the main features of this gene family's origin and diversification.

Firstly, the TMC gene family is ancestral to eukaryotes because we can identify TMC genes in different lineages of Unikonta and Bikonta, which are the two main branches of the eukaryotic tree. A close affinity of the TMC proteins with OSCA/TMEM proteins has been proposed (MURTHY *et al.* 2018) despite low sequence similarity (BALLESTEROS *et al.* 2018). We find that the percent identity between predicted TMC proteins analyzed here is higher than between TMC proteins and TMEM. For example, the gap-normalized percent amino acid identity in a pairwise alignment of human Tmc1 with the single *Naegleria gruberi* Tmc sequence is 17.3%, versus 12.7% (~5% less) with human Ano3/TMEM16C (Supporting File S2). Moreover the identity between Tmc1 and TMEM16C goes away in a multiple sequence alignment suggesting it is inflated by the extra degree of freedoms allowed in a gapped pair-wise alignment.

Secondly, in the context of the ancient eukaryotic provenance of the TMC family, we find an intriguing and suggestive pattern of TMC gene loss as follows. We find that the *Tmc* gene exists as a single copy in the genome of the non-amoebozoan slime mold *Fonticula alba* and a few early-branching fungal lineages, altogether representing the clade of Holomycota. One of these fungal lineages, *Rozella allomycis*, belongs to the Cryptomycota, a phylum notable for its absence of a chitinous cell wall typically present in most other fungi (JONES *et al.* 2011). The absence of a cell wall allows the Cryptomycota to maintain a phagotrophic lifestyle (JONES *et al.* 2011). The only other fungal lineages with a TMC gene are from Blastocladiomycota,

suggesting that TMC genes were lost in all of Ascomycota, Basidiomycota, and Zygomycota, for which many genomes have been sequenced.

Physiological incompatibility of Tmc function with certain cell walls is further supported by a similar distribution of Tmc genes in Viridiplantae (Fig. 3 green clade). We find that a single Tmc gene can be found in a chlorophyte (*Coccomyxa subellipsoidea*), a charophyte (*Klebsormidium nitens*), and in the multicellular plants of a liverwort (*Marchantia polymorpha*), a moss (*Physcomitrella patens*), and a lycophyte (*Selaginella moellendorfii*). The moss *Physcomitrella* and the lycophyte *Selaginella* represent a nonvascular land plant and a vascular land plant most closely-related to the clade composed of ferns, gymnosperms, and angiosperms, which correspond to the crown group having evolved lignin-based secondary cell walls (SARKAR *et al.* 2009). In addition, both the *Physcomitrella* and *Selaginella* genomes lack many of the *CESA* genes responsible for the synthesis of plant cell wall components (SORENSEN *et al.* 2010). Thus these TMC distributions in both plants and fungi suggest that rigid cell walls potentially interfere with physical environmental coupling to TMCs.

Thirdly, while we find TMC genes as a single copy in some lineages of the fungal and plant kingdoms, in *Naegleria gruberi*, and in the filozoan *Capsaspora owczarzaki*, we find that *Tmc* genes uniquely underwent a number of duplications during the early holozoan radiation (or earlier with corresponding losses in fungi). A complementary way of stating this finding is that TMC gene duplications can be found only within Choanozoa (choanoflagellates + Metazoa) (Fig. 3). The choanoflagellates *Monosiga brevicollis* and *Salpingoeca rosetta* have at least three TMC genes each, which we have labeled *Tmc-X*, *Tmc-Y*, and *Tmc-Z* for ease of reference here (Fig. 3). Remarkably, only ctenophores appear to have genes from the *Tmc-X* clade, which is a basally-branching Tmc clade that is sister to all of the remaining TMC genes from all of Opisthokonta. Only choanoflagellates and Holomycota have genes in the *Tmc-Y* clade. Last, except *Capsaspora*, whose single TMC gene is of uncertain affinity to the well supported *Tmc-X/Y/Z* clades, all of the remaining TMC genes are choanozoan genes from the *Tmc-Z* clade.

Fourthly, *Tmc-Z* apparently underwent a key duplication ("*Tmc123*" + "*Tmc48756*") in the stem-metazoan lineage with a corresponding loss of one of the paralogs ("*Tmc123*") in ctenophores. Alternatively, this duplication is actually a stem-benthozoan duplication that occurred after ctenophores split off from the rest of Metazoa. These two *Tmc-Z* subclades are so named here for their relationship to the vertebrate TMC1/2/3 ("*Tmc-Z1*") and TMC4/8/7/5/6 ("*Tmc-Z2*") paralogy groups.

Fifthly, we find that the *Tmc-Z2* gene (*Tmc48756*) underwent a Eumetazoa-defining duplication that unites Placozoa, Cnidaria, and Bilateria (Fig. 3). Thus, in this stem-eumetazoan lineage we see that the *Tmc-Z2* bifurcates into the sister-clades of *Tmc487* and *Tmc56*. This duplication likely excludes the possibility that Porifera or Ctenophora are more closely related to any single lineage within Eumetazoa. This contrasts with the stem-ctenophore lineage, in which the single *Tmc-Z2* gene independently duplicated several-fold to produce four ctenophore-specific duplications (*Tmc-α*, *Tmc-β*, *Tmc-γ*, and *Tmc-δ*) (Fig. 3, lineages in shades of yellow, gold, and mustard). This ctenophore specific repertoire is present in two different classes and three different orders of ctenophores. In Class Tentaculata, we have four *Tmc48756* genes from each of *Mnemiopsis leidyi* (Order Lobata) and *Pleurobrachia bachei* (Order Cydippida). In class Nuda, so named because of a complete (derived) loss of tentacles, we have only three *Tmc48756* genes from *Beroë abyssicola* (Order Beroida) because a representative gene from the *Tmc-γ* clade could not be identified.

Many metazoan sub-clades (*e.g.*, Ctenophora, Lophotrochozoa, and Vertebrata) can be defined by clade-specific duplications. For example, lophotrochozoans share a duplication of *Tmc487* into *Tmc487a* + *Tmc487b*, while gnathostomes share a duplication of vertebrate *Tmc12* into *TMC1* + *TMC2* and *Tmc48* into *TMC4* + *TMC8*. If we include the cyclostomes (hagfish + lamprey), then all vertebrates share the duplication of *Tmc123* into *Tmc12* + *TMC3*, and *Tmc487* into *Tmc48* + *TMC7*. Thus, the "canonical" eight gene vertebrate repertoire represented by *TMC1–TMC8* is more correctly characterized as a gnathostome-specific *TMC*

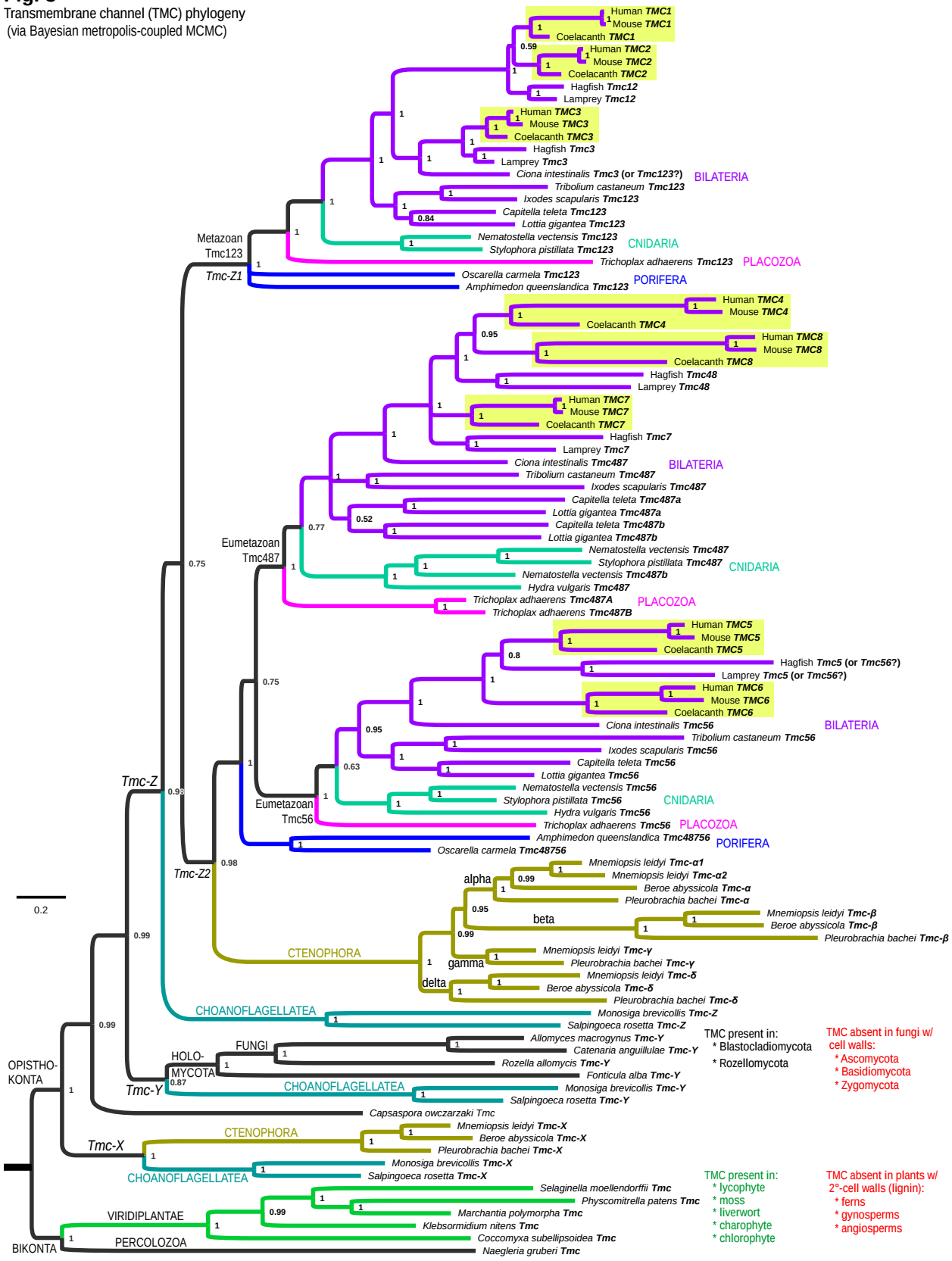
repertoire because cyclostomes are united in sharing only some of the duplications seen in humans (see Fig. 3).

Considering the *Tmc* gene duplications that are ancestral to Choanozoa and those specific to metazoan phyla, this phylogeny, based on the large TMC protein spanning ten transmembrane domains, unites Placozoa, Cnidaria, and Bilateria into a single Eumetazoa in the following ways. First, of greatest significance, is the shared derived eumetazoan duplication of *Tmc48756* into *Tmc487* and *Tmc56*. Second, is the close placement of Porifera as the sister group of Eumetazoa in both the *Tmc123* and *Tmc48756* subclades. Third is the unique situation that ctenophores possess TMC genes from an ancestral *Tmc-X* clade that were apparently lost in a stem-benthozoan lineage while also possessing an expansive repertoire via ctenophore-specific duplications (*Tmc-α*, *Tmc-β*, *Tmc-γ*, and *Tmc-δ*). These latter duplications are most parsimonious if they predate the eumetazoan duplication within the Tmc-Z2 clade. Below, we speculate on the significance of the evolution of mechanotransduction in connection with the evolution of diverse 'body plans' during the metazoan radiation.

Figure 3. Independent duplications of *TMC* genes define separate clades for eumetazoans versus ctenophores. A phylogenetic tree of the mechanotransducing transmembrane channels (TMC) delineates early metazoan diversification as a branching of only three lineages: Eumetazoa (magenta Placozoa, teal Cnidaria, and violet Bilateria), Porifera (blue), and Ctenophora (gold shades). Each of the eight *TMC* orthogroups specific to gnathostomes are highlighted in yellow. This tree is based on a data set composed of 777 trimmed alignment columns and is rooted between unikonts (labeled "Opisthokonta" due to the apparent loss in Amoebozoa) and bikonts. Eumetazoan lineages share a duplication corresponding to the Tmc487 and Tmc56 TMC clades, while ctenophores share independent duplications of an ancestral Tmc48756 gene. The ctenophores share with choanoflagellates a more ancient Tmc duplication (labeled Tmc-X here).

Fig. 3

Transmembrane channel (TMC) phylogeny
(via Bayesian metropolis-coupled MCMC)

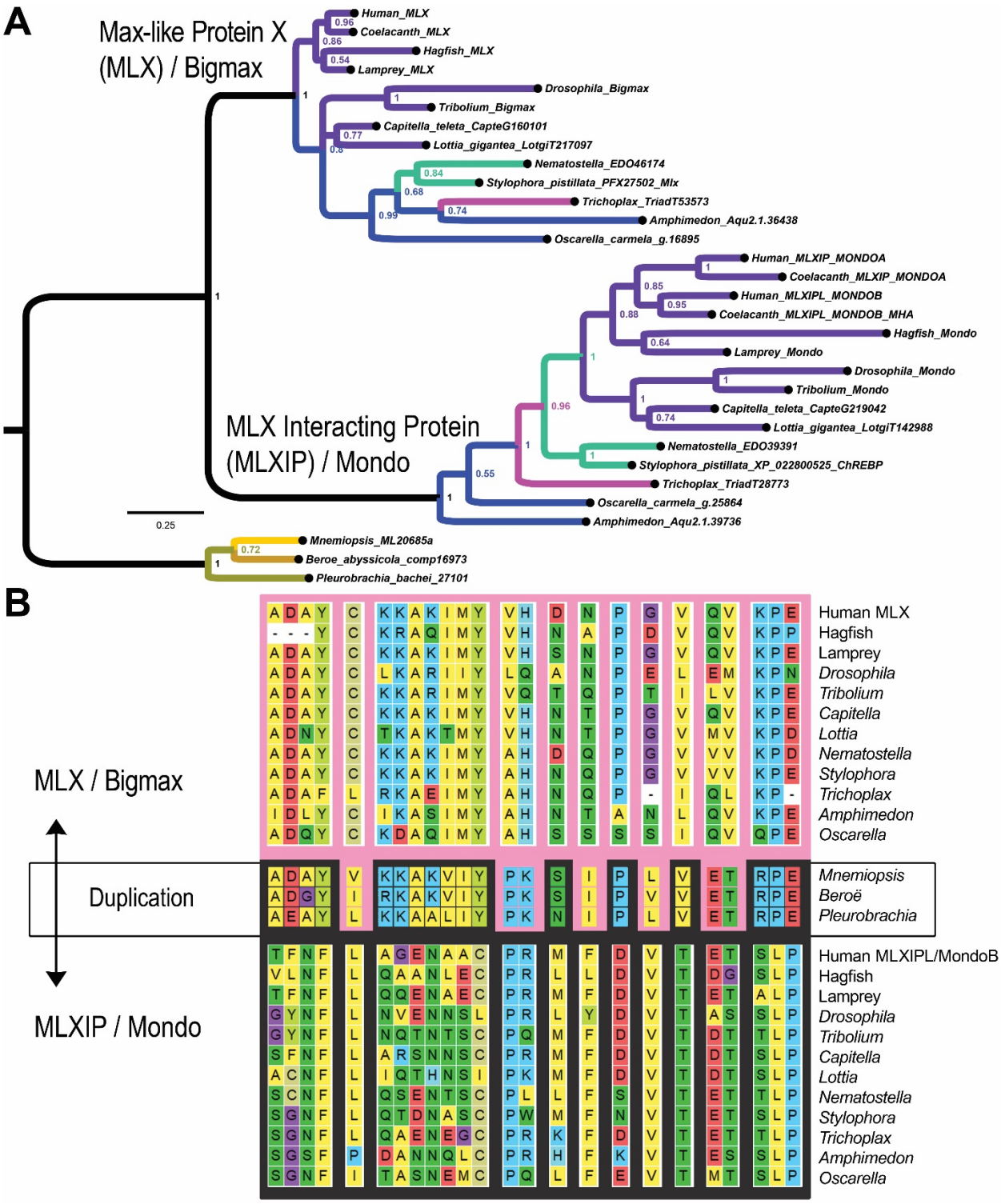


A bHLH-ZIP duplication unites Benthozoa

We find that a new gene family from the bHLH-ZIP superfamily originated in the stem-metazoan lineage most likely from a duplication of the more distantly-related Max bHLH-ZIP gene (Fig. 4). This gene occurs as one gene copy in all ctenophores but as a pair of duplicated genes in Porifera and Eumetazoa (Fig. 4A). The pair of paralogous genes corresponds to *Max-like* X (*MLX*)/*bigmax* and *MLX Interacting Protein* (*MLXIP*)/*Mondo*. Mondo paralogs are also known as the carbohydrate response element binding protein (ChREBP) (YAMASHITA *et al.* 2001; IIZUKA *et al.* 2004; HAVULA AND HIETAKANGAS 2018). The progenitor gene likely encoded a bHLH-ZIP homodimeric transcription factor (TF) in the stem-metazoan lineage, continuing as such into modern ctenophores, but evolved into the MLX:MLXIP heterodimer found in all other animals (Bigmax:Mondo in *Drosophila*, MLX:MondoA or MLX:MondoB in gnathostomes). Figure 4B shows that the bHLH domain from ctenophores shares residues with both subfamilies (pink vs. black background in Fig. 4B). In the Discussion we speculate on a possible role for this gene duplication in a lifecycle synapomorphy for the proposed clade of Benthozoa.

Figure 4. *MLX*/*bigmax* and *MLXIP*/*Mondo* encode a bHLH-ZIP heterodimer originating in a stem-benthozoan gene duplication. (A) Phylogenetic analysis of a bHLH-ZIP gene originating in the stem-metazoan lineage (possibly from a duplication of the distantly-related *Max* gene) followed by a second duplication after the divergence of the ctenophore lineage. Color coding of lineages follows Figure 2 except when topology precludes coloring sister stem lineages. This tree is rooted with ctenophores as the outgroup lineage. This tree is based on a data set composed of 263 alignment columns. **(B)** Shown are a subset of alignment columns in which the ctenophore residues more closely resemble either the MLX sequences (top pink) or the MLXIP/Mondo sequences (bottom dark gray). The MLXIPL/MondoB representative sequence is shown for humans.

Fig. 4


A
Max-like Protein X (MLX) / Bigmax
Human_MLX
Coelacanth_MLX
Hagfish_MLX
Lamprey_MLX
Drosophila_Bigmax
Tribolium_Bigmax
Capitella_teleta_CapteG160101
Lottia_gigantea_LotgiT217097
Nematostella_EDO46174
Stylophora_pistillata_PFX27502_MIx
Trichoplax_TriadT53573
Amphimedon_Aqu2.1.36438
Oscarella_carmela_g.16895
Human_MLXIP_MONDOA
Coelacanth_MLXIP_MONDOA
Human_MLXIPL_MONDOB
Coelacanth_MLXIPL_MONDOB_MHA
Hagfish_Mondo
Lamprey_Mondo
Drosophila_Mondo
Tribolium_Mondo
Capitella_teleta_CapteG219042
Lottia_gigantea_LotgiT142988
Nematostella_EDO39391
Stylophora_pistillata_XP_022800525_ChREBP
Trichoplax_TriadT28773
Oscarella_carmela_g.25864
Amphimedon_Aqu2.1.39736
MLX Interacting Protein (MLXIP) / Mondo
0.25
Mnemiopsis_ML20685a
Beroe_abyssicola_comp16973
Pleurobrachia_bachei_27101
B
MLX / Bigmax
Duplication
MLXIP / Mondo
Human MLX
Hagfish
Lamprey
Drosophila
Tribolium
Capitella
Lottia
Nematostella
Stylophora
Trichoplax
Amphimedon
Oscarella
Mnemiopsis
Beroë
Pleurobrachia
Human MLXIPL/MondoB
Hagfish
Lamprey
Drosophila
Tribolium
Capitella
Lottia
Nematostella
Stylophora
Trichoplax
Amphimedon
Oscarella

Discussion

By identifying and phylogenetically analyzing small paralogy groups that predate a eumetazoan super-clade composed of Bilateria, Cnidaria, and Placozoa, we find a handful of gene duplications that are consistent with Ctenophora being the sister metazoan lineage to all other animals (Figs. 2, 3 and 4). We discuss the significance of the different gene families that we identified and the possible relevance to metazoan biology.

We find that the transmembrane channel (TMC) gene family continued to duplicate and diversify for several phyla throughout metazoan evolution. For example, we see that the cyclostomes (hagfish and lampreys) branched early in the vertebrate tree prior to a number of duplications that occurred only in the stem-gnathostome lineage (*Tmc12* → *TMC1* + *TMC2*; *Tmc48* → *TMC4* + *TMC8*; *Mondo* → *MondoA*/*MLXIP* + *MondoB*/*MLXIPL*; and *CNIH23* → *CNIH2* + *CNIH3*). Nonetheless, cyclostomes and gnathostomes share the vertebrate-specific duplications corresponding to *Tmc12*, and *Tmc48* + *TMC7*. Thus like the vertebrate clade and the subclade, many other metazoan phyla can be defined solely on the basis of *Tmc* gene duplications (Fig. 3). We thus propose that the evolutionary diversification of the Tmc channels throughout Metazoa, including the independent diversification within Ctenophora (Fig. 3), must have been under selection of two principal forces. The first is the changing physical constraints and sensorial opportunities associated with the evolutionary diversification of animal body plans themselves. The second factor is the evolutionary diversification of specialized cell types within their epithelial sensoria.

Mechanosensory, chemosensory and photosensory responses are universal among single and multicellular organisms and can be related to the evolution of specific proteins enabling already single cells to respond to mechanical, chemical and photic stimuli. For example, opsin proteins evolved in single-celled ancestors of metazoans (FEUDA *et al.* 2012; ARENDT 2017) and many single cell organisms can sense light, gravity and several chemical stimuli with dedicated sensors (SWAFFORD AND OAKLEY 2018). While the history of chemical and

photic senses and the formation of specialized cell types and integration into appropriate organs in metazoans has been driven by the molecular insights into the molecular transducers (ARENDT *et al.* 2016), mechanosensory transduction has seen less progress due to uncertainty of consistent association of a specific mechanotransducer channel across phyla (BEISEL *et al.* 2010). On the one hand, mechanical sensation is clearly present in all single cell organisms to function as safety valves to release intracellular pressure sensed as tension in the lipid bilayer and was proposed as a possibly unifying principle of mechanosensation (KUNG 2005). Follow up work showed a multitude of channels associated with mechanosensation (BEISEL *et al.* 2010; DELMAS AND COSTE 2013) arguing against a single unifying evolution of mechanotransduction. Indeed, several families of mechanosensory channels have been identified whereby pores open as a function of lipid stretch or tethers attached to extra-or intracellular structures (COX *et al.* 2018b; COX *et al.* 2018a)

Simply speaking, no single molecule has been identified to be associated with all mechanotransduction across phyla such as the ecdysozoan TRP channels also found in bony fish but absent in mammals (BEISEL *et al.* 2010; COX *et al.* 2018b; QIU AND MULLER 2018). Likewise, the ubiquitous Piezo mechanotransduction channels in Merkel cells (DELMAS AND COSTE 2013; RANADE *et al.* 2015; COX *et al.* 2016) were hypothesized to be the mechanotransduction channel of hair cells (ARENDT *et al.* 2016) but have meanwhile been found not to be directly associated with the mechanotransduction process (WU *et al.* 2017). Molecular analysis of mammalian mutations meanwhile has focused on the family of TMC (transmembrane channels) as possibly involved in hair cell mechanotransduction (DELMAS AND COSTE 2013; PAN *et al.* 2013) and replacement of mutated TMC can restore hearing (ASKEW *et al.* 2015; SHIBATA *et al.* 2016; YOSHIMURA *et al.* 2018). More recently, molecular analysis has established that the TMC forms a mechanosensory pore as a homodimer with each subunit having ten predicted transmembrane domains (PAN *et al.* 2018). However, the detailed transmembrane protein dimer is unclear as other proteins are needed to transport the TMC

channels to the tip (PACENTINE AND NICOLSON 2019) and the entire complex of the vertebrate mechanosensory channel and its attachment to intra- and extracellular tethers remains unresolved (QIU AND MULLER 2018). To what extent TMC family channel evolution aligns with mechanosensory cell and organ evolution remains to be seen but is already indicating unique features of ctenophores at every level (FRITZSCH *et al.* 2006; FRITZSCH *et al.* 2015). Recent work also establishes that ecdysozoan *Tmc123* paralogs function in body kinesthesia (proprioception), sensory control of locomotion or egg-laying behavior via membrane depolarization, and nociception (GUO *et al.* 2016; WANG *et al.* 2016; YUE *et al.* 2018). In summary, our study further lays the groundwork for understanding the molecular history of a sensory channel family in the context of the evolution of developmental gene regulatory circuits in different animal lineages (CORBO *et al.* 1997; FRITZSCH *et al.* 2000; BEISEL *et al.* 2010; FRITZSCH AND ELLIOTT 2017; COX *et al.* 2018a).

We speculate on the possible connection of the MLX + MLXIP/Mondo duplication (Fig. 4) to a transition from a holopelagic to a biphasic pelago-benthic life cycle *in the stem-benthozoan lineage*. This particular result furthers a growing picture of metabolic and chaperone gene loss and gene innovation in early animal evolution (ERIVES AND FASSLER 2015; RICHTER *et al.* 2018). Basic-helix-loop-helix (bHLH) TFs function as obligate dimers for DNA-binding (MURRE *et al.* 1989; LASSAR *et al.* 1991; MURRE *et al.* 1991). Therefore, it is unlikely that a second MLX-related gene encoding a heterodimeric partner to the single gene found in ctenophores is artifactually missing in multiple sequenced genomes and transcriptomes (RYAN *et al.* 2013; MOROZ *et al.* 2014). It is more likely that that the single *MLX-like* gene corresponds to the predicted evolutionary intermediate gene encoding a homodimeric bHLH TF.

The MLX:MLXIP/MLXIPL heterodimeric TF acts as a transcriptional regulator of metabolic pathways (*e.g.*, lipogenesis genes) in response to variation in intracellular sugar concentrations (YAMASHITA *et al.* 2001; IIZUKA *et al.* 2004; SANS *et al.* 2006; HAVULA *et al.* 2013; HAVULA AND HIETAKANGAS 2018). In this regard it is interesting to speculate that the duplication

evolved in connection with the evolution of a biphasic pelago-benthic life cycle featuring a pelagic feeding larva and a benthic feeding adult (whether it was a motile planula or sessile adult). Pelagic larval and benthic adult feeding forms would have distinct nutritional intakes associated with dissimilar feeding strategies and dissimilar nominal parameters. This bimodal variation would have evolved on top of variation associated with just a single feeding strategy. Thus, a stem-benthozoic ancestor may have demanded additional complexity in metabolic regulation that was subsequently afforded by duplicated paralogs to expand regulation of life cycle cell forms into differentiation of different cell types (FRITZSCH *et al.* 2015).

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Author Contributions A.E. and B.F. conceived this study together; A.E. conducted the computational analyses, including the phylogenomic tree constructions; A.E. and B.F. analyzed the data; A.E. wrote the manuscript, excluding the Discussion, which B.F. helped to write; and A.E. and B.F. edited the entire manuscript.

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

Supporting Information

File S1. Zipped file containing curation documents, fasta and alignment files for all gene sets.

File S2. TMC1/TMEM pairwise alignments.

Table S1. Candidate early metazoan paralogs.