Phylogenetic analysis of higher-level relationships within Hydroidolina (Cnidaria: Hydrozoa) using mitochondrial genome data and insight into their mitochondrial transcription

Ehsan Kayal, Bastian Bentlage, Paulyn Cartwright, Angel A Yanagihara, Dhugal J Lindsay, Russell R Hopcroft, Allen G Collins

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Abstract

Hydrozoans display the most morphological diversity within the phylum Cnidaria. While recent molecular studies have provided some insights into their evolutionary history, sister group relationships remain mostly unresolved, particularly at mid-taxonomic levels. Specifically, within Hydroidolina, the most speciose hydrozoan subclass, the relationships and sometimes integrity of orders are highly unsettled. Here we obtained the near complete mitochondrial sequence of twenty-six hydroidolinan hydrozoan species from a range of sources (DNA and RNA-seq data, long-range PCR). Our analyses confirm previous inference of the evolution of mtDNA in Hydrozoa while introducing a novel genome organization. Using RNA-seq data, we propose a mechanism for the expression of mitochondrial mRNA in Hydroidolina that can be extrapolated to the other medusozoan taxa. Phylogenetic analyses using the full set of mitochondrial gene sequences provide some insights into the order-level relationships within Hydroidolina, including siphonophores as the first diverging clade, a well-supported clade comprised of Leptothecata-Filifera III-IV, and a second clade comprised of Aplanulata-Capitata *s.s.*-Filifera I-II. Finally, we describe our relatively inexpensive and accessible multiplexing strategy to sequence long-range PCR amplicons that can be adapted to most high-throughput sequencing platforms.

Introduction

Cnidaria (corals, anemones, jellyfish, hydroids) is a phylum of relatively simple aquatic animals characterized by the presence of a specific cell type, the cnidocyte, which harbors a highly specialized cellular organelle, the cnidocyst. Cnidaria encompasses five recognized classes (Daly et al., 2007): Anthozoa (stony corals, sea anemones, tube anemones, soft corals and gorgonians), Cubozoa (box jellyfish), Hydrozoa (hydroids, hydromedusae and siphonophores), Scyphozoa (the so-called true jellyfish), and Staurozoa (stalked jellyfish). Non-anthozoan cnidarians are united in the clade Medusozoa (Collins, 2002), whose members typically display a metagenetic life cycle consisting of planula larva, sessile polyp and free-swimming medusa, not all of which may be present in the life cycle of a given species. Within Medusozoa, Hydrozoa represents, to many measures, the most diverse class. Hydrozoa encompasses over 90% of medusozoan species (Daly et al., 2007), and so it is perhaps unsurprising that life cycle variation, as well as disparity of medusae, polyps, and colonies within this class far exceeds what is observed within Cubozoa, Scyphozoa or Staurozoa. An important and necessary step in understanding the evolution of the remarkable biodiversity present within Hydrozoa is a robust hypothesis of the phylogenetic relationships among its component taxa.

Recent work based on nuclear ribosomal sequences (Collins, 2002; Collins et al., 2006, 2008; Cartwright et al., 2008) and complete mitochondrial genome sequences (Kayal et al., 2013) shows that Hydrozoa consists of two main clades, Trachylina and Hydroidolina. Monophyly of the latter is also supported by phylogenetic analyses of life history and anatomical features (Marques & Collins, 2004). Trachylina is relatively poor in terms of species richness, containing roughly 150 species in four orders: Limnomedusae, Trachymedusae, Narcomedusae and Actinulida (Collins et al., 2008). The remainder of the approximately 3,350 species of hydrozoans (Daly et al., 2007) that make up the clade Hydroidolina, are classified in three orders: Anthoathecata, Leptothecata and Siphonophora (Schuchert, P. (2015). Hydroidolina. Accessed through: Schuchert, P. (2015) World Hydrozoa database at http://www.marinespecies.org/hydrozoa/aphia.php?p=taxdetails&id=19494 on 2015-07-09). Hydroidolina comprises almost all hydrozoans whose life cycle includes a benthic polypoid or hydroid stage (the exception being Limnomedusae, which is part of Trachylina). Colonial hydroid stages within Hydroidolina, especially siphonophores, tend to have greater functional specialization between zooids than other colonial members of Cnidaria (Hyman, 1940; Dunn, Pugh & Haddock, 2005; Dunn, 2009; Cartwright & Nawrocki, 2010).

Two of the three presently recognized orders within Hydroidolina (Anthoathecata, Leptothecata and Siphonophorae) have strong support for their monophyly: Leptothecata whose constituent species' hydroid phase contains a theca (with a few exceptions) and whose medusae (when present) have gonads along the radial canals (Cartwright et al., 2008; Leclère et al., 2009), and Siphonophorae, pelagic animals with a remarkable level of colony organization (Dunn, Pugh & Haddock, 2005; Cartwright et al., 2008; Dunn, 2009). In contrast, no phylogenetic analysis has provided support for the monophyly of Anthoathecata. Anthoathecata contains those species that lack a theca during the hydroid phase and whose medusae (when present) usually bear gonads on the manubrium (Cartwright et al., 2008). Yet, the absence of thecae can easily be interpreted as plesiomorphic (Cartwright & Nawrocki, 2010). Even though there has been no support for the monophyly of Anthoathecata, several likely clades have been identified within this taxonomic group Aplanulata, a group consisting of hydrozoans that lack a ciliated planula stage was introduced recently (Collins et al., 2005) and its monophyly supported in subsequent studies (Collins et al., 2006; Cartwright et al., 2008; Kayal et al., 2013; Nawrocki et al., 2013). Aplanulata contains a portion of the families whose species, in the hydroid stage, possess capitate tentacles. In the past, all hydrozoan species possessing capitate tentacles have been united within the anthoathecate suborder Capitata. However, the taxon has become restricted to a well-supported clade of non-Aplanulata species with capitate tentacles (Collins et al., 2005; Nawrocki, Schuchert & Cartwright, 2010), referred to as Capitata *sensu stricto* by Cartwright et al. (2008). The status of the anthoathecate suborder Filifera, containing species whose hydroid stage has tentacles with more or less uniform distribution of nematocysts (filiform), is even more complex, with no less than four putative clades with various levels of support recognized (Cartwright et al., 2008; Cartwright & Nawrocki, 2010).

Despite the recognition of several possible and likely clades within Hydroidolina, phylogenetic analyses have thus far suffered from low support for deep nodes representing the relationships among them. Filifera has never been recovered as a monophyletic group in any explicit phylogenetic analysis, nor has there been support for relationships among the filiferan clades, Capitata, Aplanulata, Leptothecata and Siphonophora (Collins, 2002; Collins et al., 2006, 2008; Cartwright et al., 2008; Cartwright & Nawrocki, 2010). Lack of resolution among the deep nodes of Hydroidolina hinders our understanding of their evolution. Indeed, a recent review (Cartwright & Nawrocki, 2010) highlighted the complexity of morphological characters in the evolutionary history of Hydrozoa and lamented the current lack of resolution of hydroidolinan phylogeny, particularly at ordinal and subordinal levels, which prevents a better understanding of life cycle evolution within this class.

Recent technological advances have allowed us to target the nearly complete mtDNA instead of the single-locus approaches, including barcoding, often used in systematics and biodiversity studies (Dettai et al., 2012). The small size and circular nature of the majority of animal mtDNAs make them accessible for low-budget taxonomic studies, given the availability of simple and inexpensive protocols. It is now possible to amplify the complete mtDNA using long-range PCR (Burger et al., 2007), which combined with novel high-throughput sequencing technologies, provide access to mitogenomic data for groups considered "difficult-to-sequence" at very low cost and effort (Kayal et al., 2012; Briscoe et al., 2013; Foox et al., 2015). To date, the mtDNA of 188 non-bilaterian animals has been sequenced, out of which 124 are cnidarians, mostly anthozoans.

Medusozoan mtDNA sequencing presents a unique challenge in that all medusozoans possess linear mitochondrial genomes (Kayal et al., 2012). Sequencing complete linear chromosomes using traditional long-PCR approach requires knowledge of genome organization, particularly the genes at the ends of the linear molecules. Specifically, studies have suggested that the mitochondrial genome in medusozoan cnidarians can be mono-, bi-, or octo-chromosomal (Ender & Schierwater, 2003; Voigt, Erpenbeck & Wörheide, 2008; Kayal & Lavrov, 2008; Park et al., 2012; Zou et al., 2012; Kayal et al., 2012; Smith et al., 2012). Interestingly, the linearization of medusozoan mtDNA appears to coincide with relative stability in the gene organization of most medusozoans (Kayal et al., 2012), which facilitates designing protocols for amplification and sequencing most of the coding regions of the mitochondrial chromosome(s).

We present an analysis of nearly-complete mitochondrial genome sequences from a diverse set of hydrozoan taxa in an effort to better understand the relationships within Hydroidolina. Specifically, we describe twenty-six novel, nearly-complete mitochondrial genomes from several hydrozoan orders. We first analyzed the composition and gene order of these mitochondrial genomes. We then used RNA-seq data to infer some of the mechanisms involved in mitochondrial gene expression. Finally, we used both the nucleotide and amino acid sequence data to reconstruct the evolutionary history of hydrozoans, focusing on the thus far intractable relationships within Hydroidolina.

Material and methods

Taxon sampling

We sampled species from both hydrozoan subclasses, Trachylina (three species) and Hydroidolina (twenty-three species), maximizing the coverage of hydrozoan diversity by sampling at least one species from all the currently recognized hydroidolinan clades that correspond to the orders/suborders Aplanulata, Capitata *s.s.*, Filifera I-IV, Leptothecata, Siphonophorae (Table 1). We acquired all publicly available medusozoan mitochondrial genomes through Genbank, including nineteen non-hydrozoans used as outgroup taxa (Table 1).

Obtaining nearly-complete mitochondrial genomes

We followed the protocol described in a previous study (Kayal et al., 2012) to amplify the nearly-complete mitochondrial DNA (mtDNA) of sixteen hydrozoan species. This protocol exploits the relative conservation of the gene organization within Hydrozoa to amplify the nearly-complete mtDNA in one or two pieces via long-range PCR. First, we used conserved metazoan primers to amplify and sequence regions of *cox1* and *rns* genes in all sampled taxa. For the two species of Trachylina, we also amplified and sequenced regions of *cob* and *rnl*. Finally, for several species *rns* was difficult to amplify and we sequenced *nad5* instead. We then designed species-specific and conserved primers for long-range PCR amplification as described in Kayal *et al.* (Kayal et al., 2012). We amplified the nearly complete mtDNA (encompassing most coding regions) in one or two contigs using Ranger Taq (Bioline) with a combination of one, two or three sets of primers (see Table S1 for the list of primers and lengths of long PCR amplificons per species). Long amplicons were visualized on an Agarose gel, when necessary multiple amplicons were pooled for each individual specimen, and sheared to the appropriate size-range using a Q800R sonicator (QSONICA). Sheared amplicons were processed for multiplexed double-tagged library preparation for Illumina (100 bp single-end) or Ion Torrent (200 bp single-end) sequencing using custom protocols (see Supplementary information for detailed protocols). Sequencing was performed either on one lane of Illumina HiSeq2000 platform (Illumina) at the Genomics Core Lab of the University of Alabama or using one 316 v.1 chip on the Ion Torrent Personal Genome Machine Ion platform (PGM, Life Technologies) at the Laboratories of Analytical Biology of the Smithsonian National Museum of Natural History.

Sequence assembly and annotation

Sequence reads were sorted per taxon by index and barcode using the Galaxy Barcode Splitter from the Galaxy platform (Giardine et al., 2005; Blankenberg et al., 2010; Goecks, Nekrutenko & Taylor, 2010) and Geneious v.7 (Kearse et al., 2012), respectively. Reads were trimmed and the barcode removed using Geneious before proceeding to assembly using the built-in overlap-layout-consensus assembler of Geneious v.7 and a modified version of MITObim v.1.7 (Hahn, Bachmann & Chevreux, 2013). Then, we used these consensus sequences as backbones to map the sorted and end-trimmed raw reads using both MIRA v.4 (Chevreux, Wetter & Suhai, 1999) and the built-in Geneious mapping plug-in. The final contigs covered the nearly complete mtDNAs as expected from long-range PCR amplifications.

We also probed several large sequence libraries: DNA-seq libraries obtained from a specimen of *Liriope tetraphylla* and two non-clonal specimens of *Cladonema pacificum*; RNA-seq (EST) libraries obtained from the siphonophores *Nanomia bijuga* and *Physalia physalis*, *Craspedacusta sowerbyi*, *Ectopleura larynx*, *Podocoryna carnea* and two species of *Hydractinia, H. polyclina* and *H. symbiolongicarpus* (Table 1). For these specimens, we first captured several mitochondrial regions by mapping raw reads to the mtDNA from other hydrozoan genomes with Bowtie v.2 (Langmead & Salzberg, 2012) and MIRA v.4. We then extended these contigs with several rounds of baiting (using the mirabait script from MIRA v.4) and assembly (using the overlap-layout consensus assembler in Geneious) into gapped mtDNAs for *E. larynx*, *C. sowerbyi*, *L. tetraphylla*, *P. carnea*, *H. polyclina*, *H. symbiolongicarpus*, one specimen of *P. physalis* and *C. pacificum*, as well as the nearly-complete coding regions for *N. bijuga*, and another specimen of *P. physalis*.

We identified protein genes by blasting large (>300 bp) open reading frames (ORFs) obtained via translation using the minimally derived genetic code (translation table 4 = the Mold, Protozoan, and Coelenterate Mitochondrial Code) against published hydrozoan mtDNA genomes, followed by manual annotation. Transfer RNA (tRNA) genes were identified using the tRNAscan-SE and ARWEN programs (Lowe & Eddy, 1997; Laslett & Canbäck, 2008). We identified ribosomal (rRNA) genes by similarity (BLAST searches on NCBI's GenBank) to their counterparts in published mt-genomes and delimited the ends by alignment (see below).

Sequence alignments and phylogenetic analyses

We prepared several multiple sequence alignments for phylogenetic analyses as described previously (Kayal et al., 2013). In short, the amino acid (AA hereafter) sequences of protein-coding genes were individually aligned using the L-INS-i option with default parameters of the MAFFT v.7 aligner online (Katoh & Standley, 2013) and subsequently concatenated. Nucleotide (NT hereafter) alignments for individual protein-coding genes were obtained according to their AA alignments using the online version of the PAL2NAL online program (Suyama, Torrents & Bork, 2006) and subsequently concatenated. Ribosomal genes (rRNA hereafter) were individually aligned using the online version of MAFFT with the Q-INS-i option (Katoh & Toh, 2008) and concatenated. We also created a concatenated all-nucleotides dataset consisting of NT and rRNA alignments (allNT hereafter). All concatenated alignments were filtered using Gblocks (Talavera & Castresana, 2007) with default parameters, allowing gaps in all positions, leading to alignments with 2902 positions (2501 informative sites) for AA, 9864 positions (8850 informative sites) for NT, 2154 positions (1664 informative sites) for rRNA, and 12018 positions (10773 informative sites) for allNT (Table S3, all alignments are provided as Supplementary information). We estimated the number of phylogenetically informative sites with the DIVEIN online server (Deng et al., 2010), and the saturation levels of nucleotide alignments (NT, rRNA and allNT) using the DAMBE5 software (Xia, 2013).

We performed jModelTest v.2.1.4 (Darriba et al., 2012) and ProtTest v.3 (Darriba et al., 2011) on the nucleotide and amino acid alignments, respectively, to identify the most appropriate models of sequence evolution across entire alignments for subsequent phylogenetic analyses. Phylogenetic inferences were conducted under Maximum Likelihood framework using RAxML v.8 (Stamatakis, 2014) and under Bayesian framework using MrBayes v.3.2.2 (Ronquist et al., 2012). Maximum Likelihood analyses were performed using the LG model of sequence evolution for amino acids. The General Time Reversible (GTR) models of nucleotide and amino acid evolution for all alignments were used for both Maximum Likelihood and Bayesian. Bayesian analyses consisted of two runs of 4 chains each of 10,000,000 generations using the GTR model for all alignments, sampled every 100 trees after a burn-in fraction of 0.25.

To investigate potential compositional biases in the datasets, amino acid and nucleotide composition of alignments were calculated using custom python scripts (github.com/bastodian/shed/blob/master/Python/AA-Frequencies.py and github.com/bastodian/shed/blob/master/Python/GC-Frequencies.py), and visualized in 2-

193 dimensional plots using the first two principal components as calculated by the princomp function in R version
194 2.15.1 (Team, 2014).

195 *Evaluation of competing phylogenetic hypotheses*

196 We tested 3 sets of traditional hypotheses of hydroidolinan relationships using likelihood-based topology
197 tests with the approximately unbiased (AU) tests as implemented in Consel (Shimodaira & Hasegawa, 2001).
198 Phylogenetic analyses were performed under the three following scenarios using constrained topological ML
199 searches in PhyML v. 3.1 (Guindon et al., 2010) to calculate per-site likelihoods.

200 1) Several studies have found Capitata to be the earliest branching clade within Hydroidolina (e.g. Collins,
201 2002; Marques & Collins, 2004; Cartwright et al., 2008; Cartwright & Nawrocki, 2010) while another study
202 suggested Aplanulata to be the earliest branch within Hydroidolina (Collins et al., 2006) . We compared these
203 hypotheses to our best tree to evaluate if our data were able to reject either of these alternatives. 2) Filifera was
204 traditionally viewed as being a monophyletic clade, but support for this nominal taxon has not been found so far
205 (reviewed in Collins, 2009). We calculated the best ML tree under the constraint of the monophyly of Filifera and
206 compared the resulting per-site likelihoods to those calculated from our best tree to evaluate if we can reject
207 Filifera's monophyly given our datasets. 3) Anthoathecata (Aplanulata + Capitata) is a traditional taxon within
208 Hydroidolina, a group not supported by our study and others (Collins, 2009); we compared the constrained topology
209 containing monophyletic Anthoathecata to our best tree.

Results

The mitochondrial genomes of hydrozoan cnidarians

We obtained partial or complete mtDNA from twenty-six hydrozoan species, more than tripling the number of mitogenomes available to date for this class. We found four different genome organizations in these hydrozoans (Fig. 1), three of which were described previously (Kayal et al., 2012): the trachymedusae *Geryonia proboscidalis* and *Liriope tetraphylla* have a mitochondrial genome organization similar to that known from other trachylines, *Cubaia aphrodite* (Kayal et al., 2012) and *Craspedacusta sowerbyi* (Zou et al., 2012); the mt genome organization in the aplanulatan *Euphysa aurata* is similar to those of other members of Aplanulata, *Ectopleura larynx* and *Hydra oligactis* (Fig. 1, Kayal et al., 2012); the mt-genome organization in the species *Catablema vesicarium*, *Cladonema pacificum*, *Craseoa lathetica*, *Eudendrium capilare*, *Halitholus cirratus*, *Hydractinia polyclina*, *H. symbiolongicarpus*, *Leuckartiara octona*, *Melicertum octocostatum*, *Mitrocomella polydiademata*, *Nanomia bijuga*, *Podocoryna carnea*, *Ptychogena lactea*, *Rathkea octopunctata*, *Rhizophysa eysenhardti*, S*arsia tubulosa*, and *Tiaropsis multicirrata*, as well as the partial mitogenome of *Proboscidactyla flavicirrata* are all similar to that of non-aplanulatan hydroidolinans described previously (Kayal et al., 2012). The mtDNA sequences of *Boreohydra simplex* and *Plotocnide borealis* were identical, confirming previous suggestions that these two names represent two stages in the life cycle of the same species (Pyataeva et al. accepted pending minor revision). Interestingly, the mt genome organization of this species is novel, potentially representing a transitional state between the mtDNA organization of other aplanulatan and that of non-aplanulatan hydroidolinans (Fig. 1, see Discussion).

We analyzed a large dataset of RNA-seq data (>230 M reads) from the siphonophore *Physalia physalis* and assembled the nearly-complete mt genome in multiple contigs. The mitochondrial genes represented only >7,000 reads (<0.003% of the total number of reads) of the *Physalia* RNA-seq data. We found both small and large ribosomal RNA subunits (*rns* and *rnl*, respectively) as well as the protein genes *cob*, *cox1*, and *cox2* in single-gene contigs. The other protein genes were found in collinear contigs as follows: *atp8-atp6-cox3*, *nad2-nad5*, and *nad6-nad3-nad4L-nad1-nad4* (Fig. 2A). We were not able to identify with enough confidence the two expected tRNA genes *mt-tRNA-Met* and *mt-tRNA-Thr* in this large RNA-seq dataset. Using an independently generated, smaller source of RNA-seq data (SRA Archive num. SRR871528), we assembled a more complete mt genome, confirming that the mtDNA organization in *Physalia physalis* was similar to that of the other siphonophore *Rhizophysa*

eysenhardti obtained through long-range PCR. This smaller RNA-seq dataset provided the nearly-complete mtDNA sequence, with a few scattered gaps. The low amount of sequence data for *Nanomia bijuga* did not allow us to identify all the protein genes.

Genes were found to be very similar in length among all species (varying from identical to about 5% different in length). We found the GC content to be variable among the hydrozoan species we sampled, ranging 13.1-46.9% for protein coding genes, 19.4-39.1% for rRNA genes and 22.9-47.9% for tRNA genes. The most commonly used start codon was ATG except for *atp8* in *Boreohydra simplex*/*Plotocnide borealis* (TTG) and *Tiaropsis multicirrata* (GTG); *cob* in *Tiaropsis multicirrata* (TTG); *cox1* in *Euphysa aurata* (GTG); *nad1* in *Physalia physalis* (GTG); *nad2* in *Catablema vesicarium*, *Halitholus cirratus*, and *Nanomia bijuga* (GTG); *nad3* in *Physalia physalis* (GTG); *nad4* in *Ptychogena lactea* (GTG); *nad4L* in *Nanomia bijuga* (GTG); *nad5* in *Boreohydra simplex*/*Plotocnide borealis* (TTG); *nad6* in *Catablema vesicarium* and *Nanomia bijuga* (GTG). TAA was the most commonly used stop codon for protein genes, with the exception of *nad5* and *nad6* where TAG was most often used (Table 2).

Phylomitogenomics of Hydrozoa

Our AA, NT, rRNA, and allNT analyses under both Maximum Likelihood and Bayesian frameworks did not yield completely consistent results (Fig. 3, S1-S8). Under GTR, the AA (Fig. S1, S5), NT (Fig. S2, S6) and allNT (Fig. 3, S4) Maximum Likelihood and Bayesian analyses, respectively, yielded almost identical topologies, whereas the rRNA-based topologies (Fig. S3, S7) and the AA topology assuming the LG model (Fig. S8) exhibited far lower resolution and support than all other topologies. The PCA of amino acid and nucleotide compositions (Fig. 4) of the alignments underlying our phylogenetic analyses, where taxa with similar composition cluster together, do not show evidence of strong compositional biases that may affect phylogenetic reconstruction.

Overall, we found a number of common relationships in all phylogenetic trees that were highly supported: the divergence between Trachylina and Hydroidolina within Hydrozoa, the monophyly of Leptothecata, Capitata *s.s.* and Aplanulata (Table 3). Within Hydroidolina, all analyses other than those based on just the rRNA data alone: 1) identified siphonophores as the first diverging clade in most trees; 2) supported Filifera I + Filifera II; 3) supported Aplanulata + Capitata s.s. + Filifera I-II; 4) supported Filifera III + Filifera IV, with the latter being paraphyletic

with respect to the former in many trees, and; 5) supported Leptothecata + Filifera III-IV (Table 3). No analyses recovered Capitata in its former sense [Aplanaluta + Capitata *s.s.*] nor the monophyly of Anthoathecata or Filifera. Our constraint analyses show that the placement of Capitata or Aplanulata as the earliest branching clades within Hydroidolina is rejected by both NT and allNT (NT plus rRNA) alignments, whereas both AA and rRNA data alone cannot reject these hypotheses (Table S5). The monophyly of Filifera was rejected in all cases other than for the rRNA dataset (Table S5). Lastly, the monophyly of Anthoathecata was rejected for both NT and allNT datasets while AA and rRNA alignments do not reject this traditional hypothesis (Table S5).

Discussion

The evolution of mtDNA in Hydrozoa

The gene arrangements of the newly sequenced hydrozoan mtDNAs are consistent with the three organizations recovered earlier (Kayal et al., 2012). For instance, the new trachyline mtDNAs exhibit the predicted organization of the ancestral mt-genome organization for Hydrozoa, with genes ordered into a small cluster (four genes, including the two extra protein genes *polB* and *orf314*) and a large cluster (thirteen genes) with opposite orientations (Kayal et al., 2012). We note, however, that our taxon sampling within Trachylina is still relatively limited, restricted to representatives of Limnomedusae plus the Trachymedusae *Liriope tetraphylla* and *Geryonia proboscidalis*, which have been shown to be more closely related to Limnomedusae than to other members of Trachymedusae (Collins et al., 2008). Thus, the possibility remains that other trachyline taxa (including Narcomedusae, Actinulida and other members of Trachymedusae) could exhibit an as yet unidentified mt-genome organization. New taxon sampling within Hydroidolina shows that hydroidolinan mtDNA organization is nearly identical to that so far observed in trachylines, except that they lack, and likely lost (Kayal et al., 2012), the two non-standard protein-coding genes *polB* and *orf314*. Gene organizations within Aplanulata are the most derived from the putative ancestral one for Hydroidolina, where all genes are in the same orientation but the second copy of *cox1* (which can be partial) oriented in the opposite direction to the rest of the genome (Fig. 1). Our new data are partially consistent with the proposed scenario for the evolution of the mitochondrial genome organization in Hydrozoa (Kayal et al., 2012). Specifically, the ancestral hydrozoan mtDNA contained the two extra protein-coding genes *orf314* and *polB*, which were subsequently lost in Hydroidolina before the divergence of various orders. Aplanulata displays not two, but three increasingly derived genome organizations generated by sequential gene rearrangement (Fig. 1): inversion of *rnl* and translocation of *trnW* in *Boreohydra simplex*/*Plotocnide borealis*; translocation of *trnM* in *Ectopleura larynx*, *Euphysa aurata* and *Hydra oligactis*; partitioning of the genome into two nearly equal-sized chromosomes in some species of *Hydra* (Kayal et al., 2012). We found inter-genic regions (IGRs) longer than 10 bp found after *cox2* in *Boreohydra simplex*/*Plotocnide borealis* and after *cox3* in *Ectopleura larynx*, *Euphysa aurata* and *Hydra* spp. These IGRs could conceivably be residues left from the translocations of *trnW* and *trnM*, respectively, but no obvious homology was found in our alignments (data not shown).

Expression of mtDNA genes in hydrozoans

In Metazoa, mitochondrial gene expression is thought to follow the "tRNA punctuation model", where mt genes are transcribed into polycistronic precursor transcripts (Ojala, Montoya & Attardi, 1981; Gissi & Pesole, 2003), followed by the excision of the tRNAs that release single-gene (monocistronic) mRNAs and rRNAs (Mercer et al., 2011). Unlike most animals, cnidarian mtDNAs encode either one (*trnM* in Cubozoa and Octocorallia) or two (*trnM* and *trnW* in the remaining taxa) tRNA genes. This begs the question of the mechanisms involved in the expression of mt genes for this group.

RNA-seq studies provide unique insights into the expression of genes, and we used data obtained through RNA-sequencing projects to better understand translational mechanisms of the linear mtDNAs in hydrozoans. Surveying several large RNA-seq datasets on NCBI's GenBank and one from an unpublished source (Table 1), we assembled and annotated the nearly complete mtDNA sequences for eight hydroidolinan Hydrozoa species, including three of the first four mtDNA genomes from representatives of Siphonophorae. For all non-aplanulatan hydroidoline hydrozoans species, we found no RNA-seq reads upstream of *cox2* and *rnl*, a large intergenic region (IGR) that marks the inversion of the transcriptional orientation of mitochondrial genes (Fig. 1). It was previously suggested that this IGR has the potential to fold into a stem-loop, serving as the putative mt control region (CR) in non-aplanatulan hydroidoline hydrozoans (Kayal et al., 2012); our results further support this hypothesis. In fact, two large RNA-seq runs from the filiferan *Hydractinia symbiolongicarpus* (SRA Archive num. SRR1174275 and SRR1174698) and one from *Podocoryna carnea* (SRA Archive num. SRR1796518) allowed assembling the complete mtDNA for these species excluding the CR, with an organization similar to that of other non-aplanulatan hydrozoans. This pattern suggests that the mtDNA is transcribed into two polycistronic precursor transcripts (mt pre-mRNA) with opposite orientations (Fig. 2B). Surprisingly, the CR of *Craspedacusta sowerbyi* (SRA Archive num. SRR923472) was mapped onto a few RNA-seq reads. We believe that this particular dataset contains some DNA sequences, perhaps resulting from contamination of the original cDNA libraries by mitochondrial DNA.

In siphonophores, as in other non-aplanulatan hydroidolinans, the *trnW* gene is situated between *cox2* and *atp8*, while the *trnM* gene falls between *cox3* and *nad2* (Fig. 1). While we expect the mt genome of the siphonophore *Physalia physalis* to be organized into a single chromosome similar to that of *Rhizophysa eysenhardti* as suggested by the small RNA-seq data, the partial mt genome obtained from the large RNA-seq data assembled into eight contigs (Fig. 2A). The smaller RNA-seq dataset produced six contigs, including polycistronic

325 *trnW*(3'end)-*atp8*-*atp6*-*cox3*-*trnM*-*nad2*(5'end), *nad2*(partial)-*nad5*(partial), *nad5*(3'end)-*rns*-*nad6*-*nad3*-*nad4L*-
326 *nad1*-*nad4*(partial) and *cob*(partial)-*cox1*, as well as monocystronic *cox2* and *rnl*. The failure to recover full-length
327 genes likely resulted from insufficient coverage of mt-RNAs in this dataset. For the larger RNA-seq dataset, we
328 found a different pattern of gaps, none within genes; reads span across protein gene boundaries for *atp8*-*atp6*-*cox3*,
329 *nad2*-*nad5*, and *nad6*-*nad3*-*nad4L*-*nad1*-*nad4* (Fig. 2A) with average coverage ranging from 8-223 reads per contig
330 (data not shown). The absence of any reads between these gene clusters, as well as between contigs *nad2*-*nad5* and
331 *rns* or *rns* and *nad6*-*nad3*-*nad4L*-*nad1*-*nad4* in the large RNA-seq data neither appears to be the result of insufficient
332 read depth nor is it easily explained by the highly transient nature of the polycistronic precursor (pre-mRNA)
333 transcript. The two sets of *Physalia* RNA data were produced using different approaches for capturing ESTs and
334 building the Illumina libraries, resulting in different maturation levels of the transcripts. We posit that the larger
335 RNA-seq dataset contains only mature mt-mRNAs while the smaller RNA-seq dataset has both pre- and mature
336 mRNAs. Accordingly, the pattern of mt-RNA expression is in part in accord with the tRNA punctuation model,
337 where the excision of the tRNAs would release monocistronic *cox2* and polycistronic *atp8*-*atp6*-*cox3* from the pre-
338 mRNA (Fig 2B, Step 2, black arrows). Yet, this model does not explain the bicistronic *nad2*-*nad5* nor monocistronic
339 *rns*, *cob* and *cox1*. It is possible that both the rRNAs and the tRNAs are excised, simultaneously or sequentially,
340 from the precursor transcript, releasing bicistronic *nad2*-*nad5* and monocistronic *rns* (Fig 2B, Step 2, red arrows).
341 However, an additional mechanism would need to be invoked to explain the excision of *cob* and *cox1* (as illustrated
342 by the absence of reads spanning across that gene boundary) from the polycistronic precursor transcripts. We
343 observed intergenic regions of 10 bp or longer with conserved motifs in these positions (Fig. 2, S9) with potential
344 secondary structures (Fig. S10) that could represent recognition sites for the enzyme involved in maturation of
345 mRNA (Fig. 2B, Step 2, blue arrows). This scenario is supported by the presence of IGRs before (and sometimes
346 after) mt-tRNAs. In fact, by forming short stem-loops, these IGRs might signal for the maturation of mt pre-mRNA
347 in Hydrozoa in a similar fashion as mt-tRNAs in other animals (Mercer et al., 2011).

348 *Mitochondrial view of hydrozoan character evolutionary history*

349 Using the coding regions of the mtDNA from thirty-seven hydrozoan species, including twenty-six newly
350 obtained for this study, we inferred the evolutionary history of Hydrozoa. To date, most studies of hydrozoan
351 phylogeny have relied on rRNA sequence data, providing some important insights, but no reliable inferences of

relationships among hydroidolinan taxa (Collins et al., 2006; Cartwright et al., 2008; Cartwright & Nawrocki, 2010). In our analyses, we similarly found mt-rRNA insufficient for deciphering relationships among hydroidolinan lower clades with high support (Fig. S3, S7; Table 3). The saturation test (Xia et al., 2003) suggests a high level of saturation in the rRNA alignment for 16 and 32 OTUs, while saturation levels are assumed acceptable for the other datasets (Table S2), which could explain the poor performance of rRNA. Similarly, rRNA alone did not allow discriminating among several competing hypotheses of hydroidolinan relationships while NT data did (Table S5).

Our phylogenetic analyses strongly support the monophyly of Trachylina and Hydroidolina, while rejecting Anthoathecata and Filifera as suggested by other molecular data (Cartwright et al., 2008; Cartwright & Nawrocki, 2010). Interestingly, our data support the hypothesis that Siphonophorae is the first diverging lineage within Hydroidolina (Fig. 3, Table 3) in contrast to a recent phylogenomic study that found Aplanulata to be the earliest branching clade within Hydroidolina while Siphonophorae was nested within Hydroidolina (Zapata et al., 2015). Previous studies have grouped, though with low support, Siphonophorae with either Aplanulata (Cartwright et al., 2008) or Leptothecata (Cartwright et al., 2008; Cartwright & Nawrocki, 2010), but both hypothetical positions are contradicted by our analyses (Table 3). Our competing hypothesis suggests that the unique holopelagic colonial organization of siphonophores could have been an early innovation within Hydrozoa. However, given that it is apomorphic, it could have evolved anywhere along the lineage leading from the origin of Hydroidolina to the last common ancestor of Siphonophorae.

Recent rRNA phylogenetic studies have broken Filifera into four clades (I-IV), with varying levels of support (Cartwright et al., 2008). As with our data, rRNA data revealed a clade, albeit with low support, uniting Filifera I (=family Eudendriidae), Filifera II, and Aplanulata. Similarly, our results are consistent with the rRNA-based results, again with low support, that Filifera III and Filifera IV form a clade. However, mitochondrial genome data suggest that Filifera III is embedded within Filifera IV. Studies on morphology and rRNA data have placed *Clava multicornis* within Hydractiniidae, making it a member of Filifera III (Schuchert, 2001; Cartwright et al., 2008), which is confirmed by our results. Interestingly, in our trees Filifera IV was found to include a poorly supported, but morphologically distinct, clade dubbed Gonoproxima, containing species that do not bear gonophores on the hydranth body instead budding on the hydrocauli, pedicels, or stolons (Cartwright et al., 2008; Cartwright & Nawrocki, 2010). Our taxon sampling is much more depauperate, but our analyses suggest that the positioning of the

379 gonophores may perhaps be evolutionarily too labile to be strictly used for classification, similar to the presence of
380 scattered tentacles (Schuchert, 2001).

381 The well-supported clade formed by [Aplanulata + [Filifera I + II] + Capitata] is an interesting result, but
382 our taxon sampling is too limited to make strong conclusions about whether the capitate tentacles of Aplanulata and
383 Capitata are shared derived characters (with a reversal in the lineage leading to Filifera I + Filifera II), or whether
384 they evolved independently. It is not surprising that the absence of capitate tentacles (the main uniting feature of
385 Filifera) is not revealed to be a synapomorphy.

386 Significantly more than half of the species within Hydroidolina are contained within Leptothecata, which
387 highlights the lack of taxon sampling in our analysis with just five species represented. Ribosomal analyses have
388 revealed *Melicertum octocostatum*, a species that actually lacks a theca in the hydroid stage, to be of the sister taxon
389 to the remainder of Leptothecata (Cartwright et al., 2008; Leclère et al., 2009; Cartwright & Nawrocki, 2010),
390 raising the possibility that the theca was derived within Leptothecata rather than emerging right at its base. Our
391 analyses also contain *Melicertum octocostatum* diverging early within Leptothecata, but not sister to all other
392 sampled leptothecates. Given the caveat that taxon sampling is limited, the absence of a theca in *Melicertum* is likely
393 a secondary loss. In fact, several other leptothecates show a reduced or diminutive theca into which the hydranth is
394 not able to retract.

395 **Conclusion**

396 In this study, we assembled and annotated twenty-three novel nearly-complete or complete mitochondrial
397 genomes from most orders of the class Hydrozoa, with an emphasis on the subclass Hydroidolina. Increased taxon
398 sampling revealed only one additional mitogenome organizations than those described previously for hydrozoans,
399 being consistent with the most recent overall picture of mitogenome evolution (Kayal et al., 2012). Using EST data,
400 we proposed that the mitochondrial pre-mRNA is polycistronic, with tRNAs and rRNAs likely excised
401 simultaneously during transcription following a modified tRNA punctuation model. Using both nucleotide and
402 amino acid alignments, we inferred the evolutionary history of taxa within Hydroidolina, one of the most difficult
403 questions in cnidarian phylogenetics. In contrast to previous analyses, our data yield resolved topologies and provide
404 a working hypothesis for deep hydroidolinan relationships. Specifically, mitogenome data suggest that

Siphonophorae is the earliest diverging group within Hydroidolina; a clade is formed by Leptothecata + Filifera III/IV, where Filifera IV/Gonoproxima is paraphyletic; and Aplanulata/Capitata/Filifera I + II form a clade. We conclude that mitochondrial protein coding sequence data is a pertinent marker for resolving the phylogeny of Hydrozoa. Future investigations of hydrozoans could take advantage of the highly conserved mitogenome organization and the ever-decreasing price of sequencing to obtain the complete mtDNA for massive numbers of hydrozoan samples. We are looking forward to additional studies using alternate data (nuclear genes and genomes) to test our findings.

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559 **Supplementary materials**

560 **Suppl. 1: Library preparation for multiplexed second-generation sequencing**

561 We constructed low-cost multiplexed libraries for the Illumina platform. The Illumina library preparation
562 protocol was derived from two sources (Meyer & Kircher, 2010; Rohland & Reich, 2012), is similar to another
563 recently published (Dunham & Friesen, 2013). While our libraries were designed for 100 bp single-end single-index
564 libraries for Illumina, they can easily be adapted to longer inserts as well as paired-end and double-indexing, by
565 modifying the sonication parameters for DNA shearing and the indexed adapters (see below).

566 *1) DNA shearing and sizing:*

567 Long PCR amplicons for each specimen were purified using Millipore column and combined at equimolar
568 ratios when necessary, i.e. when the mtDNA was amplified in more than one piece. Although this step is not strictly
569 necessary, it allows for the removal of primer dimers, which can otherwise take up a significant portion of the
570 libraries. Purified long-PCR amplicons were sheared to an average size of 600 bp for Illumina libraries and 200 bp
571 for Ion Torrent libraries with a QSonica Q800R Sonicator (2 min or 3 min at 30% amplitude with 10 sec on/off for
572 Illumina and Ion Torrent, respectively). The size window of sheared amplicons was verified on an Agarose gel.
573 Sheared DNA was recovered with a homemade Serapure beads suspension (Rohland & Reich, 2012) and quantified
574 using a BioTek Epoch Microplate Spectrophotometer.

575 We have found that sizing by a combination of sonication-Agarose gel-spectrophotometer yields satisfying
576 results for library preparation on most platforms and is even preferable to more sophisticated and expensive sizing
577 methods such as enzymatic shearing and use of the BioAnalyzer. They are also far less demanding in materials and
578 therefore more environmentally friendly.

579 *2) Illumina library preparation:*

580 The library preparation consisted of the combination of several previously published massively parallel
581 sequencing protocols (Meyer & Kircher, 2010; Rohland & Reich, 2012) into a double tagging approach: an internal
582 tag attached to the 3'end of the universal adapters (we called barcode) and an external tag as part of the indexed
583 adapter (we called index). This highly increased the number of samples to price ratio, but see below.

584 Each adapter was synthesized in two pieces (Table S4), with a 20 bp overlap between each half, and had an
585 over-hanging T on the 3'end of the 3'half to increase the ligation efficiency (sticky-end ligation). We prepared an
586 adapter mix containing a combination of the 3'half of a barcoded universal adapter and the 3'half of the index
587 adapter. Sheared DNAs were blunt-end repaired using the NEB Quick Blunting Kit and A-tailed using Klenow
588 Fragment (3'→5' exo–). Adapter mixes (UniAdp_barco1 & 2/UniComp_barco1 & 2 and
589 IndAdp_short/IndCompAdp, Table S4) were ligated to individual samples using T4 Ligase, followed by adapter fill-
590 in with *Bst* polymerase large fragments (barcoding). Adapters were completed by short PCR using a High-Fidelity
591 DNA Polymerase and primers (UniAdp_long and IndAdp_long1 to 20) corresponding to the 5'half of the adapters
592 with one indexed adapter per two samples (indexing). We quantified each library with Thermo Scientific SYBR
593 Green/ROX qPCR Master Mix and pooled all samples in equimolar ratio.

594 *3) Quadruple tagging protocol for Illumina paired-end sequencing:*

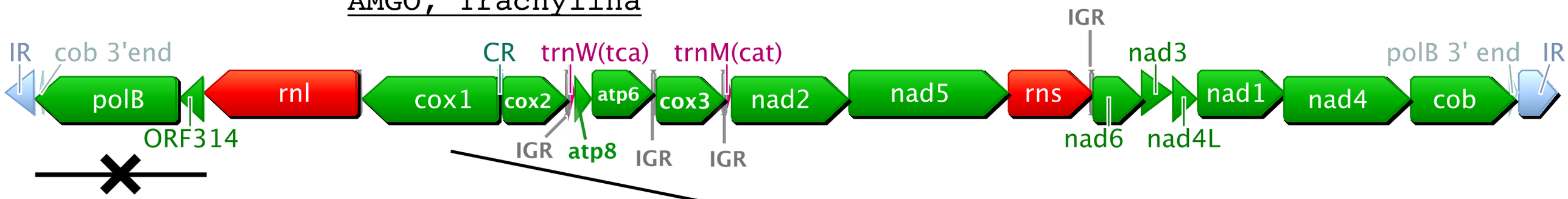
595 Illumina has recently introduced a double indexing library method, where both adapters (i5 and i7) have
596 indexes. As mentioned above, for paired-end sequencing it is possible to exponentially increase the multiplexing
597 level by introducing a tag at the 3'end of both Illumina adapters. The library preparation would be similar to the one
598 described above, with the adapter mix consisting of the combination of the 3' half of an i5 adapter and the 3' half of
599 an i7 adapter. This will provide each sample with a combination of two indexes and two barcodes. In that way, it is
600 possible to tag 600 samples with five indexes on i5 and four indexes on i7, six barcodes for i5 adapters and five
601 barcodes for i7 adapters. Such an approach reduces the cost of multiplexing by reducing the number of tags, the
602 main driver of high costs for multiplexed libraries when using standard kits. This approach will be most cost-
603 effective for barcoding purposes, where the tags can be included at the 5'end of PCR primers, hence removing the
604 substantial cost and potential errors associated with ligation.

Figure 1(on next page)

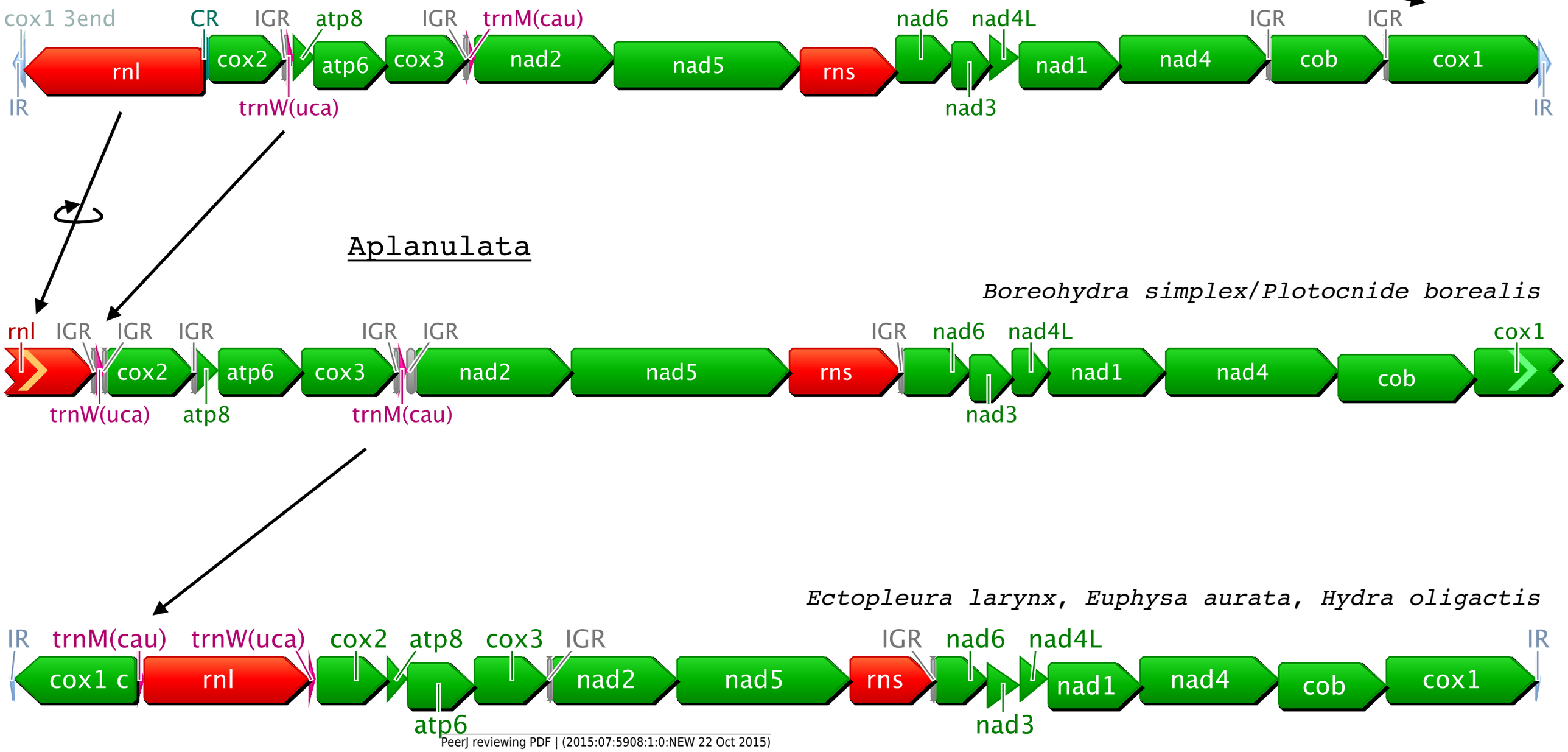
Predicted evolution of the mitochondrial genome organization in Hydrozoa (Cnidaria).

Genes are color-coded as follows: green for proteins; red for rRNAs; purple for tRNAs; light-grey for repeated regions. CR: Control Region corresponding to the inversion of transcription orientation; IR: inverted Repeat; IGR: Inter-Genic Region. AMGO corresponds to the Ancestral Mitochondrial Genome Organization as predicted in Kayal *et al.* 2013; cox1 c is a duplicated *cox1* on the other end of the mtDNA; incomplete 5'end and 3'end are represented by chevrons on the left and right side of genes, respectively.

AMGO, Trachylina



Non-aplanulatan Hydroidolina



Aplanulata

Boreohydra simplex/Plotocnide borealis

Ectopleura larynx, Euphysa aurata, Hydra oligactis

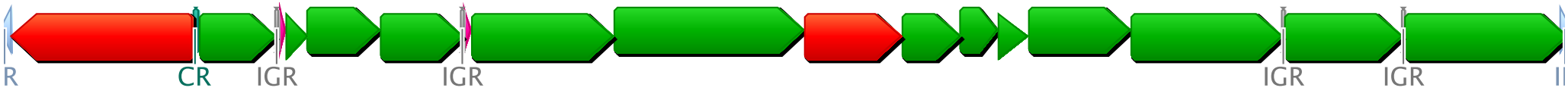
Figure 2(on next page)

Mitochondrial gene expression in non-aplanulatan Hydroidolina

A. mtDNA organization in the siphonophore Physalia physalis assembled from a large EST dataset. Grey lines correspond to the contigs assembled. Missing features (tRNAs, IGRs, CR) are shown with dotted lines. B. Predicted model of mt-mRNA expression based on findings from *P. physalis*. Color-codes are the same as Figure 1. Grey horizontal arrows are the two pre-mRNA transcripts, the larger being polycistronic. Dark vertical arrows correspond to regions of pre-mRNA excision from the “tRNA punctuation model”; red and blue arrows are the additional excision sites predicted from our model for hydrozoan mt-mRNA expression. We predict that stage 1 and 2 are simultaneous.

Physalia physalis

Model of mRNA expression in non-aplanulatan Hydroidolina



① Transcription of polycistronic pre-mRNA

② mt-mRNA maturation

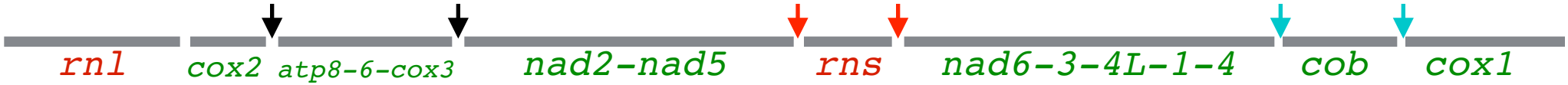


Figure 3(on next page)

Phylogenetic analysis of the allNT alignment under the Bayesian framework using MrBayes with the GTR+Γ model of sequence evolution.

Support values correspond to posterior probabilities.

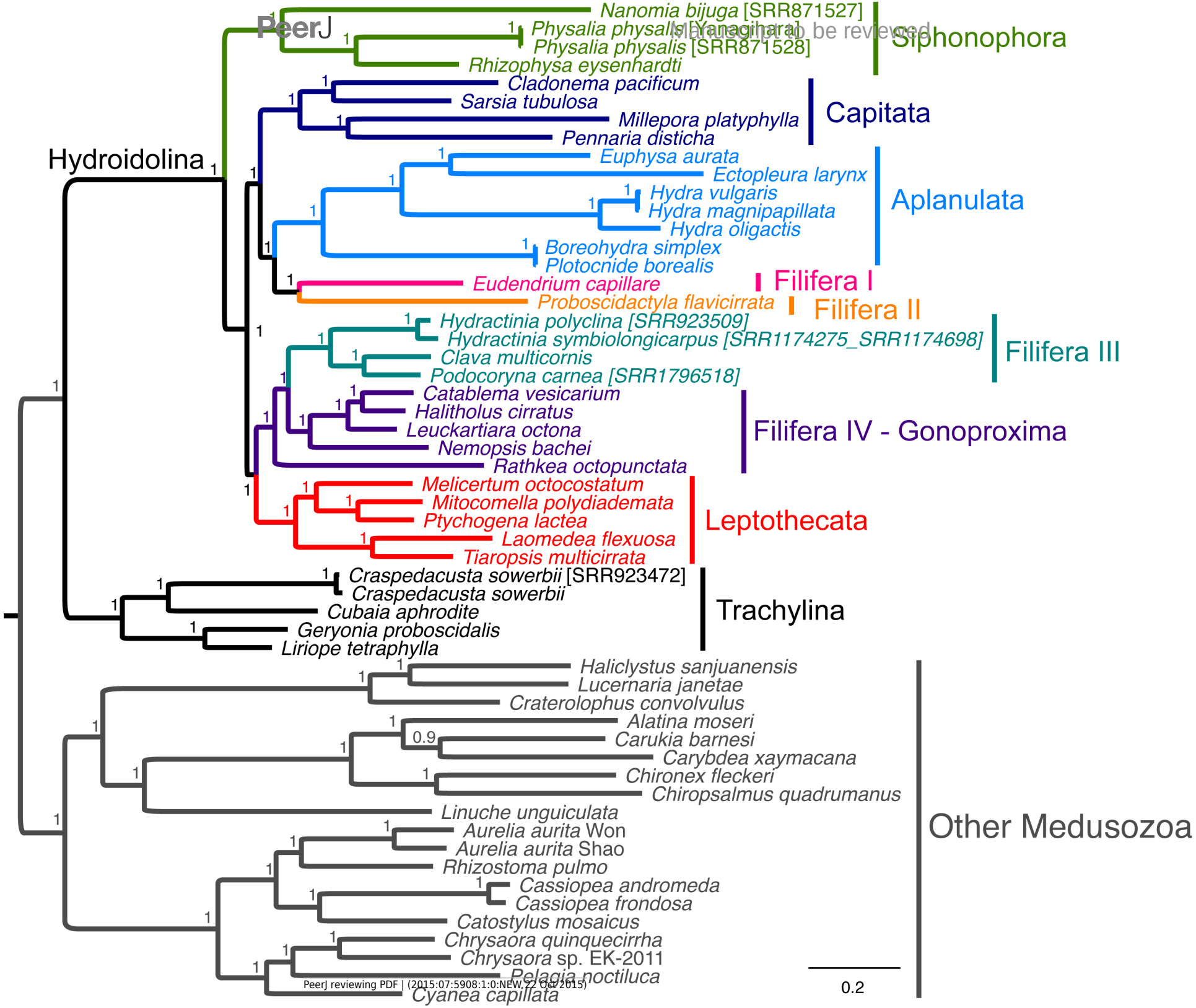


Figure 4(on next page)

2-dimensional plots of the first two principal components from the principal component analysis of the composition of the AA (A), rRNA (B), NT (C) and allNT (D) alignments.

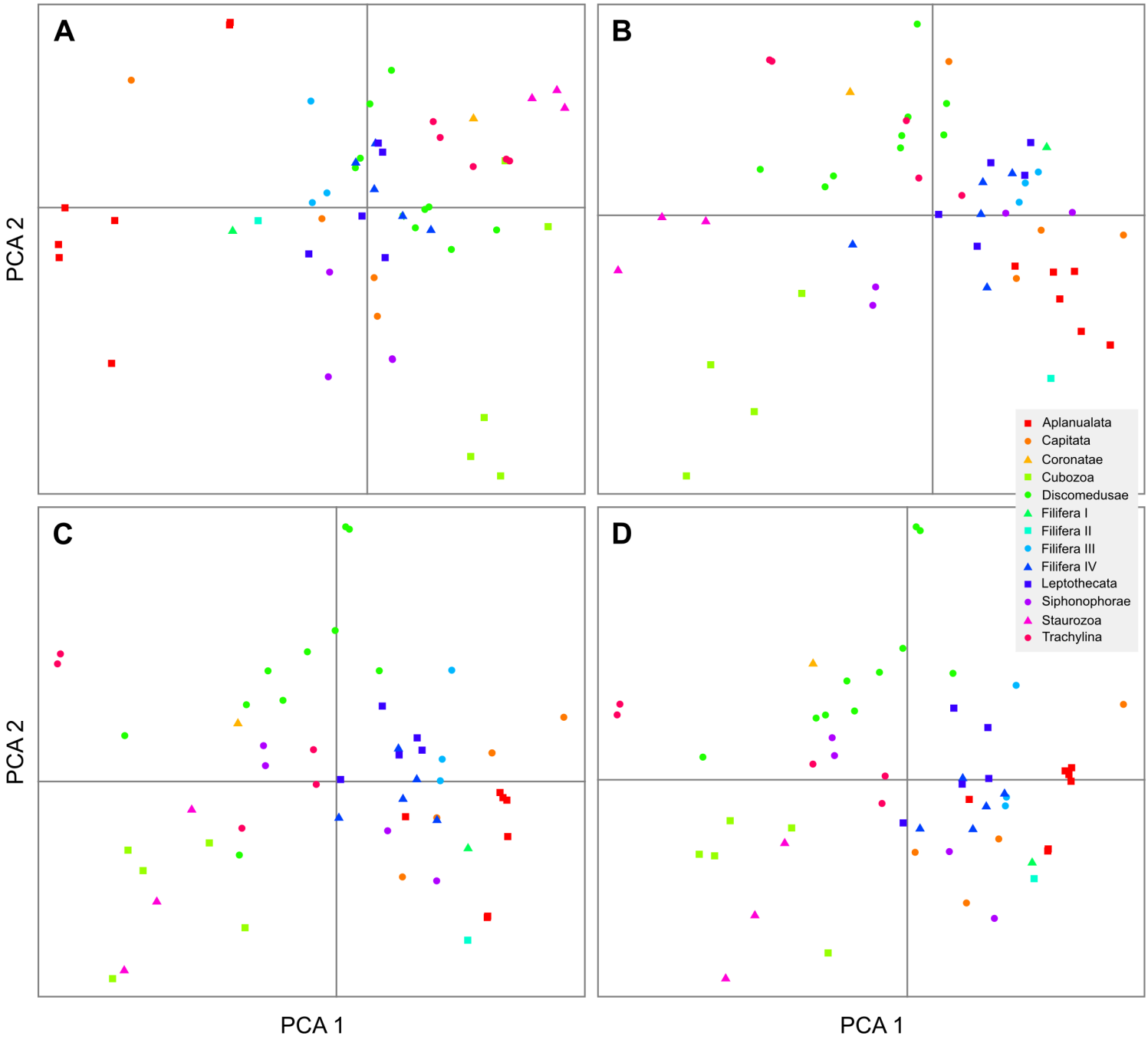

A
B
C
D
PCA 2
PCA 2
PCA 1
PCA 1
Aplanualata
Capitata
Coronatae
Cubozoa
Discomedusae
Filifera I
Filifera II
Filifera III
Filifera IV
Leptothecata
Siphonophorae
Staurozoa
Trachylina

Table 1(on next page)

List of samples used in this study.

Colore-filled taxa were obtained for this study. DNA-seq data is shaded in green; RNA-seq data is shaded in magenta long-range PCR amplified samples are shaded in cyan; * not included in phylogenetic analyses; SRR codes are GenBank Archive numbers of the runs used in this study.

Clade		Species	Voucher #	GenBank #	Note
Aplanulata		*Boreohydra simplex*	Borehydra20100904.3	KT809334	
		Ectopleura larynx		JN700938	
		*Ectopleura larynx**		*processing*	SRR923510
		Euphysa aurata	GR10-145.2	KT809330	
		Hydra oligactis		NC_010214	
		Hydra magnipapillata		NC_011220,NC_011221	
		Hydra vulgaris		HM369413,HM369414	
		Plotocnide borealis	RU087.1	KT809334	
Capitata		*Cladonema pacificum*		KT809323	Unpublished raw reads
		Millepora platyphylla		JN700943	old *Millepora* EK-2011
		Pennaria disticha		JN700950	
		Sarsia tubulosa	RU053	KT809333	
Filifera	IV	*Catablema vesicarium*	RU006	KT809324	
	III	*Clava multicornis*		NC_016465	
	I	*Eudendrium capillare*	PS101	KT809336	
	IV	*Halitholus cirratus*	GR10-115	KT809337	
	III	*Hydractinia polyclina*		*processing*	SRR923509
	III	*Hydractinia symbiolongicarpus*		*processing*	SRR1174275 & SRR1174698
	IV	*Leuckartiara octona*	PS487	KT809325	
	IV	*Nemopsis bachei*		JN700947	
	III	*Podocoryna carnea*		*processing*	SRR1796518
	II	*Proboscidactyla flavicirrata*	PS139	KT809319,KT809329	
	IV	*Rathkea octopunctata*	RU008	HT809320	
Leptothecata		*Laomedea flexuosa*		NC_016463	
		Melicertum octocostatum	RU082	KT809321	
		Mitrocomella polydiademata	RU060	KT809332	
		Ptychogena lactea	GR10-152.1	KT809322	
		Tiaropsis multicirrata	GR10-053.1	KT809326	
Siphonophorae		*Nanomia bijuga*		*processing*	SRR871527
		Physalia physalis		*processing*	SRR871528
		Physalia physalis	Angel	KT809328	Unpublished raw reads
		Rhizophysa eysenhardti	DLSI230	KT809335	
Trachylina		*Craspedacusta sowerbyi*		NC_018537	
		Craspedacusta sowerbyi		*processing*	SRR923472
		Cubaia aphrodite		NC_016467	
		Geryonia proboscidalis	BCS32a	KT809331	
		Liriope tetraphylla		KT809327	Unpublished raw reads
Discomedusae		*Aurelia aurita*		NC_008446	Shao
		Aurelia aurita		HQ694729	Won
		Cassiopea andromeda		JN700934	
		Cassiopea frondosa		NC_016466	
		Catostylus mosaicus		JN700940	
		Chrysaora quinquecirrha		HQ694730	
		Chrysaora sp. EK-2011		JN700941	
		Cyanea capillata		JN700937	
		Rhizostoma pulmo		JN700987,JN700988	
		Pelagia noctiluca		JN700949	
Coronatae		*Linuche unguiculata*		JN700939	
Staurozoa		*Craterolophus convolvulus*		JN700975,JN700976	
		Haliclystus sanjuanensis		JN700944	
		Lucernaria janetae		JN700946	
Cubozoa		*Alatina moseri*		JN642330-JN642344	
		Carukia barnesi		JN700959-JN700962	
		Carybdea xaymacana		JN700977-JN700983	
		Chironex fleckeri		JN700963-JN700968	
		Chiropsalmus quadrumanus		JN700969-JN700974	

1

Table 2(on next page)

Size, GC content and start and end codons for the genes of the newly obtained mtDNA.

B. s.: Boreohydra simplex; C. v.: Catablema vesicarium; C. p.: Cladonema pacificum; C. s. RNA:Craspedacusta sowerbyi; E.l. RNA: Ectopleura larynx; E. c.: Eudendrium capillare; E. a.: Euphysa aurata; G. p.: Geryonia proboscidalis; H. c.: Halitholus cirratus; H. p.: Hydractinia polyclina; H. s.: Hydractinia symbiolongicarpus; L. o.: Leuckartiara octona; L. t.: Liriope tetraphylla; M. o.: Melicertum octocostatum; M. p.: Mitrocomella polydiademata; N. b.: Nanomia bijuga; P. p. Y: Physalia physalis Y; P. p. SR: Physalia physalis SR; P. b.: Plotocnide borealis; P. c. ;Podocoryna carnea; P. f.: Proboscidactyla flavicirrata; P. l.: Ptychogena lactea; R. o.: Rathkea octopunctata; R. e.: Rhizophysa eysenhardti; S. t.: Sarsia tubulosa; T. m.: Tiaropsis multicirrata.

Gene		*B.s.*	*C.v.*	*C.p.*	*C.s.* RNA	*E.l.* RNA	*E.c.*	*E.a.*	*G.p.*	*H.c.*	*H.p.*	*H.s.*	*L.o.*	*L.t.*	*M.o.*	*M.p.*	*N.b.*	*P.p.* Y	*P.p.* SR	*P.b.*	*P.c.*	*P.f.*	*P.l.*	*R.o.*	*R.e.*	*S.t.*	*T.m.*
	size (nt)	705	705	705	705	705	705	705	705	705	705	705	705	705	705	705	693	705	705	705	705	?	705	705	705	705	705
atp6	GC	22.4	26.4	24.8	45.1	28.3	22.6	22	30.1	26.2	25.2	26.4	24.4	29.8	27.1	29.4	25.7	32.3	32.9	22.3	25.7	?	25.5	28.8	28.5	25.8	27.4
	Start/End	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	?	A/A	A/A	A/A	A/A	A/A
	size (nt)	198	204	207	207	207	204	213	207	204	204	204	204	207	204	204	?	>126	204	198	204	?	204	204	201	204	198
atp8	GC	13.1	22.1	16.4	39.1	21.4	15.7	14.1	29.5	18.1	17.5	17.6	19.6	30	22.1	20.6	?	20.6	24	13.1	21.6	?	22.1	24.5	18.9	20.6	21.7
	Start/End	T/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	?	?/A	A/A	T/A	A/A	?	A/A	A/A	A/A	A/A	G/A
	size (nt)	1149	1140	1140	1185	>1024	1143	1140	>787	1140	1143	1143	1140	1203	1143	1146	1164	>1016	>1133	1149	1143	>848	1146	1146	1143	1143	1146
cob	GC	25.5	27.5	27.8	46	29.7	25.9	26.3	36.5	27.3	27.5	27.8	27.6	32.3	29.3	31.8	29.2	36.5	35	25.4	29.1	25.9	27.4	33	28.4	28.6	30.6
	Start/End	A/A	A/A	A/A	A/G	A/?	A/A	A/A	A/?	A/A	A/A	A/A	A/G	A/G	A/G	A/A	A/A	?/?	?/A	A/A	A/A	A/?	A/A	A/A	A/A	A/A	G/A
	size (nt)	>712	>708	1566	1566	>1569	>884	>713	>1322	>910	1566	1566	>919	1566	>713	>699	1572	>1548	1566	>715	1566	>822	>713	>710	>711	>713	>713
cox1	GC	33	35.3	33.1	46.2	34.7	32.5	32	38	35.1	32.2	32.8	34.1	34.5	35.2	38.2	32.6	38.4	38.1	33	34.4	32.2	34.5	37.5	33.8	33.5	34.2
	Start/End	A/?	A/?	A/G	A/G	?/?	A/?	G/?	A/?	A/?	A/G	A/G	A/?	A/G	A/?	A/?	A/A	?/A	A/A	A/?	A/G	?/?	A/?	A/?	A/?	A/?	A/?
	size (nt)	726	738	738	738	>939	738	768	741	738	738	738	738	741	738	738	>720	>720	735	726	738	?	738	738	735	738	735
cox2	GC	25.8	29.5	28.6	44.3	27.6	28.6	22.3	37.2	29.7	28.1	28.2	29	32.1	30.8	34.1	31.4	39.2	38.5	25.8	29.7	?	28.9	32	32.4	29.3	29.3
	Start/End	A/A	A/A	A/A	A/A	?/A	A/A	A/A	A/G	A/A	A/A	A/A	A/A	A/G	A/A	A/A	?/G	?/?	A/A	A/A	A/A	?	A/A	A/A	A/A	A/A	A/A
	size (nt)	786	786	786	786	>778	786	786	786	786	786	786	786	786	786	786	>798	786	786	786	786	?	786	786	789	786	786
cox3	GC	26.5	29.8	30.4	45.2	30.6	28.4	24	39.6	31.7	28.5	29.1	29.5	34.6	31.6	36.5	30.3	37.8	37.5	26.3	30	?	31.7	32.8	32.6	32.1	33.3
	Start/End	A/A	A/A	A/A	A/A	A/?	A/A	A/A	A/A	A/A	A/A	A/A	A/G	A/A	A/A	A/A	A/?	A/A	A/A	A/A	A/A	?	A/A	A/A	A/A	A/A	A/G
	size (nt)	978	990	987	999	981	990	990	999	990	>972	990	990	999	987	987	990	990	990	978	990	987	990	987	987	990	990
nad1	GC	24.7	28	26.6	46.9	28.7	27.2	23.6	37	30.5	25.2	26.7	27	33.4	28.5	31.9	26.2	36.1	35.8	24.7	27.8	24	27	30.1	30.4	27.5	28.5
	Start/End	A/A	A/A	A/A	A/A	A/G	A/A	A/A	A/A	A/A	A/?	A/G	A/A	A/A	A/A	A/A	A/A	G/A	G/A	A/A	A/A	A/A	A/A	A/A	A/A	A/G	A/A
	size (nt)	1311	1362	1314	1350	>893	1353	1311	1353	1362	>1038	1362	1362	1353	1350	1356	?	>1176	1338	1311	1356	?	1356	1344	1353	1356	1347
nad2	GC	17.7	22.5	19.9	44	25.1	17.4	15.7	32.8	22.2	21	19.8	20.7	28.5	24.4	27	?	32.2	31.3	17.8	21.2	?	21.1	26.6	23.4	23.5	23.2
	Start/End	A/A	G/A	A/A	A/A	?/?	A/A	A/A	A/A	G/A	?/?	A/A	A/A	A/A	A/A	A/A	?	?/A	A/A	A/A	A/A	?	A/A	A/A	A/A	A/A	A/A
	size (nt)	360	357	357	357	357	357	357	357	357	357	357	357	357	357	357	357	357	357	360	357	357	357	357	357	357	357
nad3	GC	23.3	24.6	22.1	42.3	24.3	22.1	21.8	34.2	25.5	22.4	24.1	22.1	30.8	26.6	29.1	23.5	30	30.3	23.6	25.8	23.5	24.1	31.7	26.9	28	25.8
	Start/End	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	G/A	G/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A
	size (nt)	1455	1458	1458	1461	1458	1449	1458	1461	1458	>1251	1458	1458	1461	1455	1455	>974	>1111	>1315	1455	1458	1446	1455	1458	1461	1458	1455
nad4	GC	22.6	25	24.3	44.6	26.3	21.5	20.2	35.8	24.6	24.7	24.1	23.6	32.2	26.7	29.9	27.3	33.1	33.1	22.7	25.7	23	25.5	29.4	26.8	26.1	27.2
	Start/End	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/G	A/A	?/A	A/A	A/A	A/G	A/A	A/G	?/?	A/?	A/?	A/A	A/A	A/A	G/A	A/A	A/G	A/A	A/A
	size (nt)	297	300	294	300	300	300	300	300	300	300	300	300	300	300	300	>279	297	297	297	300	297	300	297	300	297	300
nad4L	GC	20.5	20.7	21.1	40	21.7	20.7	19.7	29.3	21.3	16.9	19	20.3	26.3	24	24.3	26.9	26.9	27.3	20.5	20.3	19.9	22	25.3	22.7	23.9	26.3
	Start/End	A/A	A/A	G/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	G/G	A/G	A/G	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A
	size (nt)	1827	1833	1830	1833	>1730	1830	1853	1833	1833	>1672	1833	1833	1833	1833	1833	>1813	>1719	1830	1830	1833	>1137	1833	1833	1830	1833	1833
nad5	GC	21.1	25.3	25.1	46.3	28.1	22.8	20.5	36.1	26.6	24.7	23.5	24.4	32.3	26	29.6	24.8	34.1	34.1	21	25.6	22.1	25	27.9	27.1	26.7	27.1
	Start/End	T/A	A/G	A/G	A/A	?/?	A/G	A/A	A/A	A/G	?/?	A/G	A/G	A/A	A/G	A/G	?/?	A/?	A/A	T/A	A/G	A/A	A/G	A/G	A/A	A/G	A/G
	size (nt)	567	561	549	564	558	552	555	564	561	564	564	561	564	564	564	552	>555	561	567	564	564	564	558	561	552	561
nad6	GC	19.2	23	21.3	44	22.4	18.8	17.5	30.3	24.1	20.2	20.6	20.9	28	25.4	25.4	20.1	32.4	33	19.2	23.8	19.1	21.6	28.5	26.6	23.7	25
	Start/End	A/A	G/G	A/G	A/G	A/A	A/G	A/A	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	?/G	?/G	A/G	A/A	A/G	A/A	A/G	A/A	A/G	A/G	A/A
rnl	size (nt)	>743	>1592	1720	>1784	>1784	>1303	>775	>600	>1590	1733	1737	>892	1768	>1591	>1581	>1759	1761	1756	>742	1704	>505	>1588	>1586	>1613	>1581	>1583
	GC	24.9	23.9	23.3	36.5	26.4	20.3	23.7	39.1	24.5	23.9	24.8	19.4	27.1	24.5	27.2	22	32.3	32.5	24.9	24.1	26.9	24.4	31.7	23.5	24.4	26.3
rns	size (nt)	930	930	912	995	>865	925	896	969	925	>910	931	928	968	921	918	>845	>873	922	930	931	906	920	902	926	915	920
	GC	21	27.8	23.6	37.6	24.9	25.3	22.5	31.3	28.1	25.6	25.6	27.6	29.1	25.7	26.6	23.2	32	31.5	21	24.9	25.1	26.5	32.9	26.6	25	29.7
trnM	size (nt)	74	71	71	71	?	71	?	71	71	?	71	71	71	71	69	?	?	69	74	67	?	69	69	69	71	69
	GC	24.3	23.9	33.8	36.6	?	26.8	?	28.2	23.9	?	29.6	23.9	26.8	26.8	30.4	?	?	37.7	24.3	28.4	?	27.5	34.8	27.5	31	30.4
trnW	size (nt)	70	70	70	71	?	70	70	71	70	?	70	70	71	70	70	?	?	?	70	70	?	70	70	70	70	70
	GC	25.7	35.7	34.3	47.9	?	32.9	30	40.8	35.7	?	34.3	41.4	39.4	35.7	32.9	?	?	?	25.7	34.4	?	28.6	44.3	28.6	34.3	35.7
	size (nt)	NA	NA	NA	354	NA	NA	NA	?	NA	NA	NA	NA	297	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
orf314	GC	NA	NA	NA	37.9	NA	NA	NA	?	NA	NA	NA	NA	22.2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Start/End	NA	NA	NA	A/A	NA	NA	NA	?	NA	NA	NA	NA	A/A	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	size (nt)	NA	NA	NA	1668	NA	NA	NA	?	NA	NA	NA	NA	1644	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
polB	GC	NA	NA	NA	41.7	NA	NA	NA	?	NA	NA	NA	NA	30.4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Start/End	NA	NA	NA	A/A	NA	NA	NA	?	NA	NA	NA	NA	A/A	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

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Table 3(on next page)

Posterior probabilities and bootstrap values for different clades within Hydrozoa.

	MB				ML				
	aa(GTR)	NT(GTR)	rRNA(GTR)	allNT(GTR)	AA(GTR)	AA(LG)	NT(GTR)	rRNA(GTR)	allNT(GTR)
Aplanulata	1	1	1	1	100	100	100	88	100
Capitata	0.95	1	1	1	NA	NA	63	94	99
Filifera	NA	NA	NA	NA	NA	NA	NA	NA	NA
Leptothecata	1	1	1	1	100	100	100	85	100
Anthoathecata	NA	NA	NA	NA	NA	NA	NA	NA	NA
Filifera I + II	0.91	1	NA	1	41	42	75	NA	80
Apla + Capit	NA	NA	NA	NA	NA	NA	NA	NA	NA
Apla + Fili I-II	0.61	1	NA	1	NA	NA	36	NA	66
Apla + Capit + Fili I-II	NA	1	NA	1	NA	NA	80	NA	81
Lepto + Fili III-IV	1	1	NA	1	91	91	91	NA	91
Sipho + Apla	NA	NA	NA	NA	NA	NA	NA	NA	NA
Sipho + Lepto	NA	NA	NA	NA	NA	NA	NA	NA	NA
Antho + Lepto	0.68	1	NA	1	NA	NA	78	NA	80

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