Phylogenomic analyses support traditional relationships within Cnidaria

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Abstract

Cnidaria, the sister group to Bilateria, is a highly diverse group of animals in terms of morphology, lifecycles, ecology, and development. How this diversity originated and evolved is not well understood because phylogenetic relationships among major cnidarian lineages are unclear, and recent studies present contrasting phylogenetic hypotheses. Here, we use transcriptome data from 15 newly-sequenced species in combination with 26 publicly available genomes and transcriptomes to assess phylogenetic relationships among major cnidarian lineages. Phylogenetic analyses using different partition schemes and models of molecular evolution, as well as topology tests for alternative phylogenetic relationships, support the monophyly of Medusozoa, Anthozoa, Octocorallia, Hydrozoa, and a clade consisting of Staurozoa, Cubozoa, and Scyphozoa. Support for the monophyly of Hexacorallia is weak due to the equivocal position of Ceriantharia. Taken together, these results further resolve deep cnidarian relationships, largely support traditional phylogenetic views on relationships, and provide a historical framework for studying the evolutionary processes involved in one of the most ancient animal radiations.

Keywords: Anthozoa, Cnidaria, Medusozoa, phylogenetics, phylogenomics, transcriptomics

1. Introduction

Cnidaria is a group of primarily marine invertebrates composed of about 11,000 described species [1] that include reef-forming corals, sea anemones, soft corals, jellyfish, marine hydroids, and freshwater *Hydra* (Fig. 1). Cnidarians are united by the presence of complex intracellular structures called cnidae, with the most universal and diverse cnidae being the stinging structures called nematocysts. The bodies of cnidarians are constructed of two epithelial layers separated by an extracellular mesoglea. They are one of the most diverse groups of animals in terms of morphology, lifecycles, ecology, and development. While they are often presented as “simple” animals, many features of presumed simplicity are actually based on misunderstandings of their biology. For example, it is often asserted that cnidarians are radially symmetrical, but most have bilateral symmetry, some have directional asymmetry, and only a subset of species have radial symmetry [2,3]. Recent analyses confirm Cnidaria as the sister group to Bilateria [4], the most intensively studied group of animals, providing an excellent outgroup for understanding cnidarian biology.

Cnidarians are divided into two groups, Anthozoa and Medusozoa (Fig. 2a). These clades are widely recovered in phylogenetic analyses of molecular data [5–7] (but see [8]) and are supported by morphological characters (e.g., [7,9]). Resolving major relationships within Anthozoa and Medusozoa has received considerable attention, but has proven to be challenging (e.g., [10–12]). At least part of that challenge is due to the ancient divergences within Cnidaria. Some fossil representatives from major cnidarian lineages from the Cambrian appear remarkably similar to extant forms [13]. The existence of these crown group Cambrian fossils suggests that multiple extant cnidarian clades already existed over 500 million years ago [14]. The ancient divergence times of the deep nodes within Cnidaria may present a particularly difficult hurdle when reconstructing higher level phylogenetic relationships within this group.

Anthozoa contains approximately 7,500 extant described species [15]. It is composed of two major groups, Hexacorallia (sea anemones, tube anemones, scleractinian corals, and black corals) and Octocorallia (soft corals, gorgonians, and sea pens). Studies using nuclear ribosomal DNA markers recover anthozoan monophyly [6,16–19]. Morphological characters that unite anthozoans include an actinopharynx, which is a tube that extends from the mouth into the gastric cavity; mesenteries, which are sheets of gastrodermal tissue that extend from the body wall into the coelenteron; and cnidae with a cap structure on the apical end of the capsule [15,20,21]. All anthozoans have bilaterally symmetric polyps [22,23]. Although these

morphological features provide compelling evidence to support monophyly of anthozoans, one cannot distinguish between apomorphic and plesiomorphic characters in the absence of a robust phylogenetic hypothesis. That is, given that Anthozoa is one of two clades of Cnidaria, polarization of cnidarian-specific characters is problematic. This issue is confounded by recent molecular phylogenetic studies using mitochondrial genome sequences that recover Anthozoa as paraphyletic, with Octocorallia as the sister taxon to Medusozoa [8,24] (Fig. 2b).

Resolving deep relationships within Anthozoa has been controversial. All hexacorals possess a distinct type of stinging organelle (cnida) called a spirocyst [25]. Hexacoral monophyly has been supported by several molecular studies [20,23,26]. These studies all recover the tube anemones, Ceriantharia, as sister to the rest of hexacorals. However, this finding has been challenged recently by Stampar *et al.* [19], who found Ceriantharia as sister to the Hexacorallia and Octocorallia clade, rendering Hexacorallia paraphyletic (Fig. 2c). Octocoral polyps have eight tentacles, eight mesenteries, and almost all species are colonial. They also have a unique gene, *mtMutS,* in their mitochondrial genome [27,28]. Several molecular studies support the monophyly of Octocorallia [16,18,20,26,29]. Although Octocorallia is traditionally divided into three groups, Pennatulacea (sea pens), Helioporacea (blue corals) and Alcyonacea (soft corals and gorgonians), Alcyonacea is likely paraphyletic, as are many of the traditionally defined groups within it [10,30]. Molecular phylogenetic studies have converged on three well-supported lineages (reviewed in [30]). The largest group, Holaxonia-Alcyoniina, includes representatives from Alcyonacea. The Calcaxonia-Pennatulacea group includes a paraphyletic Calcaxonia, with a monophyletic Pennatulacea and Helioporacea. The third group, *Anthomastus-Corallium* includes representatives from Scleraxonia and Alcyoniina.

Medusozoa comprises approximately 3,700 extant described species and is usually divided into four groups, Scyphozoa (true jellyfish), Cubozoa (box jellies), Staurozoa (stalked jellyfish), and Hydrozoa (hydroids, hydromedusae, siphonophores) [15]. Medusozoans typically, though far from universally, have a pelagic medusa stage as part of their life cycle [7,31], a linear mitochondrial DNA genome [5,32], and a hinged cap called an operculum at the apex of their nematocysts [21]. These synapomorphies are consistent with the monophyly of Medusozoa recovered by molecular phylogenetic studies using nuclear ribosomal DNA sequences [7,14,31]. Symmetry is quite diverse in Medusozoa. Different species display bilateral or radial symmetry, and some even exhibit directional asymmetry [2,3,33].

Relationships among major medusozoan lineages have received inconsistent support and some findings remain controversial. These include the rooting of Medusozoa with regard to the position of Staurozoa [7,31], and the sister relationship between Scyphozoa and Cubozoa. Maximum likelihood analyses of nuclear ribosomal sequences recover Staurozoa as the sister taxon to the rest of Medusozoa, and a monophyletic Cubozoa and Scyphozoa group as sister to Hydrozoa [7,14] (Fig. 2d). These results are contradicted by an analysis of protein coding mitochondrial gene sequences, which recovered a paraphyletic Scyphozoa and a Staurozoa and Cubozoa clade as the sister taxon to Hydrozoa [8]. In a cladistic analysis of morphological data, Marques and Collins [9] report Cubozoa and Staurozoa as sister to Scyphozoa, whereas an analysis of a corrected version of the same dataset was consistent with the results derived from nuclear ribosomal sequences [34]. Resolving the relationships among these lineages has implications for our understanding of key innovations within Medusozoa, including the origin of a pelagic medusa and associated sensory structures and swimming musculature, as well as mode of medusae metamorphosis and development.

Here, we present a broadly sampled phylogenomic analysis of Cnidaria designed to test the general framework for cnidarian phylogeny that has emerged in the past decades, and compare alternative hypotheses for remaining questions. By collecting new transcriptome data for 15 species and analysing them in conjunction with publicly available transcriptomes and genomes, we present a robust hypothesis of higher-level Cnidaria relationships.

2. Materials and Methods

(a) Taxon sampling, RNA isolation, and Sequencing

New transcriptome data were sequenced for 15 species using Roche 454 GS FLX Titanium and Illumina HiSeq 2000/2500 sequencers. Sample preparation protocol and sequencing technology for each sample are listed in Supplementary Table 1. All new data were deposited in the NCBI Sequence Read Archive (BioProject PRJNA263637). All 454 data were assembled with Newbler (version 2.5.3). Agalma (versions 0.4.0-0.5.0) [35] was used for all other analysis steps. A git repository of the analysis code is available at https://bitbucket.org/caseywdunn/cnidaria2014.

(b) Data analyses

In combination with publicly available data, sequences from 41 taxa were used for matrix construction. We sampled 1,262 genes to generate a matrix with 50% occupancy. This matrix

has a length of 365,159 aa (Fig. 3). Three taxa, *Calibelemnon francei*, *Craspedacusta sowerbii*, and *Obelia longissima*, had less than 5% occupancy and were excluded from further analyses. The primary matrix (matrix 1) used for all phylogenetic analyses therefore has 38 taxa and 54% gene occupancy. From this matrix, we constructed a reduced matrix (matrix 2) from which two poorly sampled taxa, the Cerianthid (16.6% gene sampling) and *Haliclystus sanjuanensis* (6.5% gene sampling), were also removed since they were unstable in the primary analyses. This produced a reduced matrix with 57% gene occupancy.

We inferred phylogenetic relationships using both Maximum Likelihood (ML) and Bayesian Inference (BI) approaches. For ML, we used ExaML v 1.0.12 [36] with the WAG+Γ model of amino acid substitution on the unpartitioned matrices 1 and 2. We also ran a partitioned ML analyses on matrix 1 according to results of PartitionFinder v 1.1.1 [37]. For PartitionFinder, we used genes as initial partitions, linked branch lengths across partitions, used the BIC to select among all models of amino acid substitution, and used the relaxed hierarchical clustering algorithm to search for a good partitioning scheme. Bootstrap values were estimated on the unpartitioned and partitioned analyses with 200 replicates. BI was conducted on PhyloBayes MPI v. 1.4e [38] using the CAT model of evolution [39] with the global exchange rates fixed to uniform values (CAT-Poisson). For this analysis, constant sites were removed from the alignment to improve MCMC mixing [38]. Two independent MCMC chains were run on matrix 1, adjusting the number of cycles until convergence was achieved. Convergence was determined with time-series plots of the likelihood scores, and maximum bipartition discrepancies across chains less than 0.1. Post-burn-in (50%) sampled trees were combined and summarized with a majority rule consensus tree.

(c) Hypothesis testing

We use the SOWH test [40] to evaluate two phylogenetic hypotheses: (i) Octocorallia is sister to Medusozoa (i.e., Anthozoa is paraphyletic) [8] and (ii) Staurozoa is sister to all other Medusozoa [31]. To carry out these analyses, we used SOWHAT [41] specifying a constraint tree and the WAG+Γ model on matrix 1. We used the stopping criterion implemented in SOWHAT to determine an appropriate sample size for the null distribution. The commit version at the time we ran these analyses is available at https://github.com/josephryan/sowhat/commit/e0c214e8d7756211d7cbb4a414642c257df6b411

3. Results and Discussion

Phylogenetic results are congruent across inference methods, models of molecular evolution, and partitioning schemes (Fig. 4, Supplemental Figs. 1-2). All our analyses provide strong support for the reciprocal monophyly of Anthozoa and Medusozoa, with the placement of the root for Cnidaria between these two clades. The Anthozoa/Medusozoa split is consistent with previous molecular phylogenetic studies based on rDNA sequences [6,7,32] and morphological synapomorphies [9,15]. This result is not consistent with the results of Park *et al.* [24] and Kayal *et al.* [8] which recover Anthozoa as paraphyletic using mitochondrial DNA sequences. A tree enforcing Octocorallia as sister to the Medusozoa, rendering Anthozoa paraphyletic (Fig. 2b), is significantly worse (SOWH test: n = 100, Δ-likelihood = 2523.533, p = 0) than our most likely tree (Fig. 4). This is consistent with Kayal *et al.* [8] who could not reject anthozoan monophyly using any statistical test of topology. If Anthozoa is non-monophyletic, as postulated by Kayal *et al.* [8], then those features unique to Anthozoa, including the actinopharynx, mesenteries, and the apical cap structure of nematocysts would be interpreted as either convergent in Octocorallia and Hexacorallia, or ancestral features of Cnidaria lost or transformed in Medusozoa. Our results contradict this view and confirm that these features are synapomorphies of Anthozoa.

Within Anthozoa, the monophyly of Hexacorallia has low support due to the phylogenetic instability of Ceriantharia (Fig. 4, Supplemental Figs. 1, 2), our most poorly sampled taxon within Anthozoa (16.6% gene sampling). Each analysis provides mixed support for the placement of Ceriantharia as either sister to the rest of the Hexacorallia, i.e., Hexacorallia is monophyletic (54% ML, 17% Bayes; Fig. 4, Supplemental Fig. 2), or sister to Octocorallia, i.e., Hexacorallia is paraphyletic (46% ML, 81% Bayes; Supplemental Fig. 1). Removing Ceriantharia clearly shows the monophyly of all other sampled Hexacorallia (Supplemental Fig. 3). The traditional view of hexacoral monophyly (Fig. 4, Supplemental Fig. 2) is also supported by previous molecular phylogenetic studies [6,8,23] and compelling morphological synapomorphies (discussed above). In particular, cerianthids share with hexacorals a unique type of cnida called a spirocyst [25]. A spirocyst is ontogenetically and chemically similar to a nematocyst, and is inferred to have a common origin (see [21]), but it is a single walled capsule whose internal tubule is sticky. No instances of evolutionary losses of cnidae, nematocysts included, have been reported. Stampar *et al.*, [19] also recovered a sister relationship between Ceriantharia and Octocorallia with low support considering only 28S rDNA sequences. However, due to overall better support values, Stampar *et al.*, [19] prefer the topology recovered with 16S rDNA sequences, where Ceriantharia is sister to the

rest of the Anthozoa. Our findings are inconsistent with this hypothesis. Although not discussed by Stampar *et al.* [19], their interpretation of Anthozoan phylogeny requires that spirocysts are lost in Octocorallia. The cnidome of Octocorallia includes only a limited suite of nematocysts (none of which are unique to the group: see [25]) and no single-walled cnidae, and so it is improbable that these have been transformed into another type of cnida. The alternative explanation for this feature under the preferred phylogeny of Stampar *et al.* [19] is that the spirocysts of Ceriantharia and of other Hexacorallia are convergent.

The monophyly of Octocorallia is strongly supported in all our analyses (Fig.4, Supplemental Figs 1-3). Although our sampling of octocorals is limited to four taxa, it represents the breadth of our current understanding of octocoral phylogenetic diversity [30]. Specifically, all three major clades of octocorals are represented. These are the Holaxonia + Alcyoniina clade (represented here by *Scleronephthya* and *Nephthyigorgia*), the *Anthomastus* + *Corallium* clade (represented by *Anthomastus*), and the Calcaxonia + Pennatulacea clade (represented by Keratoisidinae *sp.*). Relationships among these four taxa are congruent with recent octocoral phylogenies [10,30]. Resolution within these deep nodes suggests that this phylogenomic approach should prove valuable to reconstructing higher level octocoral phylogeny as more taxa are analyzed in future studies.

Medusozoa, comprising Scyphozoa, Staurozoa, Cubozoa, and Hydrozoa, forms a strongly supported monophyletic group (Fig. 4, Supplemental Figs 1-3). All our analyses support a sister group relationship between Hydrozoa and a clade composed of Scyphozoa, Staurozoa, and Cubozoa. This clade revives the traditional sense of Scyphozoa, prior to the elevation of Stauromedusae and Cubomedusae to distinct classes [9,42]. The only staurozoan included in our analysis, *Haliclystus sanjuanensis* (6.5% gene sampling), is the most poorly sampled taxon in our data set (Fig. 4). While all analyses place it within this clade with strong support, its position within the clade is unstable and it moves between positions as sister to Cubozoa and Scyphozoa (40% ML, 0% Bayes; Fig. 4) and sister to Cubozoa (60% ML, 100% Bayes; Supplementary Fig. 1). When the staurozoan is excluded from the analyses, the cubozoan *Alatina alata* is sister to the scyphozoans with 100% support (Supplementary Fig. 3). Collins *et al*. [7] reported Staurozoa as sister to the rest of Medusozoa, suggesting that pelagic medusae evolved after the divergence of staurozoans. Our results do not support this hypothesis and resulting scenario of medusa evolution. Enforcing the staurozoan as sister to all other medusozoans [31] (Fig. 2d) is significantly worse (SOWH test: n = 100, Δ-likelihood = 118.6461, p = 0) than our most likely tree (Fig. 4).

Instead, our results are consistent with the cladistic analysis of Marques and Collins [9] based on morphology and life history features. Characters from Marques and Collins [9] that support the clade composed of Staurozoa, Cubozoa, and Scyphozoa include radial tetramerous symmetry, medusa production involving metamorphosis of the oral end of the polyp, canal systems in the polyps, musculature organized in bundles of ectodermal origin, rhopalia or rhopalia-like structures, and gastric filaments. Characters supporting a Cubozoa + Staurozoa clade include quadrate cross section and metamorphosis of medusae without fission [9].

Recovered relationships within Hydrozoa are largely consistent with those found in previous studies [7,11], including the reciprocally monophyletic Trachylina and Hydroidolina. Trachylina is composed of Narcomedusae (represented here by *Aegina citrea*), Trachymedusae (represented here by *Halitrephes valdiviae*), and Limnomedusae (not represented). Within Hydroidolina, our sampling includes representatives of Siphonophora, Aplanulata, “Filifera” (which has previously been shown to be polyphyletic [11,43]), and Leptothecata. Relationships among the major lineages of Hydroidolina have been difficult to resolve [11,43]. The analyses presented here recovered the Aplanulata clade as sister to the rest of the sampled representatives of Hydroidolina. Given that members of Trachylina and Aplanulata are mostly solitary species (see [44]), these results may imply that coloniality in Hydrozoa evolved following the divergence of Aplanulata from the rest of Hydroidolina, as opposed to at the base of Hydroidolina as reported by Cartwright and Nawrocki [43]. It should be noted however that representatives of other colonial hydroidolinan lineages including Capitata and other Filifera were not included in this analysis, so the precise origin of coloniality within Hydrozoa awaits further sampling. The monophyly of Aplanulata and Siphonophora are strongly supported. The internal relationships of Siphonophora are in accord with previously published results [45], while those of Aplanulata differ from previous results [46] in that *Ectopleura* is more closely related to *Candelabrum* than to *Hydra.*

4. Conclusion

Although divergences within major lineages of Cnidaria likely occurred over half a billion years ago [13,14], using a phylogenomic approach this study reveals strong support for many deep nodes within the cnidarian tree of life. This represents a significant improvement from previous studies using rDNA markers which, in many cases, failed to resolve relationships between major cnidarian clades. Our study is also consistent with more traditional hypotheses of cnidarian relationships including the monophyly of Hexacorallia, Anthozoa, and a clade

composed of Staurozoa, Cubozoa, and Scyphozoa. Future phylogenetic studies with increased taxonomic sampling will continue to resolve more detailed relationships and patterns of character evolution in this highly diverse group.

Data accessibility

- Raw sequence data: NCBI Sequence Read Archive BioProject PRJNA263637, BioSample accession numbers: SAMN03418506-SAMN03418515, SAMN03453085-SAMN03453089.
- Analysis scripts, phylogenetic alignmets, tree sets, summary trees, and voucher information: https://bitbucket.org/caseywdunn/cnidaria2014. The most recent commit at the time of submission is available at: https://bitbucket.org/caseywdunn/cnidaria2014/src/333c9e4770881860f0ed09c99d86995377da2ff1
- Phylogenetic data also available at: http://dx.doi.org/10.5061/dryad.4b6d3

Competing interests

We have no competing interests.

Author contributions

PC and CWD conceived of and designed the study. FZ designed and ran analyses. FG extracted and prepared most samples for sequencing, along with SS. SAS assembled the 454 data and performed preliminary analyses. SHC performed the SOWH tests. SMS and PC generated the *Hydractinia* and *Podocoryna* data. CSM and SCF led octocoral sampling, and MD guided hexacoral sampling. CLA, AGC and PC generated the *Alatina alata* data. CM and SF provided the octocorals. MD collected most hexacorals. SHDH collected many of the medusozoans. MH assisted with data management, submission, and analysis implementation. PC, FZ, and CWD wrote the manuscript with considerable input from other authors. All authors discussed / contributed to the final manuscript version.

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manuscript.

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Supplementary Table 1

Table 1. Specimen data. Accession numbers or URLs for all data considered in this analysis, including data that were previously public and those that are newly generated here. A csv version of this table is available in the git repository (see Data accessibility).

Figures

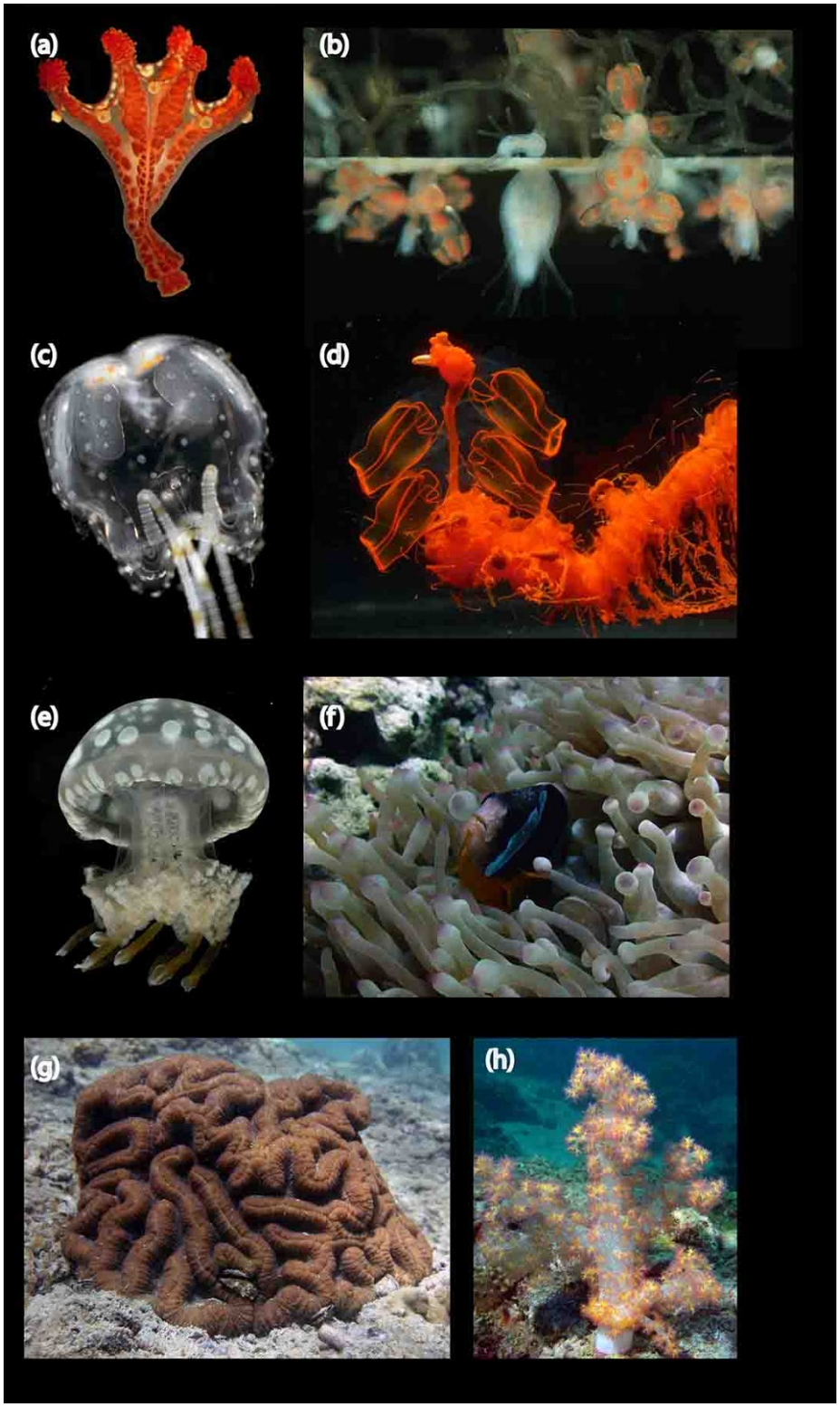


Figure 1. Photos of cnidarian representatives. (a) Staurozoa: *Haliclystus californiensis*. (b) Hydrozoa: *Podocoryna carnea*. (c) Cubozoa: *Copula sivickisi*. (d) Hydrozoa: *Marrus*

orthocanna (e) Scyphozoa: *Mastigias sp*. (f) Actiniaria: *Entacmaea quadricolor* (with anemone fish). (g) Scleractinia: Mussidae. (h) Octocorallia: *Dendronephthya sp.* Photo credits: P. Cartwright, A. Collins, M. Daly, C. Dunn and A. Migotto.

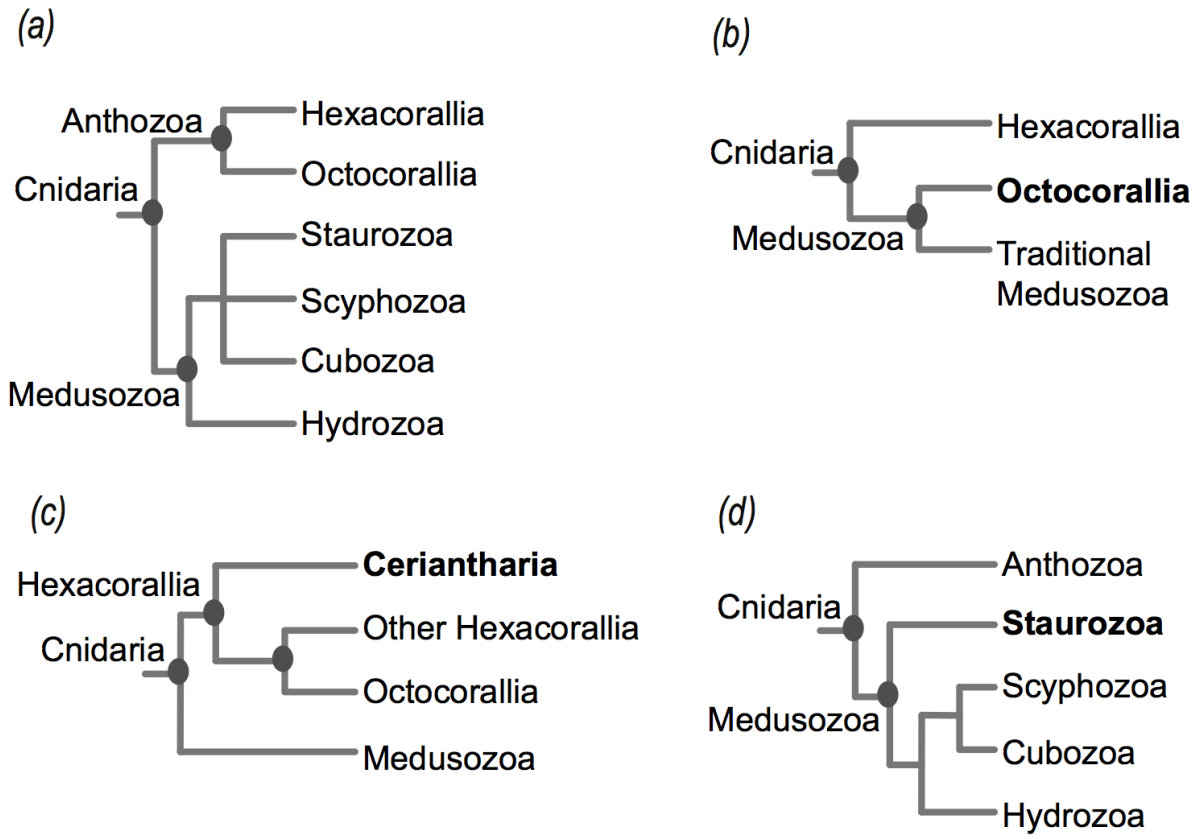


Figure 2. Alternative hypotheses for internal relationships within Cnidaria. (a) Traditional classification and relationships within Cnidaria. (b) Anthozoa paraphyletic with Octocorallia sister to the traditional Medusozoa [8]. (c) Hexacorallia paraphyletic with Ceriantharia sister to Hexacorallia + Octocorallia clade [19]. (d) Staurozoa as the sister taxon to the rest of Medusozoa [7].

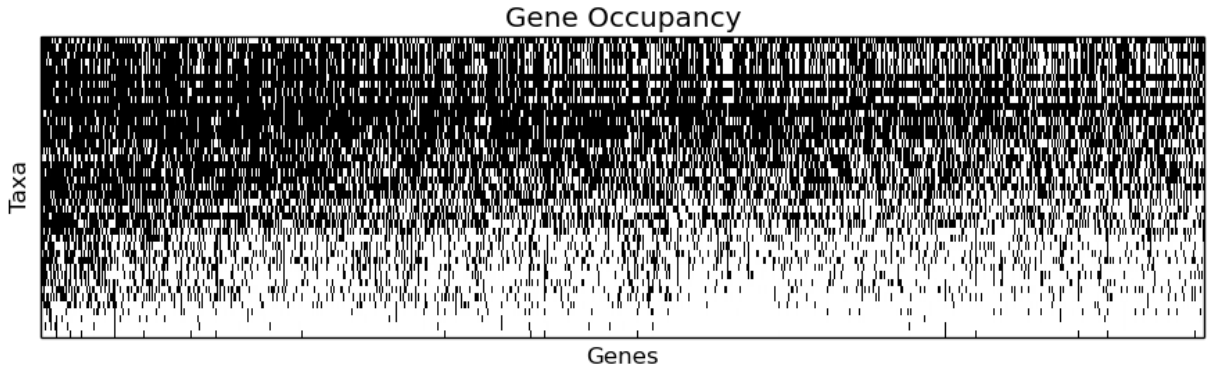


Figure 3. The 50% gene occupancy matrix. Black indicates sampled genes for each of the 41 taxa. Genes and species are sorted by sampling, with the best sampled in the upper left. The last three taxa, *Calibelemnon francei*, *Craspedacusta sowerbii*, and *Obelia longissima,* had less than 5% gene occupancy and were excluded from further analyses to produce matrix 1.

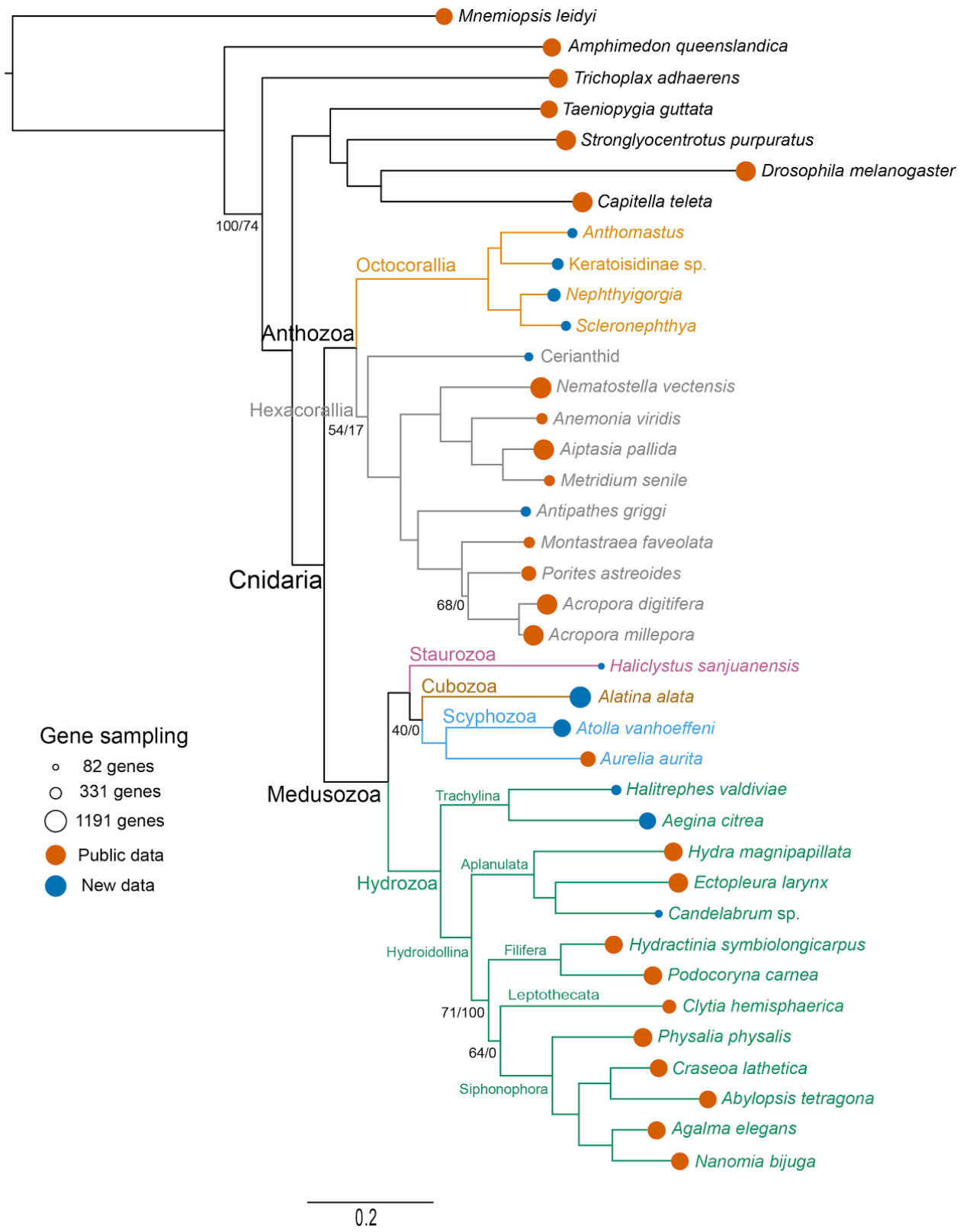
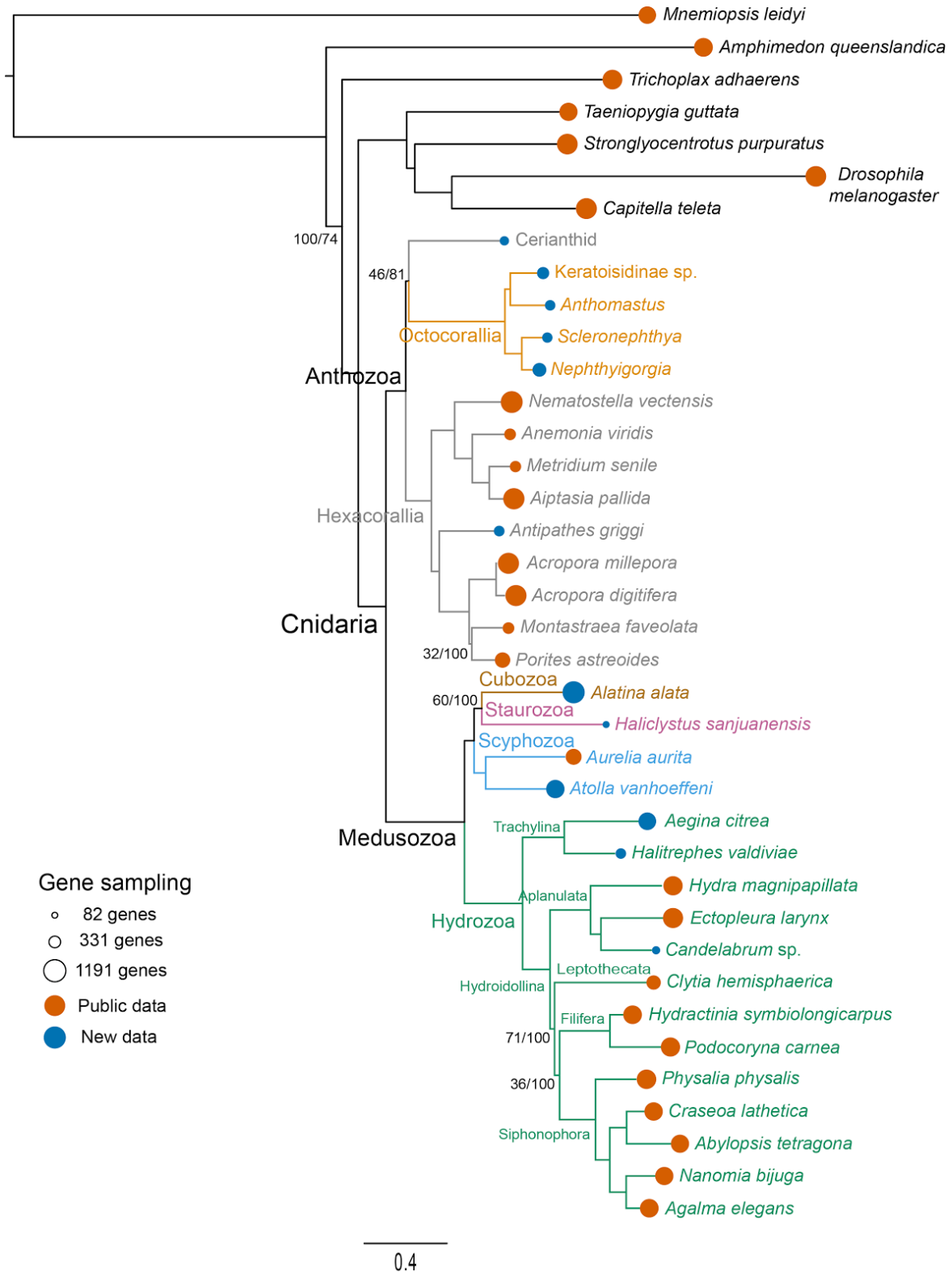


Figure 4. Rooted phylogram of the maximum likelihood (ML) analysis. Branch support values correspond to percent ML-bootstrap values/percent Bayesian posterior probabilities. No

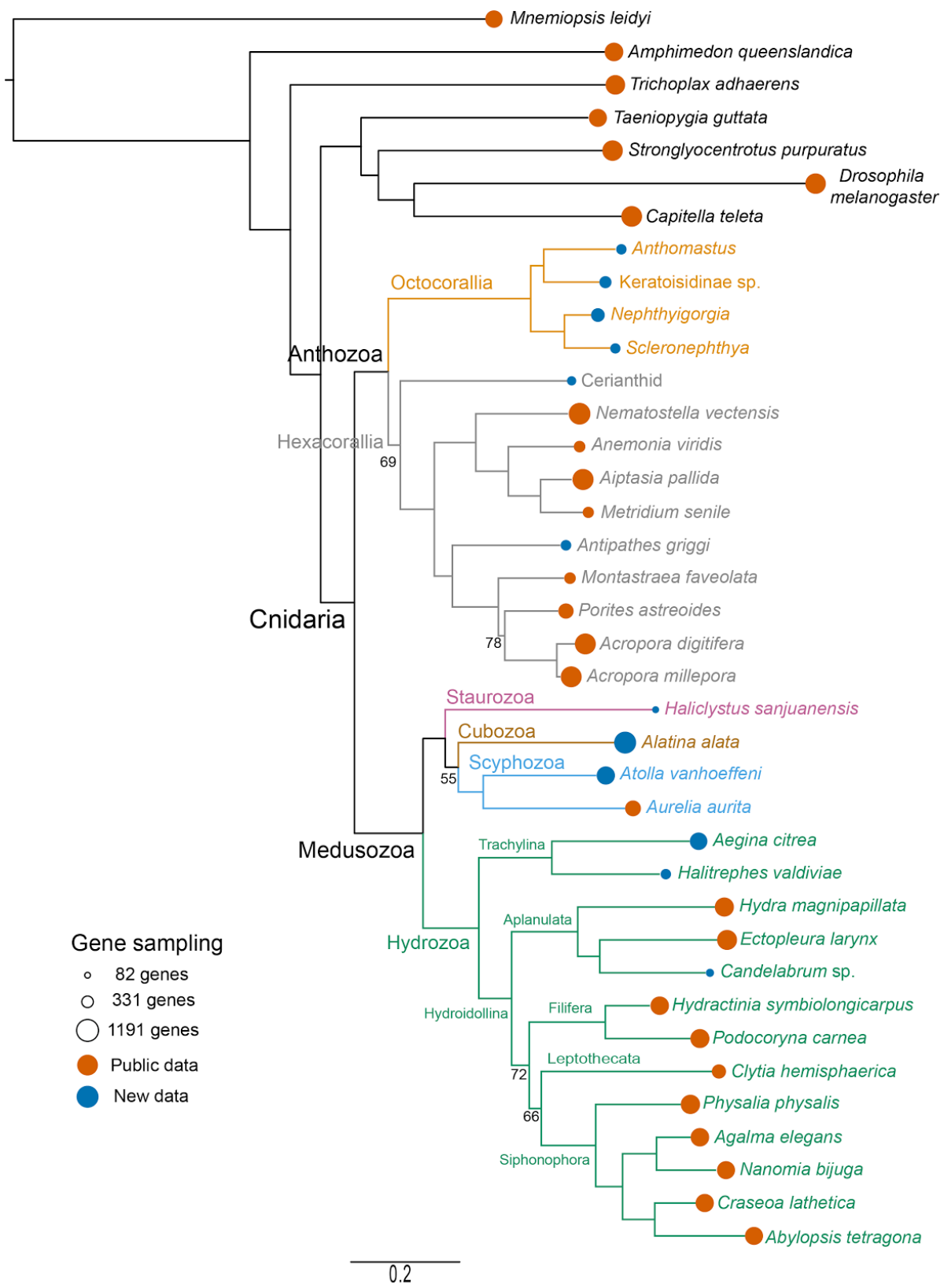
values are shown for branches with 100/100 support. The areas of the lollipops, centered on the branch tips, are proportional to the number of genes sampled.

Supplemental Figures



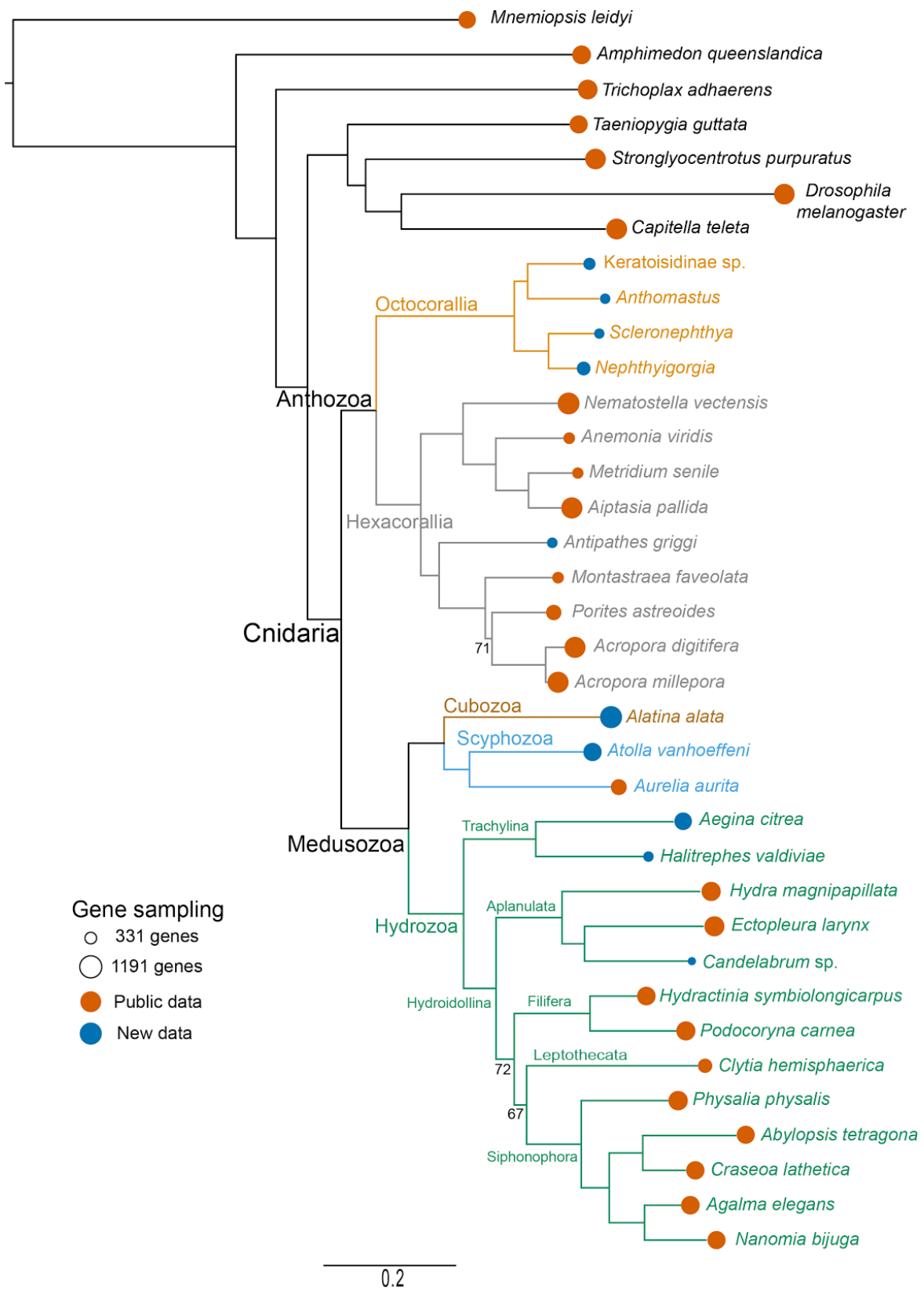
Supplemental Figure 1. Majority rule consensus rooted phylogram of Bayesian Inference

(BI) analysis. Branch support values correspond to percent ML-bootstrap values/percent Bayesian posterior probabilities. No values are shown for branches with 100/100 support. The areas of the lollipops, centered on the branch tips, are proportional to the number of genes sampled.



Supplemental Figure 2. Rooted phylogram of maximum likelihood (ML) partitioned analysis.

Branch support values correspond percent bootstraps. No values are shown for branches with 100% support. The areas of the lollipops, centered on the branch tips, are proportional to the number of genes sampled.



Supplemental Figure 3. Rooted phylogram of the maximum likelihood (ML) analysis with

unstable poorly sampled taxa (*Haliclystus sanjuanensis* and Cerianthid) removed. Branch support values correspond to percent bootstraps. No values are shown for branches with 100% support. The areas of the lollipops, centered on the branch tips, are proportional to the number of genes sampled.

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