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Expression and Localization of Carbonic Anhydrase Genes in the Serpulid Polychaete *Hydroides elegans*

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Abstract. The metalloenzyme, carbonic anhydrase (CA), catalyzes the reversible hydration of carbon dioxide into bicarbonate, and is responsible for biomineralization processes in animals. In the Annelida, the marine worms in the family Serpulidae are typified by the construction of calcium carbonate tubes. *Hydroides elegans*, a common member of warmwater biofouling communities around the world, provides an outstanding model for studies of calcification. To better understand the molecular process of biomineralization in *H. elegans*, we searched transcriptomes for CA genes at several life-history stages. Twelve CA genes were recovered in the transcriptomes. Whole mount *in situ* hybridization was performed for two of those genes on larvae and calcifying juveniles. A cytosolic CA isoform, *HeCA1*, and a secreted CA isoform, *HeCA2*, were expressed within the collar segment corresponding to the location of glands involved in formation of the calcified tube. Expression of these genes within collar segment tissues supports the role of CAs in generating bicarbonate for biomineralization processes. A phylogenetic tree of the α-CA gene family was constructed to increase understanding of CA-gene evolution within the family and its relationship to CA genes among the Metazoa.

Introduction

Biomineralization is an evolutionary adaptation by which organisms generate inorganic products in the form of ordered minerals for housing, structure, or defense. The process is important in all forms of life ranging from single-celled bacteria that produce simple calcified structures to multicellular organisms that construct complex crystalline structures like bones and shells. For marine calcifiers such as corals, bivalves, and sea urchins, both the physical and molecular processes of calcification are generally well understood. However, the Annelida, a phylum that is less well known for calcified structures, remain under-studied, especially regarding the molecular-genetic basis of biomineralization.

Numbering over 15,000 described species, annelids are diverse and ecologically important members of marine and terrestrial communities worldwide (Struck *et al*., 2011). The serpulids are a family of polychaete worms in which all species produce calcareous tubes that adhere tightly to submerged surfaces. Biomineralization in the serpulids occurs at the first thoracic segment, where two tubular glands are located along lateral sides on the ventral shield of the peristomium (Fig. 1) (Swan, 1950). The glands have been called "calcium-secreting glands" in published literature because they produce calcium carbonate ($CaCO_3$) (Hedley, 1956). Surrounding the calcium-secreting glands and lining the ventral shield are epithelial, mucus-secreting cells. While the calcium-secreting glands supply the calcareous components of the tube, as observed by Swan (1950), Hedley (1956) hypothesized that the mucus-secreting cells produce the organic matrix molecules that provide the framework in which minerals are embedded. As the products are secreted, the animal uses its collar, which folds posteriorly from the anterior edge of the first thoracic segment, to mold the components onto the anterior end of the tube until they solidify.

Although it has been shown that the metalloenzyme carbonic anhydrase (CA) is involved in molluscan and cnidarian biomineralization processes, no study has examined the expression of biomineralization genes in serpulids (Miyamoto *et al*., 1996; Moya *et al*., 2008). Carbonic anhydrase is essential for calcifying organisms because it catalyzes the

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Abbreviations: CA, carbonic anhydrase; GPI, glycophosphatidylinositol; *HeCA1*, cytosolic CA isoform of *Hydroides elegans*; *HeCA2*, secreted CA isoform of *Hydroides elegans*.

Figure 1. Adult *Hydroides elegans* removed from its calcified tube. Two calcium-secreting glands, located in the first thoracic collar segment, produce the white calcium carbonate products (arrows) that form the worm tube. Scale bar = 3 mm.

reversible hydration of carbon dioxide into bicarbonate and protons, and provides the bicarbonate necessary for calcium precipitation. Currently, there are five recognized families of CAs spanning an assemblage of taxonomic groupings: α-CA, β-CA, γ-CA, δ-CA, and ζ-CA; metazoan genes are closely associated with the α-CA family (Chegwidden *et al*., 2000). The ancestral function of α-CAs involves physiological processes including respiration, acid-base balance, and electrolyte transport; however, numerous duplication events have occurred in the α-CA family, likely a result of the enzyme being co-opted for biomineralization processes (Maren, 1967; Jackson *et al*., 2007).

The tube-building serpulid polychaete *Hydroides elegans* (Haswell, 1883) serves as a useful model to study the molecular underpinnings of annelid biomineralization. During development, *H. elegans* undergoes rapid change as it transitions from a planktonic larva to a sedentary juvenile in response to bacterial biofilms (Unabia and Hadfield, 1999). The beginning of metamorphosis marks a transitional period when larvae secrete a proteinaceous primary tube that adheres to the substratum, followed shortly thereafter by the formation of a calcified secondary tube embedded within an organic matrix (Carpizo-Ituarte and Hadfield, 1998). Even though the small worm is less than 200 microns long at this stage, secondary tube accretion rates can exceed 1.5 mm d^{-1}, and aggregations of *H. elegans* can quickly overwhelm the hulls of naval vessels in as little as two months in tropical harbor embayments like Pearl Harbor, Hawaii (Nedved and Hadfield, 2009).

Studying the presence and localization of CA genes during juvenile calcification stages will provide important insight into biomineralization processes in the serpulids. Their transparent tissues, ease in culturing, and ability to calcify make juvenile *H. elegans* ideal for studying annelid calcification processes through molecular techniques such as *in situ* hybridization. The goals of the research described here were twofold: 1) to determine the gene sequences for CA from transcriptomes developed for *H. elegans*, and 2) to identify where expression of CA genes occurs within tissues of competent larvae and calcifying juveniles of *H. elegans*.

Materials and Methods

Animal collection and larval-juvenile culture

Vexar screens (Conwed, Minneapolis, MN) bearing adult specimens of *Hydroides elegans* were collected from Pearl Harbor, HI, and maintained in outdoor aquaria with running seawater at the Kewalo Marine Laboratory, Honolulu, HI. Adults were removed from their tubes to induce spawning. Embryos and developing larvae were reared at a density of 5–10 larvae ml^{-1} in 0.22-μm-filtered seawater (FSW). Daily water changes and feeding with the unicellular alga *Isochrysis galbana* Tahitian strain (6.0×10^4 cells ml^{-1}) were performed until larvae reached competency 5 days post-fertilization (Nedved and Hadfield, 2009). Competent larvae were induced to settle on glass slides that had been submerged in flowing seawater for 3 weeks to allow time to develop a suitably inductive biofilm. Post-metamorphic juveniles were allowed to form calcified tubes for periods of 6 h or 10 days, with daily FSW changes and feeding (3.0×10^5 cells ml^{-1} for animals <3-days-old; 1.0×10^6 cells ml^{-1} for animals >3-days-old). Individuals were removed from their tubes by inserting an eyelash brush at the anterior aperture and gently pushing the animal out through the posterior end. The eyelash brush was made with a single human eyelash attached to the end of a 13-cm (3-mm diameter) wooden stick, using fingernail polish as adhesive.

Generation, assembly, and annotation of transcriptomes

Total RNA was extracted from both precompetent and competent larvae and adults, using an RNeasy kit (Qiagen, Valencia, CA). Each developmental stage was kept separate from the others; concentration and quality of the RNA were analyzed with an Agilent RNA 2100 Bionalyzer (Agilent Technologies, Santa Clara, CA). Samples were sent to the University of Utah's Microarray and Genomic Analysis Core Facility, where mRNA was separated from total RNA and then reverse transcribed into cDNA using a TruSeq RNA Sample Prep Kit v2 (Illumina, San Diego, CA). Libraries were paired-end sequenced on an Illumina HiSeq 2000 platform. Three replicates for each stage generated 101,539,704 paired-end reads. *De novo* assembly using the Trinity platform (Broad Institute, Cambridge, MA) on the paired-end reads generated 158,314 transcripts (Grabherr *et al*., 2011). Annotation was carried out by BLAST (Basic

Table 1

Oligonucleotide primer sequences for HeCA1 *and* HeCA2

Primer	Sequence (5′–3′)
HeCA1-F	AGAACCTACAATCCGAACATGCCT
HeCA1-R	GCGTTCATCTGTGCCTCAGTGAC
HeCA2-F	TGTATCAGTCCCTCCGTATT
HeCA2-R	CGAGTCGTATCCATGGAATT

HeCA, *Hydroides elegans* carbonic anhydrase; F, forward; R, reverse.

Local Alignment Search Tool) searches of transcripts against SWISS-PROT, TrEMBL, and nr (non-redundant) databases; and GO (gene-ontology) terms were assigned based on matches.

Carbonic anhydrase (CA) gene identification and cloning

In addition to searching the annotations with CA-specific identifiers, we used CA homologs identified from the literature to search the assembled transcriptomes of *H. elegans* by TBLASTN (translated nucleotide BLAST databases). *Hydroides elegans* carbonic anhydrases 1 and 2 (*HeCA1* and *HeCA2*, respectively) were 2 of 12 *HeCA* genes identified from recovered contigs in the transcriptomes. We generated cDNA libraries using the Advantage RT-for-PCR Kit (Clontech Laboratories, Mountain View, CA) from mixed life-history stages (competent larvae, juveniles, and adults), and successfully amplified a 738- and a 900-base-pair (bp) fragment for *HeCA1* and *HeCA2*, respectively, using oligonucleotide primers (Table 1). Isolated fragments were cloned into the pGEM-T (Promega, Madison, WI) vector. Plasmids were sent to the University of Hawai‘i at Mānoa ASGPB (Advanced Studies in Genomics, Proteomics and Bioinformatics) facility for Sanger sequencing, and screened to ensure that the correct inserts had been cloned.

Whole mount in situ *hybridization*

Competent larvae and 6-h and 10-day-old juveniles of *H. elegans* were relaxed in 7.5% magnesium chloride for 10 min, and fixed overnight at 4 °C in 3.7% formaldehyde in FSW. After fixation, specimens were washed with phosphate-buffered saline (PBS), dehydrated with methanol, and stored at –20 °C until further use. Sense and antisense, digoxigenin-labeled riboprobes were developed using the MEGAscript kit (Ambion, Austin, TX) from the cloned fragments for *HeCA1* and *HeCA2*. Whole mount *in situ* hybridization was performed according to a published protocol for *H. elegans,* with a riboprobe concentration of 1 ng μl^{-1} and a hybridization time of 48 h at 68 °C (Seaver and Kaneshige, 2006). Specimens were cleared, transferred to 80% glycerol solution, and mounted on glass slides. Samples were observed using differential interference contrast (DIC) microscopy on a Zeiss Axiophot microscope and photographed with a Zeiss AxioCam HRc camera (Carl Zeiss AG, Oberkochen, Germany).

Sequence and phylogenetic analyses

Le Roy *et al*. (2014) performed an α-CA phylogeny based on 137 complete or nearly complete genomic and transcriptomic amino acid sequences accumulated from the NCBI (National Center for Biotechnology Information), JGI (Joint Genome Institute), and SpBase (Cameron *et al.*, 2009). Twelve CA genes, which we identified in the transcriptomes of *H. elegans*, were added to these 137 sequences. Analysis of the amino acid sequences from *H. elegans* was performed using SMART, SignalP ver. 4.1, and TMHMM ver. 2.0 sequence prediction software (Schultz *et al*., 1998; Krogh *et al*., 2001; Petersen *et al*., 2011). Sequences were also analyzed by running conserved domain searches on NCBI (Marchler-Bauer *et al*., 2014). Coding sequences used in the α-CA phylogeny were aligned with Geneious ver. 7.1.5 using ClustalW (gap opening: 10; gap extension: 0.2) and trimmed with TrimAl to remove non-conserved regions (Capella-Gutierrez *et al*., 2009; Kearse *et al*., 2012). ProtTest ver. 2.4 determined a model of protein evolution (LG+G model) under the Akaike Information Criteria (AIC) (Abascal *et al*., 2005). Maximum likelihood analysis was performed using RAxML (Stamatakis, 2006) with 1000 bootstrap replicates.

Results

Characteristics of HeCA *sequences*

Twelve carbonic anhydrase (CA) genes were identified from transcriptomes of *Hydroides elegans,* and named *HeCA1, HeCA2, HeCA3, HeCA4, HeCA5, HeCA6, HeCA7, HeCA8, HeCA9, HeCA10, HeCA11*, and *HeCA12* (Table 2). Based on BLASTX searches against GenBank and conserved-domain searches on NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), the complete open reading frames (ORFs) were determined for 8 of the 12 sequences (Table 2). The remaining 4 sequences, without complete ORFs, included 2 with missing start codons (*HeCA4* and *HeCA9*) and 2 with missing stop codons (*HeCA3* and *HeCA12*) (Table 2). Because the 4 partial sequences contained all of the CA conserved domains and spanned nearly the entire ORF, they were included in the phylogeny. *In silico* analysis revealed 7 signal-peptide-containing genes and 3 with GPI-linked (glycophosphatidylinositol) domains (Table 2). There were no predicted transmembrane regions in any of the complete *HeCA* sequences, based on two different prediction-modeling programs (SMART and TMHMM). In addition, two gene fragments

Table 2

Carbonic anhydrase (CA) gene characteristics in Hydroides elegans

CA	Accession	Amino Acid Length	ORF	Signal Peptide	GPI Domain	Terminal Transmembrane Domain
HeCA1	KX129935	272	Full	No	No	No
HeCA2	KX129936	315	Full	Yes	No	No
HeCA3	KX129937	304	Partial	Yes	No	No
HeCA4	KX129938	266	Partial	N/A	No	No
HeCA5	KX129939	285	Full	Yes	No	No
HeCA6	KX129940	307	Full	Yes	Yes	No
HeCA7	KX129941	314	Full	Yes	No	No
HeCA8	KX129942	332	Full	Yes	Yes	No
HeCA9	KX129943	321	Partial	N/A	No	No
HeCA10	KX129944	314	Full	Yes	Yes	No
HeCA11	KX129945	305	Full	No	No	No
HeCA12	KX129946	329	Partial	No	No	No

GPI, glycophosphatidylinositol; *HeCA*, *Hydroides elegans* carbonic anhydrase; ORF, open-reading frame.

were successfully PCR-amplified using *HeCA1* and *HeCA2* oligonucleotide primers. Closer examination of the *HeCA1* and *HeCA2* sequences revealed that *HeCA2* contained a signal peptide, but *HeCA1* contained no recognized signal-peptide domains (Fig. 2). Both *HeCA1* and *HeCA2*, when compared to homologous sequences from other metazoans, contained characteristic α-CA domains, QSPINI, GSEH, GLAVL, and GSLTTP, and included three histidine residues that are essential for regeneration of the zinc active-site (Fig. 2).

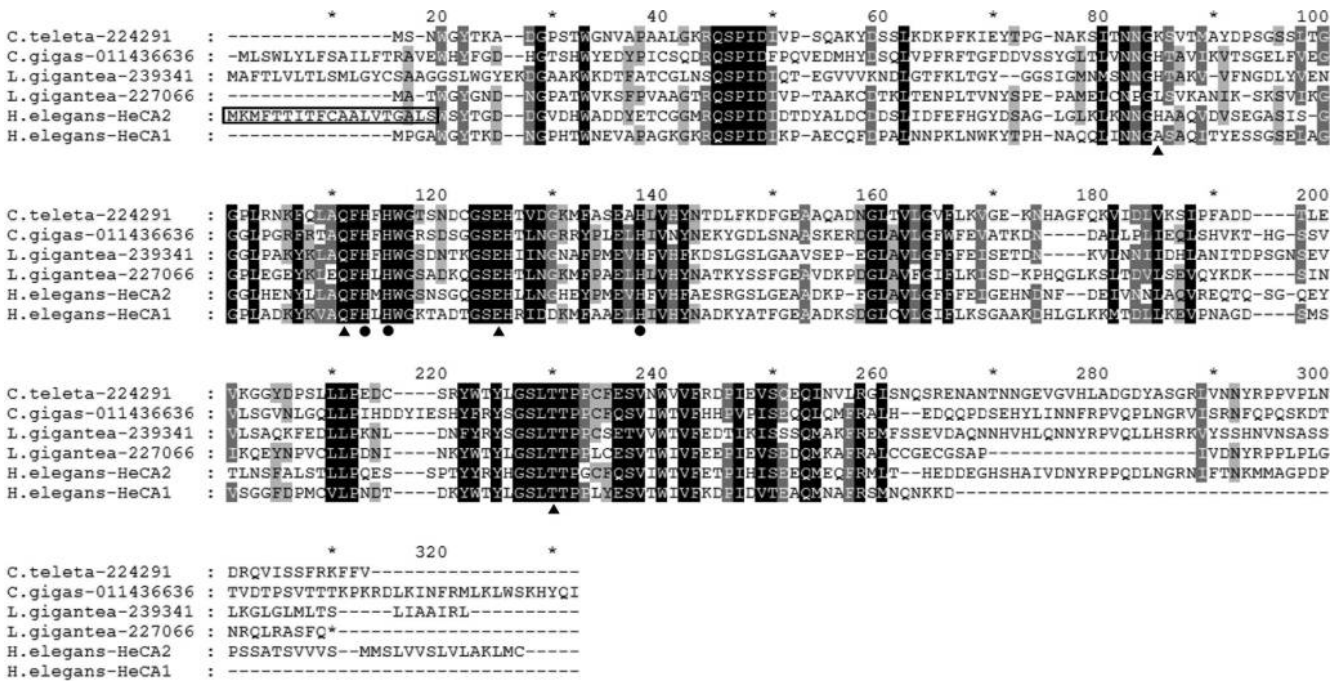

Figure 2. ClustalW alignment of 6 α-CA amino acid sequences, two from *Hydroides elegans* (NCBI: KX129935, KX129936), two from *Lottia gigantea* (JGI: 239341, 227066), one from *Crassostrea gigas* (NCBI: 011436636), and one from *Capitella teleta* (JGI: 224291). Amino acid conservation percentages are indicated by shading: 100% conservation (black), 80% conservation (dark gray), 60% conservation (light gray), and 0% conservation (white). Black box indicates signal peptide sequence. Circles below sequences denote three histidine residues involved in the regeneration of the zinc active-site, and triangles indicate active site residues associated with catalytic activity of the enzyme.

In situ *hybridization expression patterns of* HeCA1 *and* HeCA2

Expression occurred in competent larvae and 6-h and 10-day-old juveniles using *HeCA1* and *HeCA2* antisense probes (Fig. 3). Using *HeCA1* and *HeCA2* sense probes, no expression occurred in any of the three stages. Competent larvae of *H. elegans* are free-swimming planktotrophic animals with a ciliated prototroch and metatroch and a collar (Fig. 3C). Expression of *HeCA1* and *HeCA2* was restricted to two symmetrical points along lateral sides of the collar segment, during the competent larval stage (Fig. 3A, B). As larvae undergo metamorphosis, they lose their trochs and develop collar structures and branchial rudiments (Fig. 3F). Slight differences in gene expression patterns between *HeCA1* and *HeCA2* became apparent by the 6-h juvenile stage, but remained localized to the anterior region of the animals for both genes (Fig. 3D, E). *HeCA1* was restricted to symmetrical points (Fig. 4A, B), while *HeCA2* was expressed throughout the entire first thoracic segment and collar, and in parts of the branchial rudiments (Fig. 4C, D). Differences in expression patterns between the 2 genes became more pronounced in 10-day-old juveniles, which have fully developed branchial tentacles, collar, and abdominal segments (Fig. 3I). Expression of *HeCA2* was present in the branchial tentacles, first thoracic segment, and abdominal segments, while *HeCA1* was expressed in the first thoracic segment and lower abdominal segments (Fig. 3G, H). Closer examination of the collar segment revealed similar expression patterns between *HeCA1* and *HeCA2;* they were enhanced at two locations where the calcium-secreting glands are located (Fig. 5A, B).

Phylogenetic analysis

To assess the evolutionary relatedness of α-CA genes, a multi-taxon protein phylogeny was constructed using maximum-likelihood analysis on 149 genomic and transcriptomic sequences (Fig. 6). As shown in the phylogeny presented by Le Roy *et al*. (2014), the α-CA family divides into an early branching and monophyletic poriferan clade in addition to two α-CA secondary clades. The two secondary clades are categorized by their subcellular localizations into cytosolic-mitochondrial type α-CAs and secreted and membrane-bound type α-CAs. The cytosolic-mitochondrial clade contains 5 α-CA sequences (*HeCA1*, *HeCA7*, *HeCA9*, *HeCA11*, and *HeCA12*) from *H. elegans*, which are polyphyletically arranged in 4 clades (Clade I–Clade IV) (Fig. 6). Clade I contains *HeCA1*, *HeCA12*, and CAs from 3 non-annelidan species, *Lottia gigantea, Acropora millepora,* and *Branchiostoma floridae*; Clade II contains *HeCA12* and CAs from 2 other annelid species, *Riftia pachyptila* and *Capitella teleta*; Clade III contains *HeCA7* and CAs from 2 other species, *Lottia gigantea* and *Capitella teleta*; and Clade IV contains *HeCA9* and CAs from 3 non-annelidan species, *Trichoplax adhaerens*, *Acropora millepora*, and *Branchiostoma floridae*. The secreted and membrane-bound secondary clade contains the remaining 7 α-CA sequences (*HeCA2*, *HeCA3*, *HeCA4*, *HeCA5*, *HeCA6*, *HeCA8*, and *HeCA10*) from *H. elegans*. In addition, sequences from *H. elegans* are grouped monophyletically within a single clade (Clade V) and include *Strongylocentrotus purpuratus*, *Lottia gigantea*, *Capitella teleta*, *Trichoplax adhaerens*, and *Acropora millepora*.

Discussion

Recognizing that the enzymatic processes in biomineralization are broadly conserved within Metazoa (Le Roy *et al*., 2014), we hypothesized that carbonic anhydrase (CA) genes were expressed in tissues involved in biomineralization in tube-building serpulids. Carbonic anhydrase genes were seen as logical candidates for providing the molecular basis for biomineralization in annelids, because the enzyme readily hydrolyzes carbon dioxide to form bicarbonate—a precursor of calcium carbonate ($CaCO_3$) precipitation. The hypothesis was supported by identification of 12 CA genes in the transcriptomes of *Hydroides elegans* and by localization of two of those genes in the first thoracic segment, where calcification occurs, by *in situ* hybridization.

During the larval stage, *H. elegans* carbonic anhydrases 1 and 2 (*HeCA1* and *HeCA2*) exhibited identical expression patterns within the collar segment, signifying gene activation during a non-biomineralization stage. Divergence in expression patterns occurred following metamorphosis, with branchial rudiment staining in the 6-h juveniles and expression of the transcripts in lower abdominal tissues by the 10-day-old juvenile stage. We hypothesize that protein products are involved in different functions depending on location found. For instance, staining that occurred within the collar segment corresponded to the location where Hedley (1956) first described the calcium-secreting glands in serpulids. But expression within branchial tentacles, where gas exchange occurs in serpulids (De Cian *et al*., 2003), indicates involvement in respiration processes. While both genes are localized to the collar segment in all three stages, they also had diffuse expression patterns within abdominal segments that are likely not consistent with functions involved in biomineralization. Through duplication events, CA genes may have been co-opted for biomineralization from their ancestral roles, accounting for the multiple sequences discovered within the transcriptomes, and tissue expression seen in different tissues within *H. elegans.*

Enhanced expression of *HeCA1* and *HeCA2* in the first thoracic segment, where tube formation is known to occur, however, is consistent with biomineralization functions. In serpulids, biomineralization is thought to occur either by generation of $CaCO_3$ within the lumen of the calcium-secreting

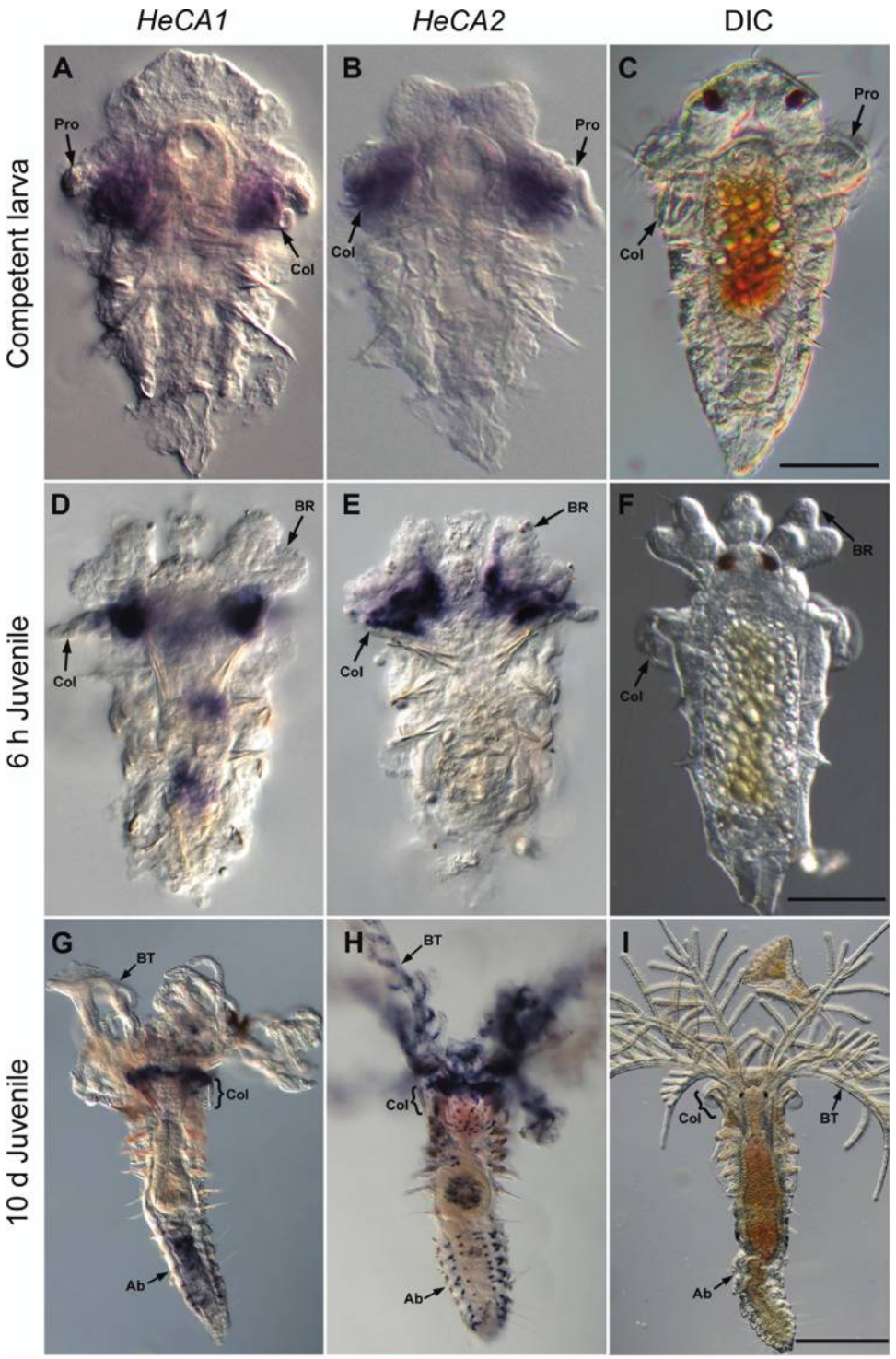

Figure 3. Expression of *HeCA1* and *HeCA2* in competent larvae and 6-h and 10-day-old juveniles of *Hydroides elegans.* Purple staining indicates expression of gene products. Expression of *HeCA1* and *HeCA2* is localized to the collar segment in (A)(B) competent larvae, and in (D)(E) 6-h juveniles. In 10-day-old juveniles, (G) expression of *HeCA1* is restricted to the first thoracic segment and lower abdominal segments; (H) *HeCA2* is expressed within branchial tentacles, collar, and abdominal segments. Reference photographs taken by differential interference contrast (DIC) microscopy are in the right column (C)(F)(I). BR, branchial rudiments; BT, branchial tentacles; Col, collar; Pro, prototroch. Scale bars = 50 μm (C), 75 μm (F), and 250 μm (I).

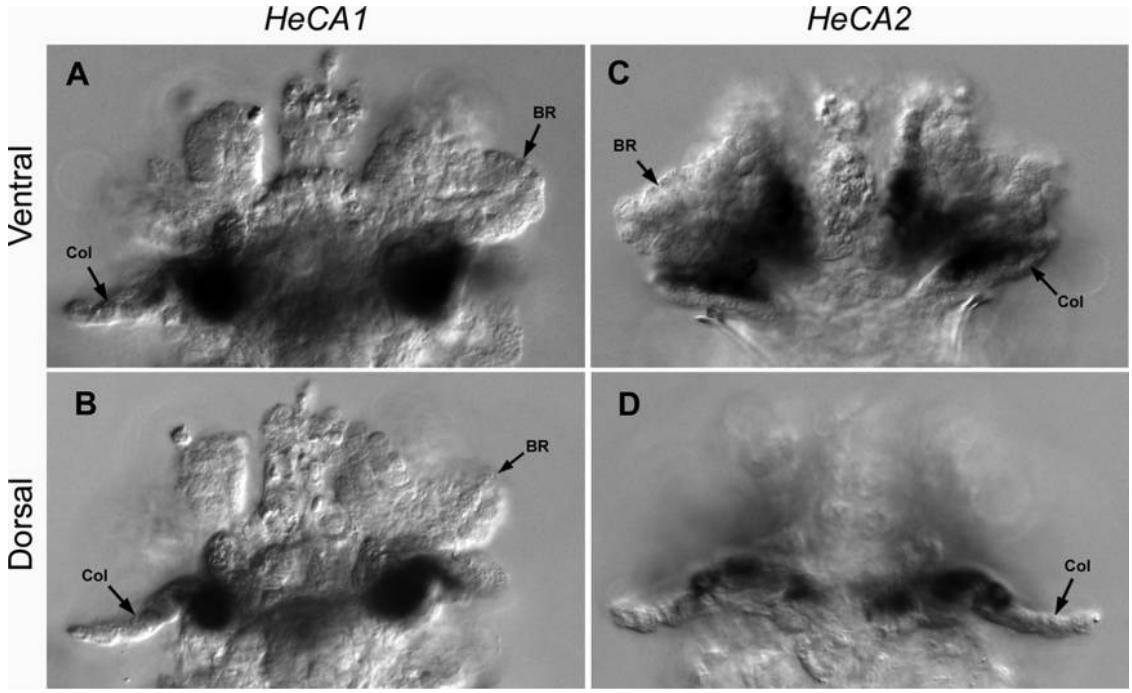

Figure 4. Expression of *HeCA1* and *HeCA2* in 6-h juveniles of *Hydroides elegans*. BR, branchial rudiments; Col, collar. (A)(B) Ventral and (C)(D) dorsal sides for *HeCA1* and *HeCA2*. Expression of *HeCA1* is restricted to the (A) collar segment in the ventral plane, which is also seen from the (B) dorsal side. *HeCA2* expression is localized to the base of the branchial rudiments, as seen from the (B) ventral side, and along the length of the collar segment when examined from the (D) dorsal side.

glands (Vinn *et al*., 2009) or from calcified intracellular granules (Neff, 1969). In our phylogeny, the genes *HeCA1* and *HeCA2* were shown to be part of the cytosolic and secreted α-CA clades, respectively. Cytosolic CA enzymes are involved in coral biomineralization, where they function by hydrolyzing metabolic carbon dioxide and transporting bicarbonate to the sites of calcification (Bertucci *et al*., 2011). In *Hydroides elegans*, HeCA1, which is found in the tube-forming collar segment, may hydrolyze metabolic carbon dioxide in the cytosol of cells in the calcium-secreting glands, followed by transport of the bicarbonate ions to the lumen of the glands where $CaCO_3$ precipitation occurs. However, secreted enzymes play a prominent role in molluscan biomineralization processes, and are found in the extrapallial space formed between the mantle tissues and shell (Miyamoto *et al*., 1996; Hattan *et al*., 2001), indicating that enzymatic bicarbonate production is extracellular. In *H. elegans*, *HeCA2*, also located in the collar segment, may be

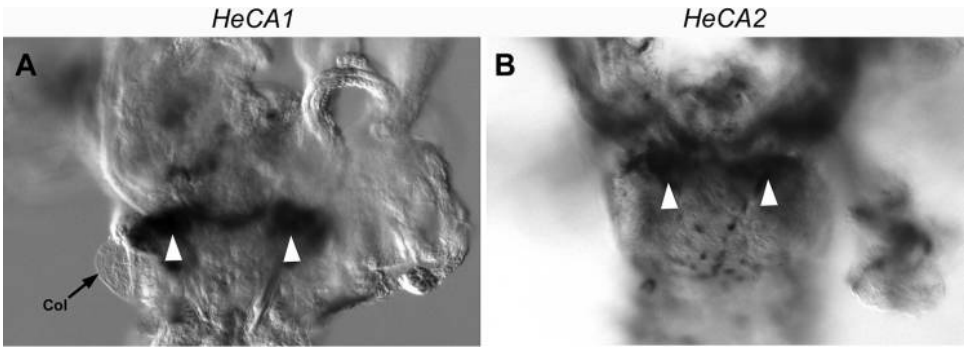

Figure 5. Expression in the collar segment of *HeCA1* and *HeCA2* in 10-day-old juveniles of *Hydroides elegans*. BT, branchial tentacles; Col, collar. Staining is restricted to symmetrical points along the base of the collar segment in both (A)(B) *HeCA1* and *HeCA2*. *HeCA2* expression is also present in the (B) branchial tentacles.

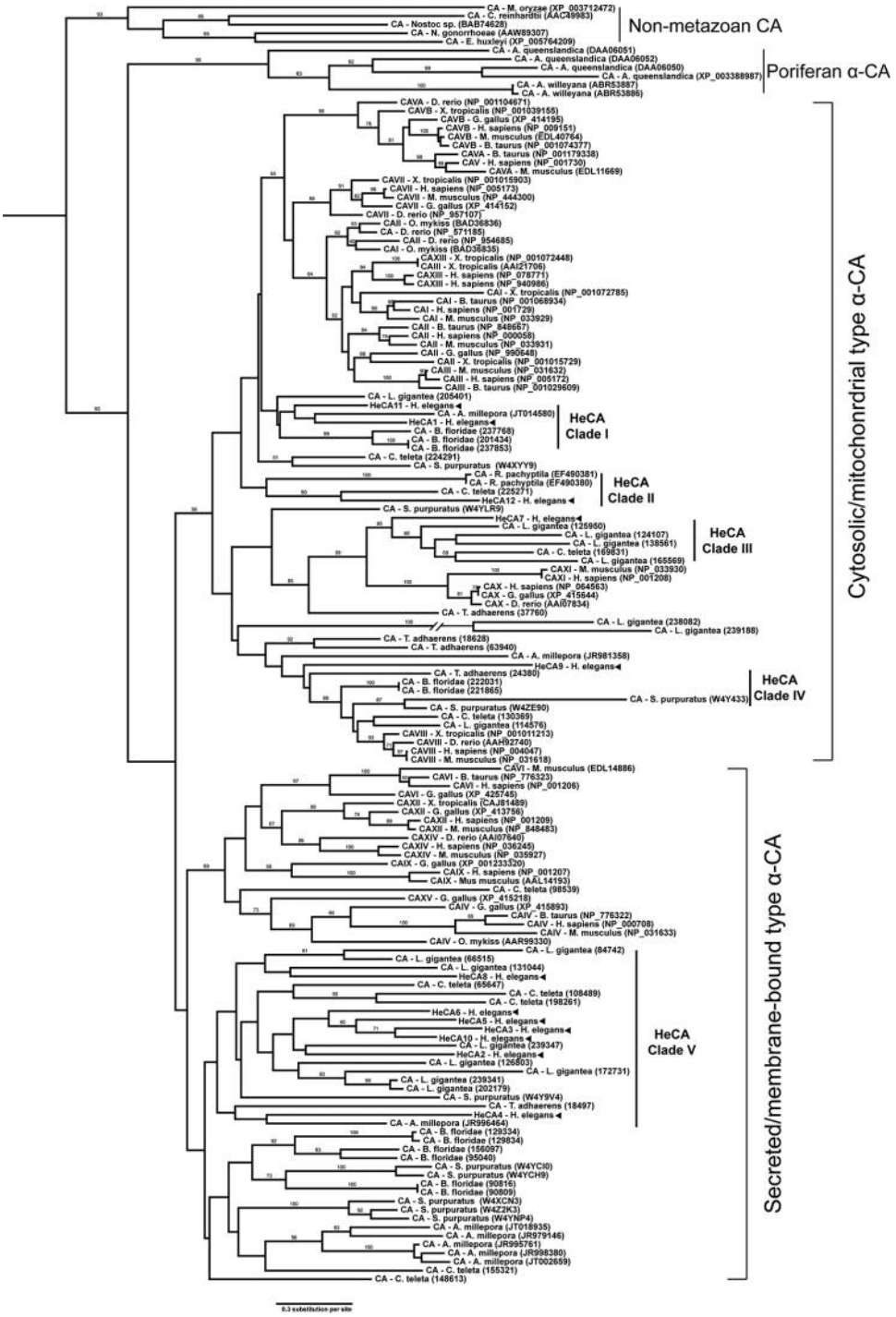

Figure 6. Phylogenetic analysis of the α-CA family using maximum likelihood (ML), with bootstrap values located at node bases. Bootstrap values <50 were not included in the tree. Arrows denote sequences from *Hydroides elegans,* and brackets correspond to clades within which *HeCA* sequences are grouped. Sequences used in the phylogeny included *Homo sapiens*, *Gallus gallus*, *Mus musculus*, *Danio rerio*, *Bos taurus*, *Xenopus tropicalis*, *Branchiostoma floridae*, *Oncorhynchus mykiss*, *Strongylocentrotus purpuratus*, *Capitella teleta*, *Riftia pachyptila*, *Acropora millepora*, *Trichoplax adhaerens*, *Amphimedon queenslandica*, and *Astrosclera willeyana*. Out-group species included *Emiliania huxleyi*, *Chlamydomonas reinhardtii*, *Nostoc* sp., and *Neisseria gonorrhoeae*.

secreted into the gland lumen where it provides the catalytic activity for $CaCO_3$ precipitation, similar to molluscan CAs. Future studies employing thin-section, *in-situ* hybridization on the first thoracic segment may reveal subcellular localization of these two CA genes. In addition, inhibition experiments using an effective CA inhibitor like acetazolamide would help assign specific functions to the genes presented in this study.

In the phylogenetic assessment presented here, and in most other phylogenies, sponges are the sister group to the Eumetazoa. The early branching position for the poriferan clade has previously identified the phylum as the ancestral progenitor for subsequent diversity in the α-CA family (Jackson, 2007). Interestingly, α-CA sequences in our tree did not group by traditional taxonomy, but by subcellular localization, in agreement with patterns reflected in similar published phylogenies (Moya *et al*., 2012; Le Roy *et al*., 2014). The sequences that were aligned in the present phylogeny were the same sequences used in the Le Roy *et al.* (2014) α-CA phylogeny, and our tree resulted in the same two secondary metazoan clade groupings, which were separated into a cytosolic-mitochondrial clade and a secreted and membrane-bound clade. The former contains CA isoforms that are present within the cytosol or in the mitochondria, while the latter represents enzymes either secreted into extracellular space or embedded within the cell membrane (Chegwidden *et al*., 2000). Interestingly, *HeCA7*, which contains a signal peptide, would presumably be grouped within the secreted and membrane-bound clade, but is grouped in the cytosolic-mitochondrial clade in the phylogeny. Endocytosis of the protein could account for a secreted protein being grouped within the cytosolic-mitochondrial clade, but sequencing or assembly error could also account for the discrepancy exhibited in the tree. Expression studies were performed for *HeCA1* and *HeCA2*, because they were found grouped within the secreted and membrane-bound and cytosolic-mitochondrial clades, respectively. However, future studies might examine the other 10 *HeCA* genes to see if similar expression patterns are observed for those sequences also.

Determining the ancestral source of biomineralizing CA genes in *H. elegans*, and presumably all serpulids, was made difficult by low bootstrap values and lack of taxonomic distribution exhibited in the tree. The large number of phyla with few constituent genera represented likely contributed to the low bootstrap values. Nevertheless, it was apparent that CA genes in the annelid *Capitella telata*, a non-calcifying polychaete, shared little homology with CA genes from *H. elegans.* Of the 14 CA genes identified in the genome of *Capitella teleta*, only a single sequence (JGI: 225271) was orthologous to a sequence from *H. elegans*. This large discrepancy in gene homology between the two polychaete genera may indicate either that a novel function for biomineralization arose from duplicated annelid genes in serpulids, or the form of CAs in serpulids was uniquely inherited from the last common ancestor to possess a biomineralizing α-CA. The accumulation of more CA sequences from species in underrepresented taxa, especially from other annelid species, will not only help resolve the tree, but allow us to determine the evolutionary origin of genes responsible for CA biomineralization.

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