

# The expression of neural and developmental genes in *Pleurobrachia bachei*

Ian Mahone<sup>1,4</sup>, Billie J. Swalla<sup>1,3</sup>, Andrea Kohn<sup>1,2</sup>, Leonid Moroz<sup>1,2</sup>

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<sup>1</sup>Friday Harbor Laboratories, University of Washington, Friday Harbor, WA 98250

<sup>2</sup>Department of Neuroscience, University of Florida, Gainesville, FL 32611

<sup>3</sup>Department of Biology, University of Washington, Seattle, WA 98195

<sup>4</sup>Department of Natural Sciences, Michigan State university, MI 48825

Contact information:

Ian Mahone

[mahoneia@msu.edu](mailto:mahoneia@msu.edu)

(262) 705-5785

### Abstract

*Brachyury* and *Forkhead* are transcription factors that play an important role for development within organisms. Ctenophores are basal metazoans with little known about what kind of gene they have or where these genes are expressed. For these reasons, *brachyury* and *forkhead* are used as markers in the ctenophore *Pleurobrachia bachei*. These genes were cloned and then used for in situ hybridizations in adults. This revealed distinctive expression patterns for *brachyury* in the esophagus and stomach, while *forkhead* was expressed in the tentacles and comb rows of the animal. Not to mention, that through genomics and bioinformatics the *forkhead* genome has been discovered. Revealing that there is 24 *forkhead* genes within the genome of ctenophores.

### Introduction

Ctenophores (phylum *Ctenophora*) are aquatic invertebrates that have gelatinous transparent bodies. The ctenophore, *Pleurobrachia bachei*, is an ambush predator that catches food with its tentacles and carries the prey to the mouth by rotating its body rapidly, which causes the tentacle bearing the food to swipe across the oral region (Wrobel, Mills 1998). As in most ctenophores, *Pleurobrachia bachei* are known to express specific transcription factor proteins that bind to regulatory regions to control gene expression (Latchman DS, 1997). Gene expression gives the cell control over structure and function. These transcription factors have orthologs within many species spanning a multitude of phyla. Two of these transcription factors, *Forkhead* and

*Brachyury*, are the focus of my research on the ctenophore species *Pleurobrachia bachei*.

### Forkhead

*Forkhead (FOX)* proteins are a family of transcriptions factors that play a significant role in regulating the expression of genes involved in cell growth, proliferation, differentiation and longevity (Jung JW et al, 2011). The gene accountable for the forkhead phenotype (ocular anterior segment malformations and glaucoma) was cloned in *Drosophila melanogaster* in 1989 (Lehmann OJ et al, 2003). Unfortunately, the sequence was not conserved with any protein motif known at the time (Weigel D et al, 1989). Weigle and Jäckle (1990), noticed a similarity of the central 110 amino acids between *D. melanogaster* and mammalian Fox proteins (Weigel D et al, 1990). This domain was identified as forkhead due to the characteristic of the DNA-binding motif (Häcker U et al, 1992). Current studies in humans suggest that the *FOX* genes are downstream targets of the hedgehog signaling pathway, which plays a role in the development of basal cell carcinomas (Muy-Teck Teh et al, 2002). One gene, *FOXJ2/3*, is tightly controlled by microRNA-103(miR-103) to regulate the process of erythropoiesis (Yang GH al, 2009).

*FOX* gene have also been identified in the invertebrate ctenophores. Previous studies identified the organization of axial characteristics of ctenophores by simply isolating a forkhead gene of the Brain Factor 1(BF-1) family.(Yamada A, Martindale MQ, 2002). Even though ctenophores maybe the most basal metazoan, they express forkhead transcription factors showing that they are closely related to the common ancestor of humans (Yamada A, Martindale MQ, 2002). Ctenophore *FOXJ2/3* studies

could be implemented to inform human *FOX* gene studies on the evolutionary origin of the gene.

### *Brachyury*

*Brachyury*, a T-box complex gene, has a distinctive DNA binding domain found in transcription factors known to be important to development (Edwards et al, 1996). *Brachyury* was first discovered in 1927 in a study describing how mutations effects both homozygotes embryonic viability and heterozygotes tail development. When vertebrate subjects from the mutated gene were homozygous, they died from forming an improper axial and posterior mesoderm structure, while the heterozygous mutants with shorter tails lived (Holland et al, 1995; Papaioannou and Silver, 1998). These results demonstrated *brachyury* affects the posterior development of the mesoderm during gastrulation and is well conserved in the germ layer of vertebrates (Scholz and Technau, 2003). *Brachyury* also induced the formation of mesoderm in animal cap assays (Martinelli et. al, 2003). In invertebrate chordate ascidians, *brachyury* is expressed in fate-restricted notochord cells (Yasuo and Satoh, 1993 and 1994). Yamada et al. (2010) has shown that ctenophores require *brachyury* for the morphogenetic movements that form the blastopore and stomodaeum but is not involved in endomesodermal formation. Although *brachyury* is important in mesoderm formation in invertebrates, its role in basal metazoans remains unknown. Investigation into the role of *brachyury* would aid in the understanding of development in ctenophores.

## Materials and Methods

### *In-situ hybridization:*

For day one, fix whole specimens in 4% paraformaldehyde in Filtered Sea Water (FSW) overnight (O/N) at 4°C. Place no more than 10 animals in 50ml conical tube. To mix, hold on side and rotate gently. Then for day two, rinse 3 x for 10 min in PTW at Room Temperature. To mix, hold on side and rotate gently. Wash in 1:1 Methanol/ PTW 10 min at Room Temperature. Store in 100% MeOH at -20°C for 2 hours up to a week. Place on its side in the freezer to allow animals to be separated. On the third day, rehydrate specimen for 10 minutes in MeOH/PTW 3:1, 1:1, 1:3, 0:1 at Room Temperature. Wash in 1:1 solution of hybridization buffer and PTW for 15 min. at Room Temperature. Incubate (prehybridize) in HB buffer for 1 hour at 60°C. Incubate (hybridize) in HB with DIG-RNA probe O/N at 60°C. Then for day four, wash in HB for 30 min at 60°C. Wash in 1:1 HB/PTW for 30 min. at 60°C. Wash in PTW for 30 mins. at Room Temperature. Block in 10% Goat Serum(GS) for 60 min at Room Temperature. Incubate in anti-DIG 1/2000 at 4°C O/N. Finally for day 5, wash 4 x 30 min in PBS Room Temperature. Make detection buffer and aliquot 1ml into clean well for each sample. When ready to develop, add 20 uL of NBT/BICP mmix until dissolved. Should be yellow in color. NOW add samples. Put on ice and cover with foil. Add animal into .5x PBS for 5 min wash. Then put into 4% paraformaldehyde and EtOH. Add animal into 100% Ethanol (can store for 2 weeks at this stage). Then place animal into a new EtOH well, and cut into desired pieces. Place animal into Methyl Salicylate in a new well, try to stretch flat

(repeat if necessary). Place animal onto slide, remove methyl silylate that remains.

Then place a few drops of Permunt onto slide, place cover slip on slide.

#### *Alignments and Gene Tree Construction:*

Using the *Homo sapien* protein sequences from the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/>) to find the homologues within *Pleurobrachia bachei*. From there, I used the Moroz/Swalla Lab's gene database sever to acquire the conserved protein sequences of *P.bachei*. With these sequences, I ran the BlastP against the NCBI database to gain conserved sequences from across the Metazoa for the gene tree comparisons. Lastly, I used MEGA 5 software to align my sequences and obtain a minimum evolutionary gene tree with having the bootstrap values below 50% collapsed.

#### Results

The expressions pattern seen in the figures below show that there are consistencies within the *in situs* that were done. For *Brachyury* in figure 1, 2, and 3 the gene is being expressed (shown in purple) within the esophagus and stomach.



Figure 1. Esophagus of Adult *P. bachei* at 4x

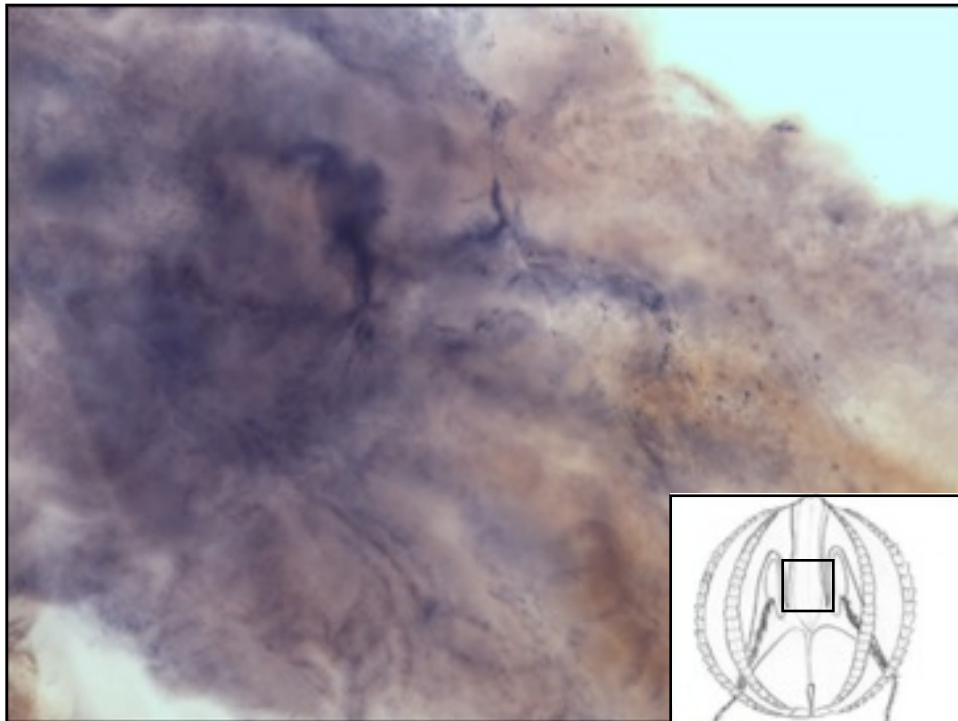


Figure 2. Stomach of adult *P. bachei* at 10x

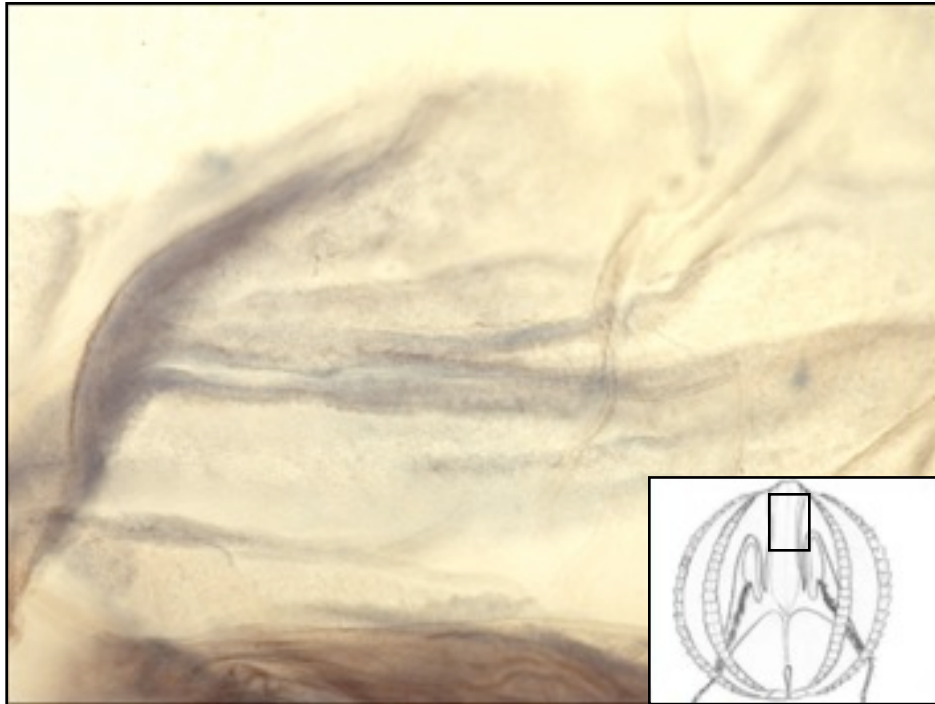


Figure 3. Stomach of adult *P. bachei* at 20x



Futhermore, in figure 4, it shows the results of a gene tree of the gene *brachyury* in *Ctenophores*.

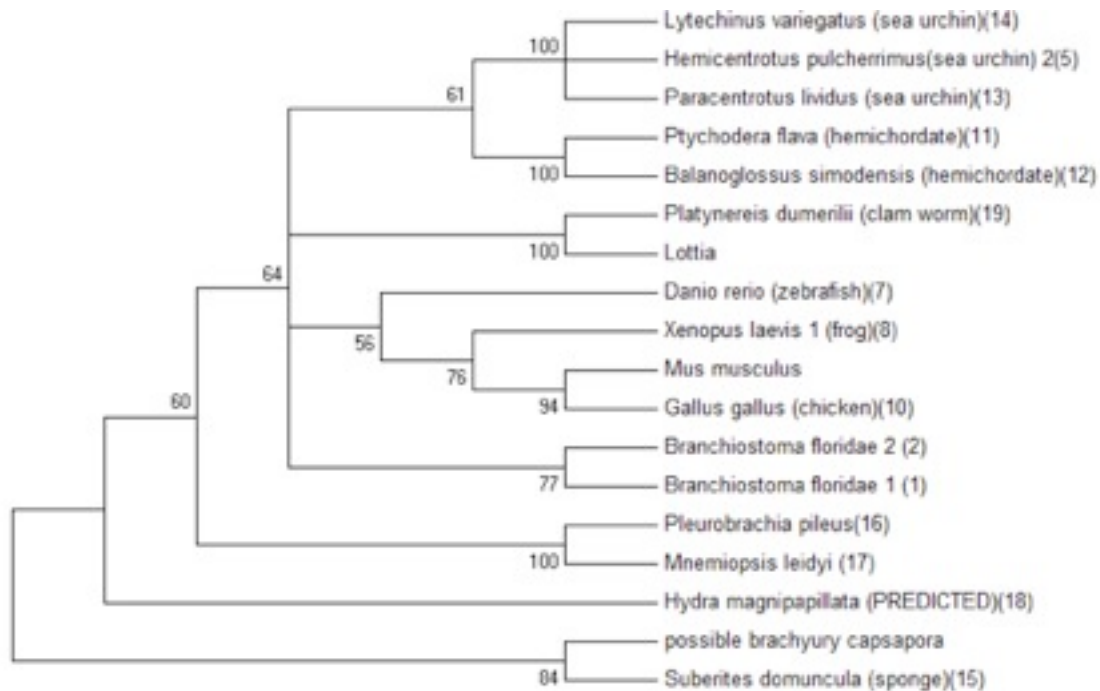


Figure 4. Gene tree for *brachyury* protein. The parenthesized numbers at the end of the species names are referencing a file used to organize the sequences, and not important to understanding the tree. All bootstrap values below 50% are collapsed.

For forkhead, it seems that when doing *in situ*'s that it the area that would be expressed constantly would be the comb rows along with the tentacle bulb, which can be seen in figure 5-10.

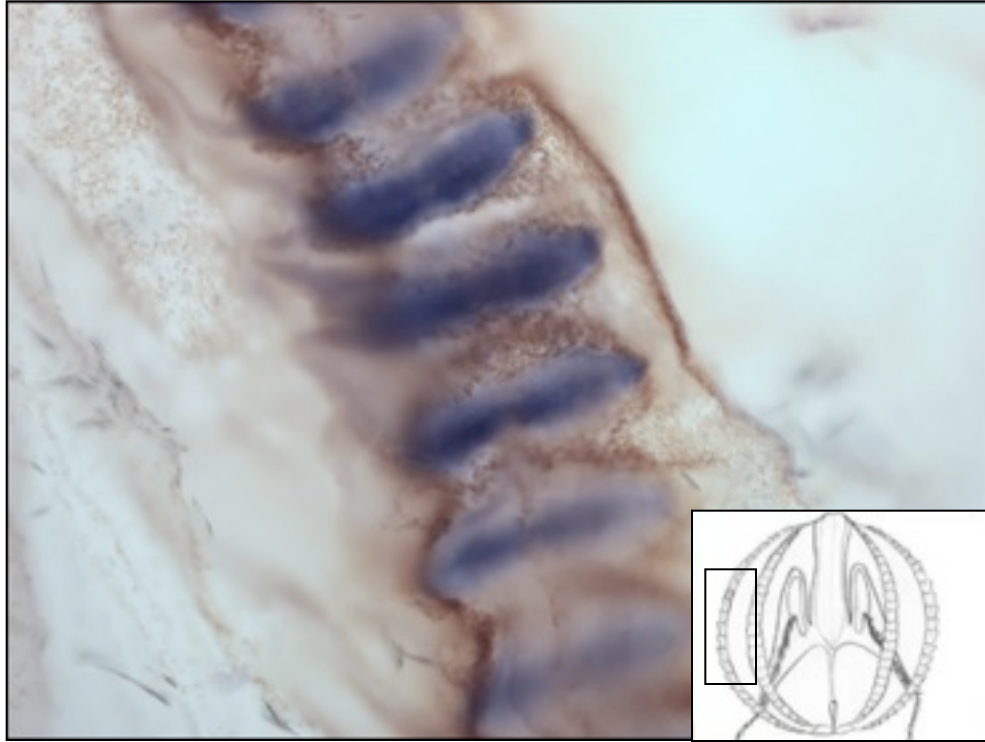


Figure 5. Comb rows of adult *P.bachei* at 10x

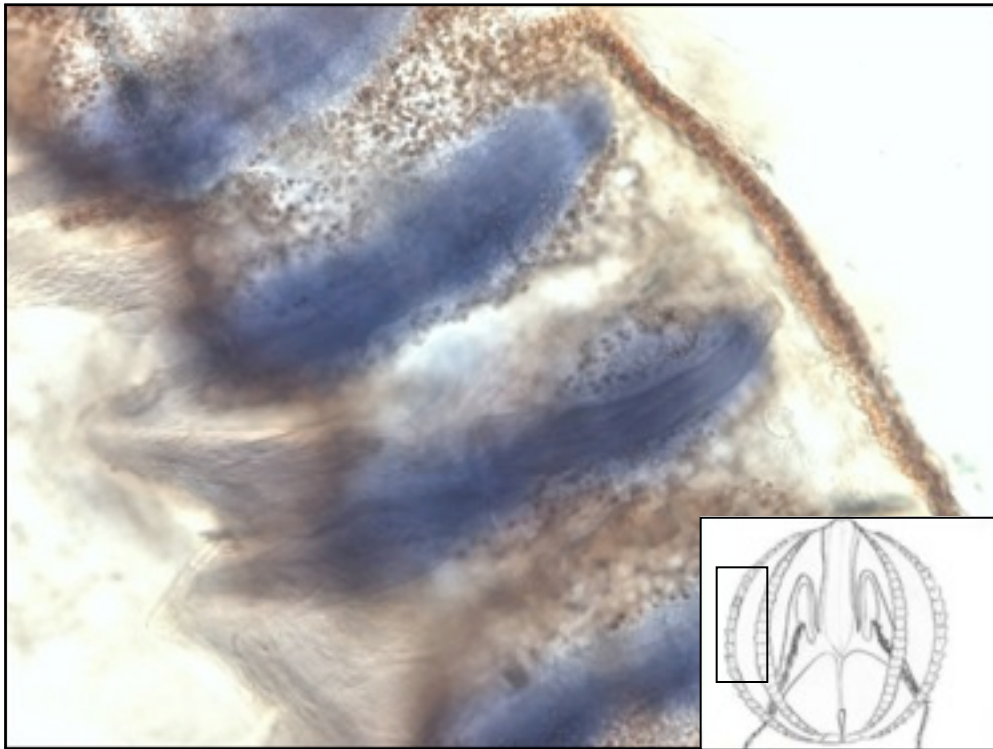


Figure 6. Comb rows of adult *P.bachei* at 20x

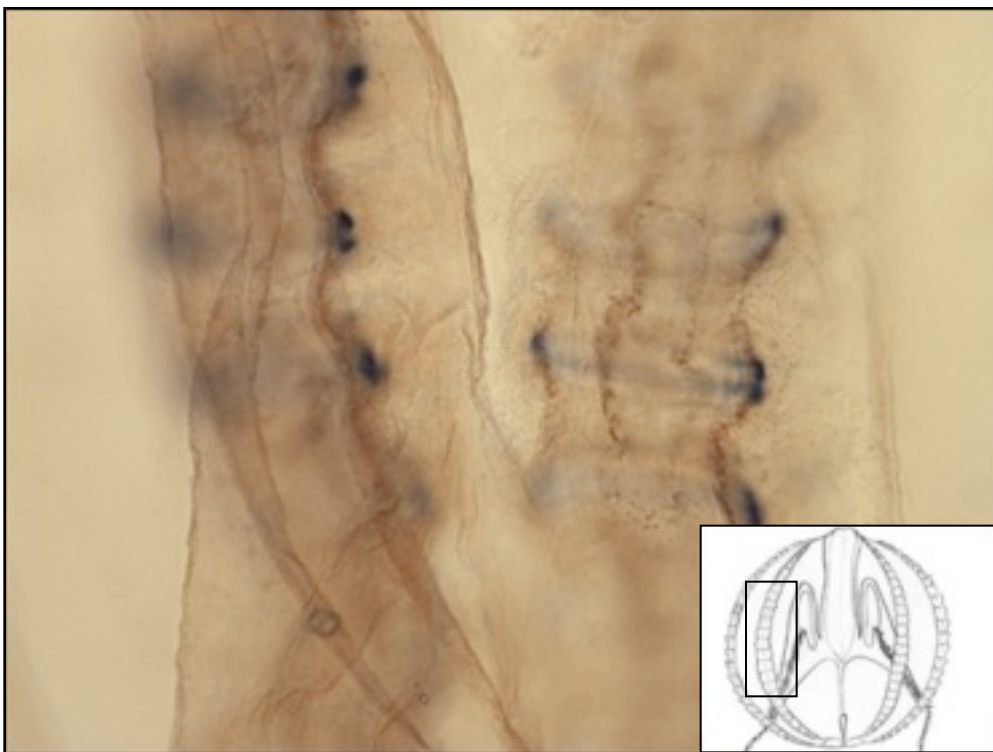


Figure 7. Comb rows of adult *P.bachei* at 10x

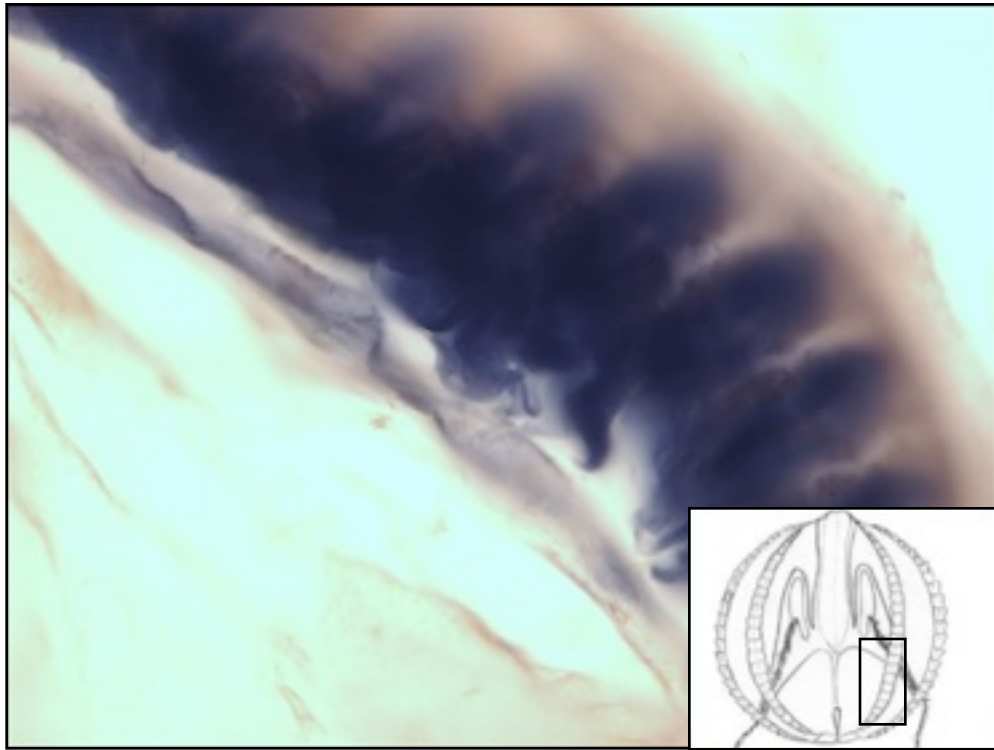


Figure 8. Comb rows of adult *P.bachei* at 10x

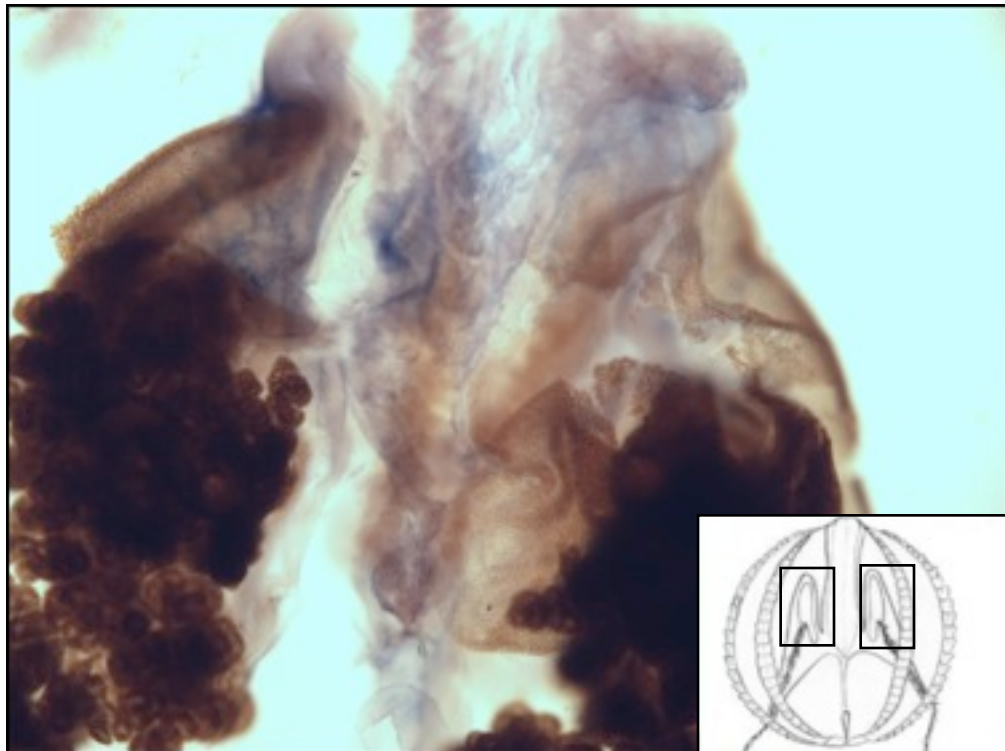


Figure 9. Tentacle bulb of adult *P.bachei* at 4x

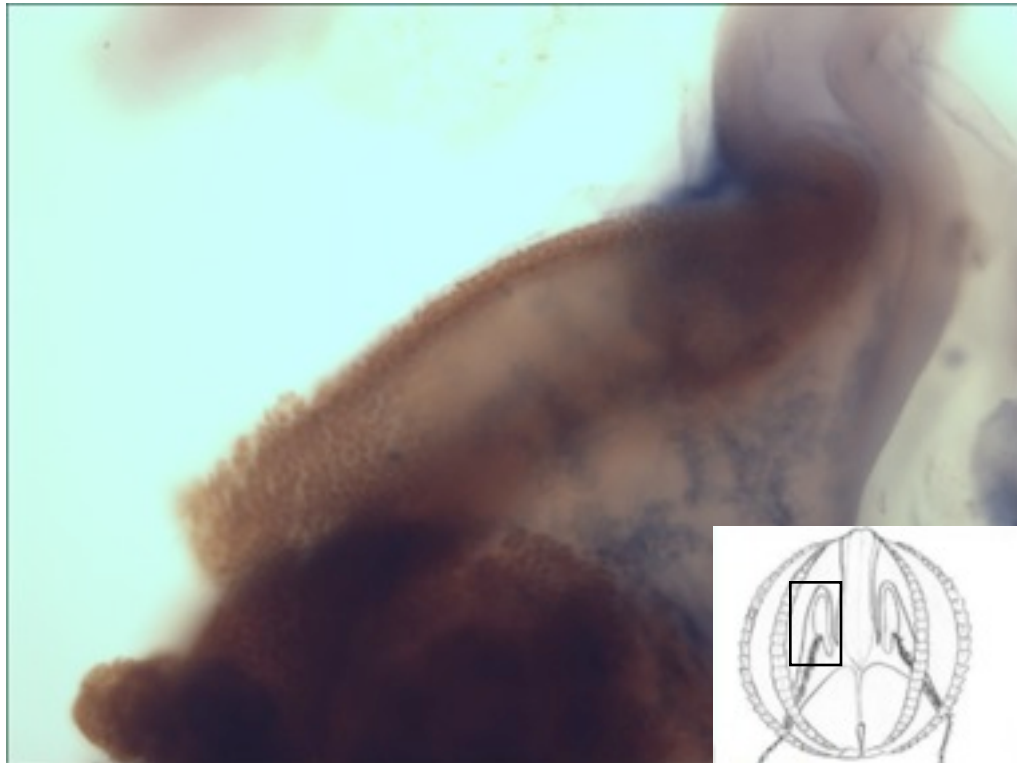


Figure 10. Tentacle bulb of adult *P.bachei* at 4x

Furthermore, in figure 11 and 12, it shows the results of a gene tree of the gene *forkhead* in *Ctenophores*.

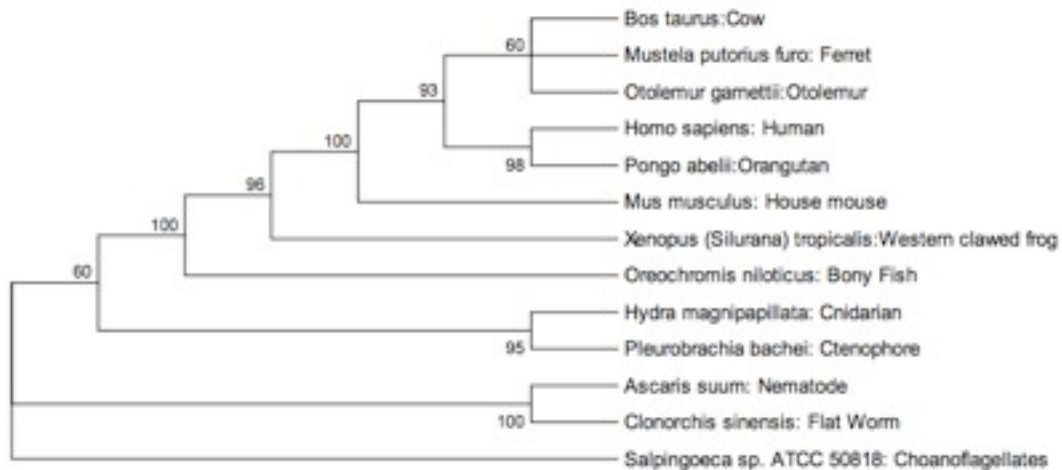


Figure 11. Gene tree for *forkhead* protein. Having a grouping of vertebrates and mammals on one end and basal invertebrates on the other side. Having the traditional group of cnidarians and ctenophores and a monophyletic group of nematodes and flat worms. All bootstrap values below 50% are collapsed.

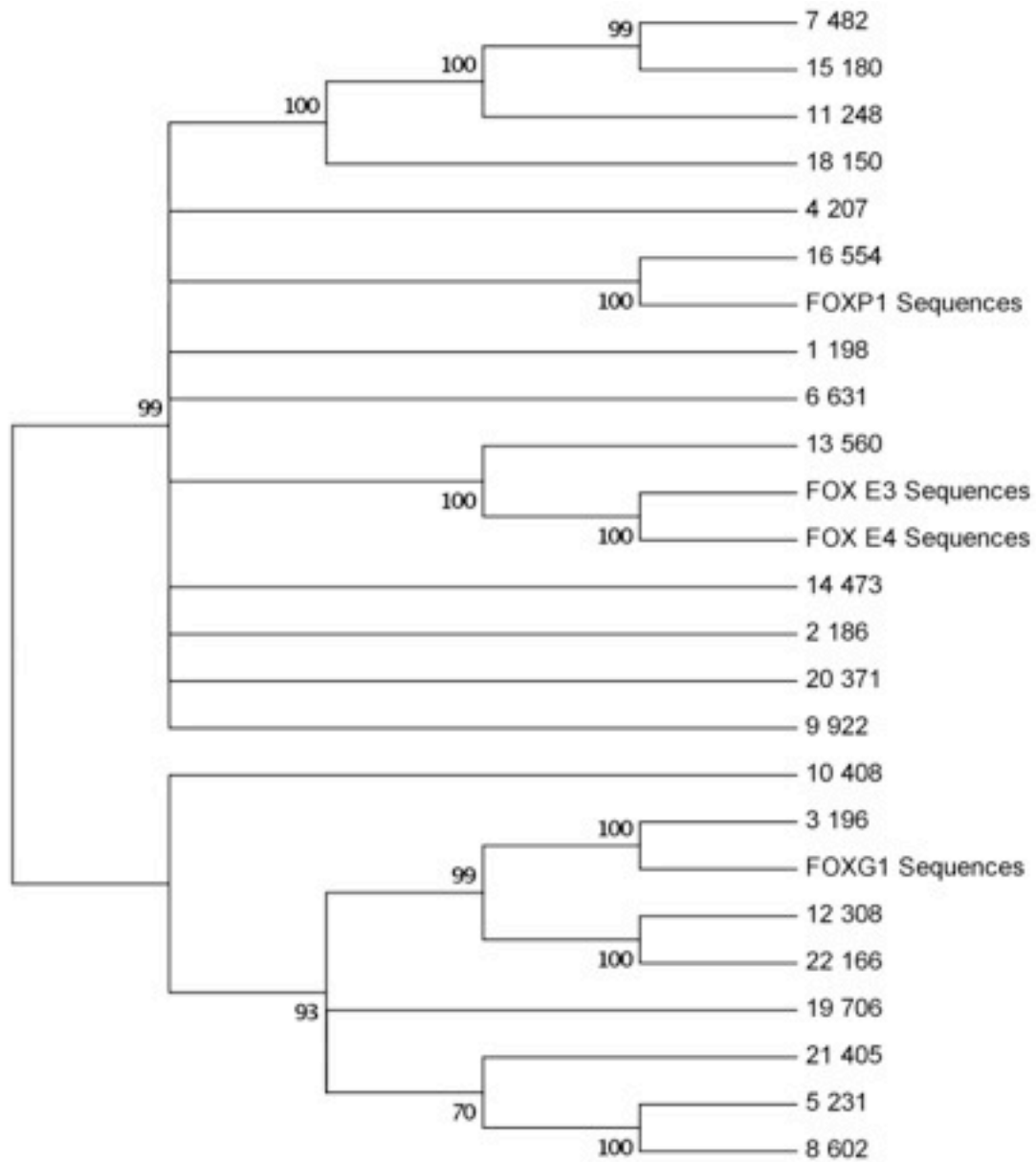


Figure 12. Gene tree for *forkhead* genome. Showing that is 25 genes within the *forkhead* genome of ctenophores.

## Discussion

### *Gene tree:*

#### *Forkhead* gene tree

The *Forkhead* gene tree of *FOXJ2/3* is grouping the mammals and vertebrates on one side, while group the basal invertebrates on side of the tree. Also showing the traditional group of ctenophores and cnidarians because they are both sister groups from one of another. Not to mention, the tree is showing the monophyletic group of nematodes and flat worms, but this group is problematic because its the main branch that it connecting them both has a bootstrap of 60. Meaning that the sole reason why the are connected could be due to long branch attraction.

Furthermore, the other gene tree shows how many *forkhead* genes are within the genome of ctenophore. There is 25 genes within the tree, but when aligning the genes in MEGA I saw that *FOXE3* sequence and *FOXE4* sequence were the same. Rendering the number of genes within the genome to be 24 instead of 25. Also the gene tree of the genome show that there is a split between all the sequences, but more research would have to done to show why they are divided.

#### *Brachyury* gene tree

The *brachyury* tree seems to be broken up into three groups. The first group is the deuterostomes, followed by the vertebrates, with the last group being the basal invertebrates. The gene tree groups the ctenophores together, but as more derived than

sponges. Since there is some debate on which group is more basal, this is an interesting result, but not an informative result. First, the bootstrap values are too low that even where the values are not collapsed, the topology is not entirely correct. Furthermore, as a gene tree the information contained within it is limited to the point where it is difficult to infer a phylogeny from a single gene. Finally, while representatives of the sponges and non-metazoans were included, but these alignments were not very good, and were only kept for the sake of comparison. These two species were probably placed as the most basal because they had the most divergent sequences, and thus had the greatest distance between the other species. When in all reality, divergence does not imply that a group is basal. For these reasons, the constructed gene tree should be looked at as perhaps a informative for grouping phyla, but not for inferring broader phylogenies.

#### *Expression Patterns:*

##### *Brachyury* Genes in *Pleurobrachia bachei*

It is interesting that *brachyury* is even expressed within the adults of the *Pleurobrachia bachei*, for the simple fact that the majority of research today suggests that the main function of this gene is a developmental role. Therefore so there is very little known about how this gene acts within adults, perhaps suggesting that *brachyury* has more than just the one function of being important in early morphogenetic movements (Yamada et al. 2010). Giving the possibility that *brachyury* is labeling the endoderm in *P.bachei* when they are young and when the embryo goes through gastrulation it helps form the esophagus and stomach. Since they need their esophagus



and stomach to digest prey, it is vital for them to maintain the expression of this gene to continue to survive. This could be a possible reason for the gene being expressed in adults and the properties that *brachyury* has in early developmental functions.

### *Forkhead Genes in *Pleurobrachia bachei**

Our results show that there are 24 *Forkhead* genes in *Pleurobrachia bachei*. These results suggest that ctenophores have an expansion of this type of transcription factor. There are at least two possibilities that can be explored because these results. One is that ctenophores have specialized tissues that has not been previously appreciated. A related second possibility is that predators have a more complex genomic underpinning of tissues than filter feeders do. This hypothesis would suggest that ctenophores have retained a complex genomic circuitry that has been lost in sponges and placozoans.

The *forkhead* gene family is still unexplored, which leaves many possibilities for future studies. *Forkhead J2/3* was expressed in the *P. bachei* comb rows. Since the comb rows are for motility purposes, it must be continually renewed. Expression was seen in the edges of the comb rows where the stem cells for the comb rows are located. Ctenophores are known to be constantly moving their comb rows for hours on end. Therefore, the comb rows can deteriorate and the expression of *forkhead J2/3* is necessary to either regrow cells or keeping them from deteriorating. *Forkhead* plays a key role in regulating cell growth and longevity in mammals, so this is a possibility (Jung JW et al, 2011).

### *Conclusions*

The things that I can conclude at this point in my research is that ctenophores have and express the genes *brachyury* and *forkhead*. Also there is 24 *forkhead* genes within the genome of ctenophores. However, further research needs to be done to find what functional role that these genes play within ctenophores. Namely, the embryonic stage, to see, on a developmental level, what these genes can do.

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