Gene Expression of Na^+/K^+ ATPase in *Haminoea japonica*

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Introduction

Gene flow is important in maintaining genetic variation and replenishing locally disrupted populations. Because of the importance of gene flow, predicting dispersal patterns and connectivity in marine ecosystems is essential for effective conservation and management (Gray 1997, Lipcius et al. 2008). Most benthic marine invertebrates disperse only during a pelagic larval period, which varies in length between and among species (Krug 2009). Since an organism with a relatively long Pelagic Larval Duration (PLD) can potentially travel farther than an organism that settles sooner, PLD was initially used as an indicator of connectivity (Shanks et al. 2003). However, results from studies on a variety of invertebrates show that PLD is not a consistent predictor of connectivity (Lester and Ruttenberg 2005, Shanks 2009). These results suggest that chemical and environmental signals may induce larval settlement and metamorphosis sooner than expected (Strathmann et al. 2002, Weersing and Toonen 2009, Kelly and Palumbi 2010).

Excess K^+ ions act as a chemical signal that induces metamorphosis in many marine invertebrates (Baloun and Morse 1984, Yool et al. 1986, Hadfield and Pennington 1990, Degnan et al. 1997). K^+ ions enter the cell against an ionic gradient through the actions of the $Na^+ K^+$ ATPase carrier protein. The ionic gradient regulates cell volume, provides the electrochemical gradient for the uptake of ions, and maintains osmotic balance (Yool et al. 1986, Marsh et al. 2000). Since these functions are critical for basic physiological functions of the cell, it is expected that the $Na^+ K^+$ ATPase gene would be expressed constitutively. A study on sea urchins however, found that the gene was expressed only for a 16 hour period during 80 hours of development (Marsh et al. 2000). This study was designed to investigate the expression of the $Na^+ K^+$ ATPase alpha subunit 1 gene throughout the larval development of *Haminoea japonica* using *in situ* hybridization. *H. japonica* is a poecilogonous marine gastropod that produces lecithotrophic larvae as well as juveniles from the same egg mass. Metamorphosis inducers for *H. japonica* include extracts from prey algae, egg mass jelly, and excess K^+ ions (Gibson 1995). Because $Na^+ K^+$ ATPase is instrumental in the uptake of K^+ ions, an *in situ* hybridization study on the $Na^+ K^+$ ATPase gene could contribute to the understanding of the mechanism of ionic metamorphosis induction, which is needed to make more accurate predictions of dispersal.

Haminoea slugs are classified as Opisthobranchs, which are a subgroup of the Euthyneurid gastropods. Although the Euthyneurids have generally been accepted as a monophyletic group, Dinapoli (2010) classified them as paraphyletic based on several nuclear and mitochondrial markers. Molecular studies have also shown Opisthobranchs to be a paraphyletic group (Dinapoli and Klussmann-Kolb 2010). The addition of the *Haminoea* $Na^+ K^+$ ATPase gene to gene databases can potentially contribute to the refining of phylogenetic hypotheses and the understanding of evolutionary gain and loss of traits. Although I was unable to complete the *in situ* hybridization study, I successfully sequenced the *H. japonica* $Na^+ K^+$ ATPase gene and generated a gene tree for $Na^+ K^+$ ATPase in related lophotrochozoans.

To visualize changes in the developing larvae, I photographed *H. vesicula* larvae that had been preserved and stained with phalloidin and 4',6-diamidino-2-phenylindole (DAPI) at three

intervals after hatching. Phalloidin, a fluorescent derivative of the *Amanita phalloides* mushroom, absorbs blue light and emits green light when bound to actin (Wulf et al. 1979). DAPI is a fluorescent stain that binds to adenine-thymine rich regions of DNA and will absorb ultraviolet light and emit blue light (Kubista et al. 1987).

Materials and Methods

DNA extraction and gene amplification

Eight adult *H. japonica* slugs and egg masses were collected from Spencer's Spit on Lopez Island, Washington. I selected one small adult slug for RNA extraction. The slug was relaxed in a 1:1 solution of 7.5% $MgCl_2$ and seawater and its head removed with a razor blade. I extracted RNA from the fresh head tissue using a Qiagen RNeasy® kit. The extracted RNA was then reverse transcribed to cDNA using an Ambion RETROscript® kit. After reverse transcription, I amplified the gene using a polymerase chain reaction (PCR) with degenerate oligonucleotide primers. After amplification, I performed gel electrophoresis on the PCR product and removed and purified the band containing the $Na^+ K^+$ ATPase gene using an Illustra® GFX PCR DNA & Gel Band purification kit. Next, I performed a TOPO® cloning reaction for transformation of the purified DNA into chemically competent TOP10F' One Shot® *E. coli* cells. After transformation, I plated the cells onto agar plates treated with kanamycin and x-gal and allowed the cells to incubate at 37° C for 12 hours. Several white colonies grew on the plate, indicating successful transformation. Ten of the transformed colonies were then transferred to separate tubes and cultured in a shaking 37° C incubator in 3 ml LB medium and 50 μg/ml kanamycin. After incubating for 12 hours, DNA was purified using a 5 Prime FastPlasmid Mini Kit® and sent to Genewiz® for sequencing.

Data Analysis

The resulting sequences were analyzed using Geneious version 5.1.6 (Drummond AJ 2011) and translated into amino acid sequences using MEGA version 5.05 (Tamura et al. 2011). A gene tree was generated to compare the sequence of $Na^+ K^+$ ATPase from *H. japonica* with sequences of other lophotrochozoans from the NCBI database. The evolutionary relationships were inferred using the Maximum Likelihood method with MEGA 5.05 based on the JTT-matrix-based model (Jones et al. 1992) (fig. 1).

If I had proceeded with *in situ* hybridization, I would have synthesized RNA probes from the purified DNA sequence. The probes would have been used to reveal expression of the $Na^+ K^+$ ATPase gene in larvae that had been preserved at regular intervals throughout development.

Fixation and staining of H. vesicula *larvae*

Haminoea vesicula egg masses at varying stages of development were collected from False Bay, San Juan Island, Washington. Egg masses were kept in bag-filtered seawater at 20° C and observed for signs of hatching. Larvae were preserved two hours, three days, and five days after hatching. At each interval, 20-40 larvae were added to a 1.5 ml microcentrifuge tube and

relaxed in a 1:1 solution of 7.5% magnesium chloride in seawater for 15 minutes. I then replaced the seawater with 4% paraformaldehyde in filtered seawater and allowed the larvae to fix for about 12 hours at -20° C. After fixation, I washed the preserved larvae three times in phosphate buffered saline (PBS) for ten minutes each, added phalloidin in 200 µl PBS, and allowed larvae to incubate in a dark drawer for about one hour. The larvae were then washed in three ten-minute PBS rinses, after which they were placed on a microscope slide with DAPI mounting medium and observed and photographed at 40× magnification through a fluorescence microscope (fig. 2).

Results

Na^+ K^+ ATPase gene tree

The Na^+ K^+ ATPase Maximum Likelihood tree shows expected taxonomic relationships. The *H. japonica* gene formed a monophyletic clade with the gene from the sea hare, *Aplysia californica,* another Opisthobranch. These were grouped within the gastropod clade, which formed a branch of the molluscs group (fig.1). The sequence from the *H. japonica* gene formed a longer branch than that of *A. californica,* suggesting that there were more mutations in *H. japonica* than *A. californica,* and that *A. californica*'s sequence is closer to that of the ancestral gene.

DAPI and Phalloidin staining

Larvae preserved and stained two days after hatching did not show any fluorescence, indicating that both DAPI and phalloidin failed to bind. Larvae preserved three days after hatching did not show phalloidin binding, but a small region of fluorescence was observed under ultraviolet light, indicating DAPI binding. In larvae preserved five days after hatching, the region of DAPI fluorescence was greater than it had been at three days, and phalloidin fluorescence was visible as a region of green fluorescence (fig.2).

Discussion

Because it evolves slowly, the Na^+ K^+ ATPase gene is useful for the reconstruction of deep level phylogenies but it may not be as effective for distinguishing between more recently diverged species (Anderson et al. 2004). There were no polytomies in the mollusc nodes of the tree, suggesting that this gene was an adequate marker for the mollusc species used in the study. However, including additional species might reduce resolution and require a faster evolving marker, such as a mitochondrial gene (Kyle and Boulding 2000). Since the gene tree generated by this study included only a small sample of gastropod species, the inclusion of additional species will improve its usefulness as a tool for inferring the evolution of traits and the grouping of species.

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References

Anderson, F. E., A. J. Córdoba, and M. Thollesson. 2004. Bilaterian phylogeny based on analyses of a region of the sodium–potassium ATPase ?-subunit gene. Journal of molecular evolution 58:252-268.

Baloun, A. J., and D. E. Morse. 1984. Ionic control of settlement and metamorphosis in larval Haliotis rufescens (Gastropoda). The Biological Bulletin 167:124.

Degnan, B. M., D. Souter, S. M. Degnan, and S. C. Long. 1997. Induction of metamorphosis with potassium ions requires development of competence and an anterior signalling centre in the ascidian Herdmania momus. Development Genes and Evolution 206:370-376.

Dinapoli, A., and A. Klussmann-Kolb. 2010. The long way to diversity-Phylogeny and evolution of the Heterobranchia (Mollusca: Gastropoda). Molecular Phylogenetics and Evolution 55:60-76.

Drummond AJ, A. B., Buxton S, Cheung M, Cooper A, Duran C, Field M, Heled J, Kearse M, Markowitz S, Moir R, Stones-Havas S, Sturrock S, Thierer T, Wilson A. 2011. Geneious v5.4 Available from http://www.geneious.com/.

Gibson, G. 1995. Why be choosy? Temporal changes in larval sensitivity to several naturally-occurring metamorphic inducers in the opisthobranch Haminaea callidegenita. Journal of experimental marine biology and ecology 194:9-24.

Gray, J. S. 1997. Marine biodiversity: patterns, threats and conservation needs. Biodiversity and Conservation 6:153-175.

Hadfield, M. G., and J. T. Pennington. 1990. Nature of the metamorphic signal and its internal transduction in larvae of the nudibranch Phestilla sibogae. Bulletin of Marine Science 46:455-464.

Jones, D. T., W. R. Taylor, and J. M. Thornton. 1992. The rapid generation of mutation data matrices from protein sequences. Computer applications in the biosciences: CABIOS 8:275.

Kelly, R. P., and S. R. Palumbi. 2010. Genetic structure among 50 species of the northeastern Pacific rocky intertidal community. PLoS One 5:e8594.

Krug, P. J. 2009. Not my" type": Larval dispersal dimorphisms and bet-hedging in opisthobranch life histories. The Biological Bulletin 216:355.

Kubista, M., B. Aakerman, and B. Norden. 1987. Characterization of interaction between DNA and 4', 6-diamidino-2-phenylindole by optical spectroscopy. Biochemistry 26:4545-4553.

Kyle, C. J., and E. G. Boulding. 2000. Comparative population genetic structure of marine gastropods (Littorina spp.) with and without pelagic larval dispersal. Marine Biology 137:835-845.

Lester, S. E., and B. I. Ruttenberg. 2005. The relationship between pelagic larval duration and range size in tropical reef fishes: a synthetic analysis. Proceedings of the Royal Society B: Biological Sciences 272:585.

Lipcius, R. N., D. B. Eggleston, S. J. Schreiber, R. D. Seitz, J. Shen, M. Sisson, W. T. Stockhausen, and H. V. Wang. 2008. Importance of Metapopulation Connectivity to Restocking and Restoration of Marine Species. Reviews in Fisheries Science 16:101-110.

Marsh, A. G., P. K. K. Leong, and T. Manahan. 2000. Gene expression and enzyme activities of the sodium pump during sea urchin development: implications for indices of physiological state. The Biological Bulletin 199:100.

Shanks, A. L. 2009. Pelagic larval duration and dispersal distance revisited. The Biological Bulletin 216:373.

Shanks, A. L., B. A. Grantham, and M. H. Carr. 2003. Propagule dispersal distance and the size and spacing of marine reserves. Ecological Applications 13:159-169.

Strathmann, R. R., T. P. Hughes, A. M. Kuris, K. C. Lindeman, S. G. Morgan, J. M. Pandolfi, and R. R. Warner. 2002. Evolution of local recruitment and its consequences for marine populations. Bulletin of Marine Science 70:377-396.

Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular biology and evolution

Weersing, K., and R. J. Toonen. 2009. Population genetics, larval dispersal, and connectivity in marine systems. Marine Ecology Progress Series 393:1-12.

Wulf, E., A. Deboben, F. A. Bautz, H. Faulstich, and T. H. Wieland. 1979. Fluorescent phallotoxin, a tool for the visualization of cellular actin. Proceedings of the National Academy of Sciences 76:4498.

Yool, A. J., S. M. Grau, M. G. Hadfield, R. A. Jensen, D. A. Markell, and D. E. Morse. 1986. Excess potassium induces larval metamorphosis in four marine invertebrate species. The Biological Bulletin 170:255.

Figures

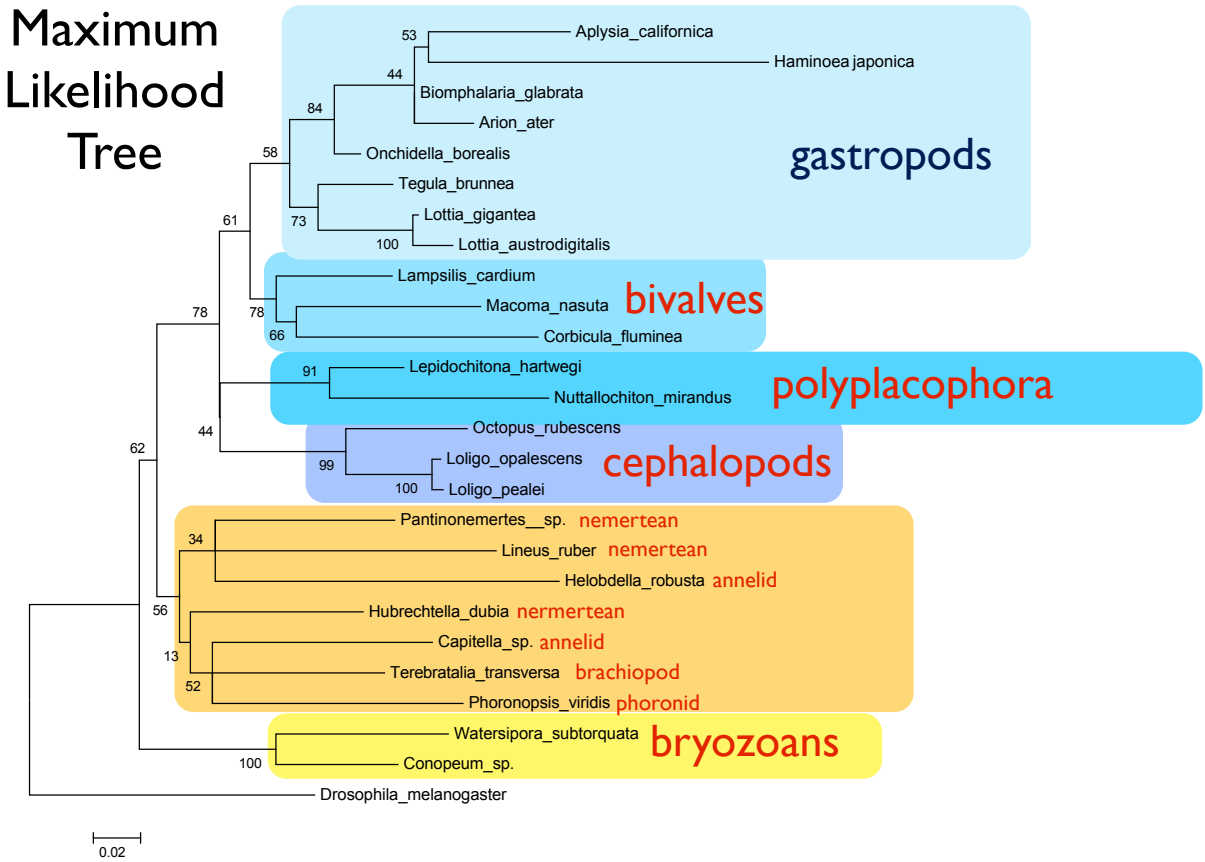

Figure 1. Maximum Likelihood tree for select lophotrochozoans generated on MEGA 5.05. based on the JTT-matrix-based model (Jones et al. 1992)

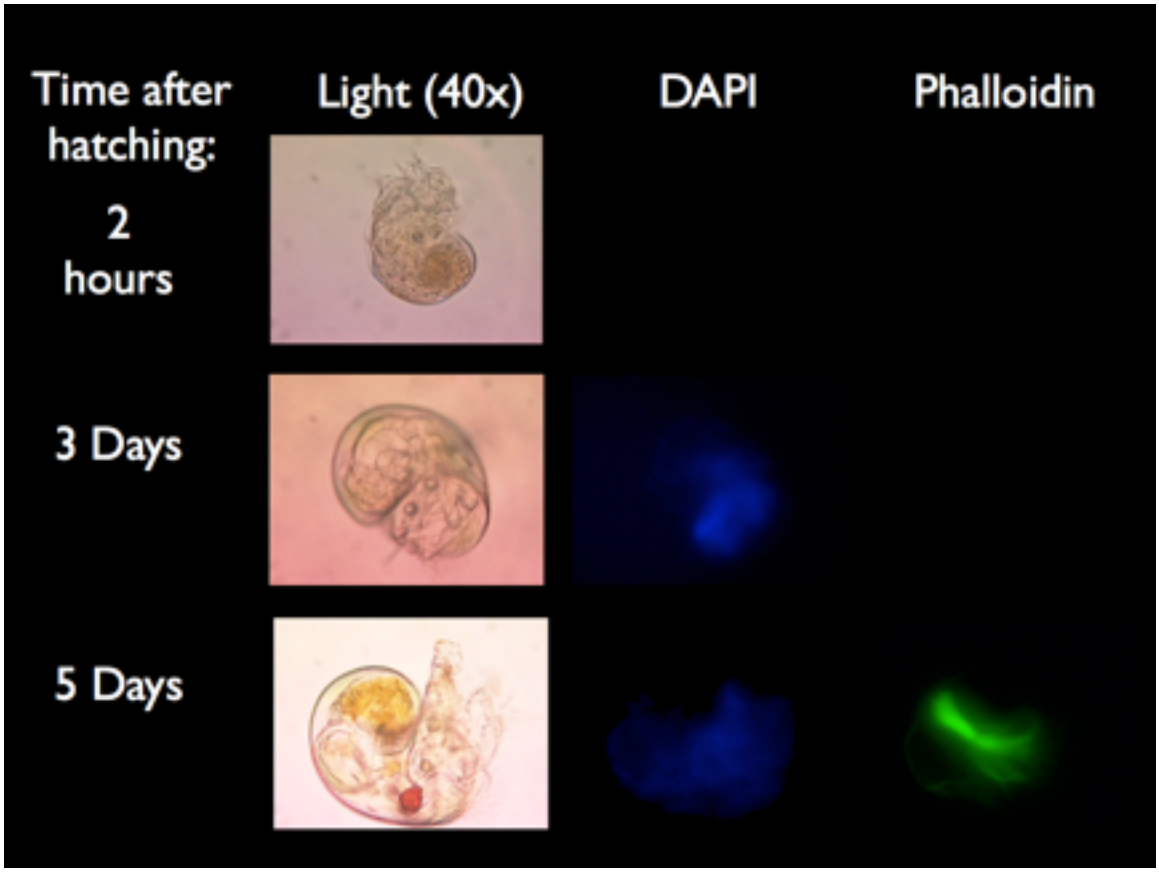

Figure 2. *Haminoea vesicula* larvae (40×) stained with DAPI and phalloidin at two hours, three days, and five days after hatching.