

**Searching for *Slit* and *Robo* in the Ascidians, *Boltenia villosa*  
and *Molgula oculata***

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**Abstract:**

The axonal guidance molecules *slit* and *robo* are known to interact producing a repulsive signal to the growing processes of nerve cells during development. These signals contribute to the proper ‘wiring’ of nervous systems. Ascidians possess comparatively simple nervous systems, yet still require mechanisms to establish proper synaptic connectivity. In this paper I attempted to identify and clone *slit* and *robo* from two ascidians, *Boltenia villosa* and *Molgula oculata*. I was unable to clone *slit* or *robo* sequences from either of these organisms, but was able to analyze the sequences from the *Molgula* transcriptome sequences. I constructed gene trees for both genes with available Genbank sequences. These trees grouped vertebrates together, but in both cases the high sequence divergence in the invertebrates resulted in a weak phylogenetic signal leading to a polytomy among the invertebrates.

**Introduction:**

All nervous systems, whether simple or complex, require mechanisms of patterning during their development. Given the number and precision of synaptic connections in even comparatively simple nervous systems, the challenge of pattern formation appeared early in metazoan evolution. (All metazoans, save sponges, have nerve tissue.) Neural patterning is therefore a fundamental issue in developmental biology, and when set in an evolutionary context, the question of how patterning mechanisms initially evolved, and were modified over time is an important one to answer. Much developmental biology has been done using animal models such as the sea urchin, worm (*Caenorhabditis*), frog (*Xenopus*), chicken and mouse, among others. These studies have given us significant insights into mechanisms of development, as well as a large suite of molecules involved in these processes. To gain a clearer picture of the evolutionary history of these mechanisms, we can look for common and divergent developmental processes and molecules allowing us to infer how developmental mechanisms evolved.

Nervous systems are constructed with networks of nerve and support cells (*i.e.*, glia). How these networks are established during development is a significant developmental question. This proposal addresses the establishment of neural connectivity in the nervous system of a group of marine invertebrates, the ascidians, within an evolutionary context by looking for molecules known to function in the guiding of nerve cell processes during development.

*Neural Network Formation – An Overview:* Neuroanatomist Ramon y Cajal was the first to suggest that developing nerve cell processes were guided by chemotactic factors in their microenvironments – a hypothesis which has proven remarkably accurate.<sup>1</sup> As neurons extend neurites towards their synaptic targets, these growing processes are

directed by chemical factors which signal through membrane receptors on the neurite's growth cone. This structure functions as a biological navigator reading the chemical map which directs the neurite to its synaptic target. Molecules known to participate in the patterning of neural networks include the netrins and their receptors DCC and the UNC5s, the semaphorins and their receptors, plexins and neuropilins, as well as their cofactors, the membrane bound Ephs and ephrins, and the slits and their robo receptors, the molecules of especial interest in this proposal.<sup>2,3</sup>

Despite complex signaling, the core pathways triggering axon guidance molecules and their receptors converge on common effector molecules.<sup>4</sup> Whether the signal is attractive or repulsive, the effectors act by reshaping the growth cone's cytoskeleton. Repulsive cues trigger the collapse of the cytoskeleton; attractive cues result in an enlargement of the actin cytoskeleton pushing the growth cone towards the source of the chemical cue. The Rho family of GTPases (RhoA, Rac, Cdc42) are major effectors for this cytoskeletal remodelling.<sup>5</sup>

*Slits/Robos*: The slit/robo signaling complex is best known for its role in inhibiting commissural axons in the mammalian spinal cord from re-crossing the midline.<sup>6</sup> However, robo was initially discovered in an invertebrate – *Drosophila melanogaster* – during a screen for mutants showing nerve cord defects.<sup>7</sup> A robo ortholog is also present in the nematode, *Caenorhabditis elegans* (*Sax-3*).<sup>8</sup> Robo receptors have also been cloned from other organisms including zebrafish, chicks and mice.<sup>9</sup> Slit is the primary ligand of robo receptors, as was first demonstrated in *Drosophila*.<sup>10</sup> Slits are proteolytically cleaved into two fragments, the larger of which (N-terminal fragment) interacts with Robo receptors.<sup>11</sup> The repulsive signaling role of slit/robo in regulating neurite growth near commissures in the nervous system appears to be conserved from *C. elegans* through *Homo sapiens*.<sup>12</sup> Work involving slit and robo mutants in both *Drosophila* and vertebrate model organisms (e.g., mouse) has shown that in the absence of these molecules growing neurites exhibit abnormal phenotypes including stalling at, or re-crossing the midline.<sup>13</sup> There is also evidence suggesting a role for slit/robo signaling in neuronal differentiation and migration in both *Drosophila* and vertebrate model organisms.<sup>14</sup> Overall, there is strong evidence that slit/robo signaling plays an essential role in the development of neuronal connectivity in at least invertebrates and model vertebrates. Like other guidance molecules and pathways, this signaling system shows a high degree of evolutionary conservation among vertebrates but exhibits divergence between and among invertebrates both with respect to the molecules and signaling pathways involved. However, whether these same guidance molecules and pathways play important roles in establishing neuronal connectivity in non-model invertebrate organisms has received little attention. For this reason making a sweeping statement about the evolutionary conservation of slit and robo signaling is inadvisable. However, in the species studied to date (*C. elegans*, *D. melanogaster*, *M. musculus*) slit/robo signaling is repulsive, and

*Drosophila* and vertebrate slit and robo proteins have also been shown to functionally interact.<sup>15</sup>

*Research Objectives:* I attempted to identify, clone and sequence orthologs of *slit* and *robo* in the ascidians *Boltenia villosa* and *Molgula oculata*, and use *in situ* hybridization to visualize their expression pattern during the development of the larval stages (neurula stage, tailbud stage and the tadpole stage).

*Rationale for Proposal:* The aim of this research is to provide evidence that ascidians have and express a major family of axon guidance molecules during development thereby showing evolutionary conservation of a chemotactic system for establishing neuronal patterning and network connectivity. Previous research has shown that the ascidians *Ciona intestinalis* and *Oikopleura dioica* express the guidance molecule netrin in their notochord cells. Discovery of netrin in the notochord of *C. intestinalis* suggested that, as in vertebrates, the notochord may be a source of developmental signals, notably in this case for projecting neurites.<sup>16</sup> In *Oikopleura*, netrin expression is seen at the neurula stage, early hatchling stage, late tailbud stage, stage 1 and stage 2.<sup>17</sup> Given that peripheral nerves from the caudal nerve cord extend along the surface of the ventral notochord, the presence of netrin is suggestive of a role in guidance.<sup>18</sup> Eph/ephrin signaling has also been shown in *C. intestinalis*, though no clear link to growth cone guidance has been shown.<sup>19,20</sup> Semaphorin signaling, like slit/robo signaling has not been investigated in any ascidian to date. Nevertheless it should be noted that the Rho family members and their regulators (*i.e.*, RhoGEFs, RhoGAPs) have been identified in the genome of *C. intestinalis* showing key effectors used in growth cone guidance are present.<sup>21</sup>

Given the presence of axon guidance molecules and their major effectors in at least some ascidians, and the apparent conservation of growth cone guidance mechanisms, I hypothesize that slit/robo signaling may be operative during the development of the nervous systems in *Boltenia* and *Molgula*. Such a finding will be of interest to both developmental and evolutionary biologists as it will provide insight into molecular mechanism through which the 100 neuron nervous system of *Boltenia*<sup>22</sup> is assembled, as well as showing the conservation of a set of neural patterning molecules.

## **Methods**

*Animals:* *Boltenia villosa* were obtained from the docks at Friday Harbor Labs. In preparation for whole mount *in situ* hybridization work, I spawned *Boltenia villosa* by combining eggs and sperm from the animal's gonads and fixed embryos at the neurula and tailbud stages in 4% paraformaldehyde (for method, see refs. 23,24). As I was not able to advance to *in situ* work, I stopped the embryological work.

*Primers:* Slit/robo binding has low stringency as the slit isoforms will bind robo receptor isoforms owing to similarity among the isoforms. *Drosophila* has one slit gene, while vertebrates possess three. Vertebrate slit genes show about 60% sequence similarity, as do robo receptors. However, there is considerable divergence when compared to a predicted sequence for *C. intestinalis*. A search of Genbank (NCBI) revealed *robo* sequence data for the following metazoan animals: *Anolis carolinensis*, *Taeniopygia guttata*, *Mus musculus*, *Xenopus laevis*, *Danio rerio*, *Ciona intestinalis*, *Saccoglossus kowalevskii*, *Strongylocentrotus purpuratus*, *Aedes aegypti*, and *Caenorhabditis elegans*. *Slit* sequences were also identified in these same animals. There is a hypothetical slit sequence in *Nematostella*, but no robo sequence; it was therefore excluded. In addition, *slit* and *robo* sequences were obtained for *Molgula oculata* using a transcriptome provided by B.J. Swalla. This was done by blasting the *Molgula* transcriptome for *slit* and *robo* sequences; three *Molgula oculata* sequences were returned for each.

Nucleotide sequence data was converted to protein sequence and used to create alignments using *Mega* (muscle alignment tool).<sup>25</sup> These alignments revealed that despite high sequence conservation among vertebrate *slit* and *robo* sequences, there was little conservation between vertebrate and invertebrate sequences. Furthermore, sequence divergence among the invertebrates was high such that no regions of conservation on which to build primers were located. To overcome this problem, only sequences from *Ciona intestinalis* and *Molgula oculata* were used in the alignment (MacVector). The following degenerate primers were designed for *robo*:

Robo forward primer: 5' – GRGKTCCRGARCCSACCAAT – 3' ( $T_m = 59.7^\circ\text{C}$ , GC content = 60%)

Robo reverse primer: 5' – CCYTTRTASGGWCGKATGCG – 3' ( $T_m = 56.6^\circ\text{C}$ , GC content = 57.5%)

The *slit* sequences were also highly divergent between *Ciona* and *Molgula* and so three pairs of primers – one forward, two reverse – were designed to maximize the likelihood of amplifying the sequence from *Boltenia*.

Slit forward primer: 5' – TGYGRTTGYYTTTRCTWC – 3' ( $T_m = 49.7^\circ\text{C}$ , GC content = 30.4%)

Slit reverse primer#1: 5' – YTKGAYYTKAARSAWAACYTKAT – 3' ( $T_m = 47.3^\circ\text{C}$ , GC content = 41.1%)

Slit reverse primer #2: 5' – TYKGTTYTCTGWCAARTCCAA – 3' ( $T_m = 52.1^\circ\text{C}$ , GC content = 38%)

*PCR:* Genomic DNA was extracted from *Boltenia villosa* using a standard Qiagen DNA extraction protocol. cDNA from *Molgula oculata* had been previously prepared (B.J.

Swalla) and was used in these experiments. PCR was used to attempt to identify and amplify both *slit* and *robo* genes from the two ascidians. The following reaction program was run as the ‘standard’ from which additional modified program were based:

Denature, 94°C, 4min.	} 35 cycles
Denature, 94°C, 1min.	
Annealing, 47°C, 30sec.	
Extension, 72°C, 1min.	
Extension, 72°C, 10min.	
Hold, 6°C	

This reaction did not amplify either *slit* or *robo* with the template used. It was repeated and, using *Molgula* genomic DNA as a template, a faint band was observed when *slit* reverse primer #2 was used. In an attempt to amplify *slit* sequences from *Boltenia* and *robo* sequences from either organism, the annealing temperature was adjusted to 43°C. A lower temperature of 40°C with 50 cycles was also used to no avail. Finally a “touch down” PCR was used (annealing temperature of 48°C, decreasing 0.3°C for 50 cycles). No bands were seen in this reaction either. The single *slit* band obtained from *Molgula* was purified and later reamplified using PCR (*slit* reverse primer #2), following a failed transformation.

**Purification, Cloning & Sequencing:** The single band obtained for *slit* (from *Molgula* cDNA) was cut from the gel and purified using the Illustra GFX PCR Gel Purification kit. Ligation of my PCR product was done at 15°C using for 1 hour (TOPO TA dual promoter vector). Miniprep were done using the 5-Prime kit. Chemical transformation was completed in competent *E. coli* cells, and colonies were grown up on X-Gal and kanamycin-containing plates. The first transformation using the initial *slit* band resulted in negative colonies (100% blue colonies). This was confirmed by plasmid digestion with *EcoRI* which demonstrated there was no insert. Reamplification of the PCR product to increase the concentration of the product led to 4 positive plates (22 positive colonies total) out of 19 total plates during the second transformation. Digestion confirmed the presence of an insert. These cDNAs were sent for sequencing. Returned sequences were Blasted to determine if the PCR product was *slit*.

**Gene Tree Construction:** Gene trees were constructed using the alignments previously utilized to design PCR primers. The maximum likelihood statistical method of gene tree construction with 100 bootstraps was utilized for both the *slit* and *robo* trees. *Mega* software was used for generating gene trees.

## **Results:**

**Genes:** A single PCR band was obtaining using *slit* forward primer and reverse primer #2 in *Molgula oculata* following PCR conditions given in the Methods (Figure 1). No other PCR products were obtained. Transformation with this purified product yielded 4 transformed colonies. Digestion with *EcoRI* showed these colonies had taken up the vector containing the insert (Figure 2). These vectors were sent for sequencing (see,

Appendix 1). Each sequence was run through Blast that revealed that none of the inserts was a *slit* sequence. The PCR product was identified as a zinc finger protein. None of the sequenced PCR products contained the *slit* primer sequences. Therefore, I was unsuccessful at amplifying either *slit* or *robo* from the genomes of the ascidians, *Boltenia villosa* and *Molgula oculata*.

*Gene Trees:* Gene trees were also constructed for *slit* and *robo*. The *robo* tree showed the vertebrates as a monophyletic grouping with strong bootstrap values, however, the high divergence of the invertebrate sequences produced a tree with weak bootstrap values which is also inconsistent with prevailing hypotheses in metazoan phylogeny. As a consequence, a cut-off bootstrap value of 70% was utilized which maintained the vertebrate clade, but collapsed the invertebrates into a polytomy (Figure 3). The *slit* gene tree also recovered the vertebrate group, but had weaker support. As with the *robo* tree, the invertebrates formed unusual groupings with low support (Figure 4). The low bootstrap values throughout the vertebrate clade prevented collapsing the gene tree. *Caenorhabditis elegans* was used as an outgroup for both gene trees.

### **Discussion:**

*Slit & Robo:* The attempt to clone *slit* and *robo* genes in *Boltenia villosa* and *Molgula oculata* was unsuccessful. Several factors likely contributed to this failure. First, the poor sequence conservation made it very difficult to design specific primers for *Boltenia*. The sequence divergence seen in the invertebrates suggests the possibility that the *slit* and *robo* genes in *Boltenia* may be sufficiently divergent from the primers designed using *Ciona* and *Molgula* sequences that the primers would fail to hybridize with the *Boltenia* genomic DNA. Given the divergence of the *slit* and *robo* genes among the invertebrates, that the primers used would fail to amplify these sequences in *Boltenia* is not entirely surprising. That *robo* could not be visualized in *Molgula* is more interesting since primers were designed using sequence data from this organism. Primer dimers were repeatedly seen on the gels where *robo* primers were used. Thus complementarity between the primers may have prevented detection of *robo* in both *Molgula* and *Boltenia*. The primers may also have taken on a secondary structure which prevented hybridization with the template DNA. The inability of the one pair of *slit* primers to detect the target sequence in either species may be explained in the same ways. However, the other pair of *slit* primers amplified a sequence which, following sequencing, was found not to be *slit*, or *slit*-like (*i.e.*, shared no common domains). Blast analysis revealed the insert to be a zinc finger domain binding protein. The primers were degenerate and so it appears the gel band was the result of unintended primer hybridization. That I was unable to obtain this same band when I repeated the PCR with the primers under the same conditions suggest that the band obtained was possibly unrepeatable. However, as absence of evidence is not evidence of absence, we should not conclude ascidians lack axonal guidance molecules.

*Phylogeny:* The gene trees obtained did not shed new light on the evolutionary history of slit and robo molecules. As noted, in both trees the vertebrates form a monophyletic clade which is consistent with our understanding of animal phylogeny. However, the high divergence among the invertebrates gives too weak a phylogenetic signal to construct a meaningful hypothesis. This raises an interesting question: Could the function of slit and robo be different in certain invertebrates be different than it is among the vertebrates where these molecules function in neural patterning? Without functional studies, no conclusions can be drawn, however, the high divergence suggests the possibility that these molecules may function in a different context. Alternatively, we may have a case of developmental systems drift in which dissimilar molecules carry out the same function(s) in distantly related metazoans.

*Future direction:* Ascidian tadpoles have a nervous system containing approximately 100 neurons. The possession of such a system demands a developmental system for establishing its synaptic connectivity. The number of ways to establish such connectivity are limited. Features such as body plan topology, biophysical forces within the embryo, and chemical cues are key contributors in other systems and are likely to be important in ascidians as well.<sup>26,27</sup> In addition, there is evidence of classical guidance molecules in ascidians (*e.g.*, netrin) so the presence of slit/robo would not be surprising.<sup>28</sup>

However, future research must first successfully identify *slit* and *robo* sequences in *Boltenia* and *Molgula*. Given the difficulty in designing effective primers, this step may prove non-trivial. Nevertheless, I believe *slit* and *robo* are likely present in the genomes of *Boltenia* and *Molgula*, and are actively expressed during neural development.

Upon successful identification of *slit* and *robo*, I would attempt to show an overlap in the expression pattern of slit and robo from which functional signaling could be inferred.<sup>29</sup> Ideally, triple staining for *slit*, *robo* and individual neurons would be conducted. This *could* be done as previous work in ascidians has employed the monoclonal antibody UA301 to identify neurons in *C. intestinalis* and acetylthiocholine iodide to identify motoneurons in *Botryllus schlosseri*.<sup>28,29</sup> Alternatively a more general neuronal stain, cresyl violet, could be used to stain larval nerve cells. The disadvantage with this stain is that glia (present in ascidians) are also stained, but it might be interesting to try.

The ascidian nervous system consists of a central sensory vesicle in the head of the tadpole, neck ganglia and a nerve cord running down the dorsal side of the tadpole's tail. Furthermore, in 1998 Takamura published a largely anatomical study which showed that in addition to central ganglia there were two peripheral nerve clusters in the larval trunk and one in the tail.<sup>32</sup> These would be the regions in which I would expect to see an overlap in *slit/robo* staining. With respect to the temporal expression of *slit* and *robo*, I would expect the expression of these molecules to begin in or shortly after the neurula stage – when cells specified to form neural populations begin to migrate extend their

axons (see, figure 5). Since axon guidance molecules have previously been visualized in the vicinity of the notochord and dorsal nerve cord this further strengthens the case for looking carefully in these regions.<sup>33</sup> Study of the developing adult nervous system during metamorphosis is another period in which *slit* and *robo* may be expressed. Indeed, the developing adult nervous system is of greater complexity than that present in the tadpole, increasing the need for more advanced developmental systems. Therefore, both the embryonic and adult (metamorphosis stages) should be examined for evidence of slit/robo signaling.

The question of slit/robo function in ascidians also requires further study. Function can be inferred from expression patterns and knowledge of function in other systems. For example, observing slit and robo in or around the nerve cord would be consistent with a role in restricting the path of these growing processes, and therefore with a role in axon guidance. However, given the divergence of *slit* and *robo* in other invertebrates, functional studies would be necessary to confirm this. RNAi or morpholinos would be viable strategies for looking at the function of *slit* and *robo* during nervous system development in ascidians.<sup>34</sup> It may well be the case that slit and robo have different functions in these animals. Indeed, a recent paper has shown that conserved molecules can function in different ways in ascidians, so one must be careful when interpreting gene expression data and inferring function in ascidians based on the function of the same molecules in other systems (*e.g.*, in vertebrates).<sup>35</sup>

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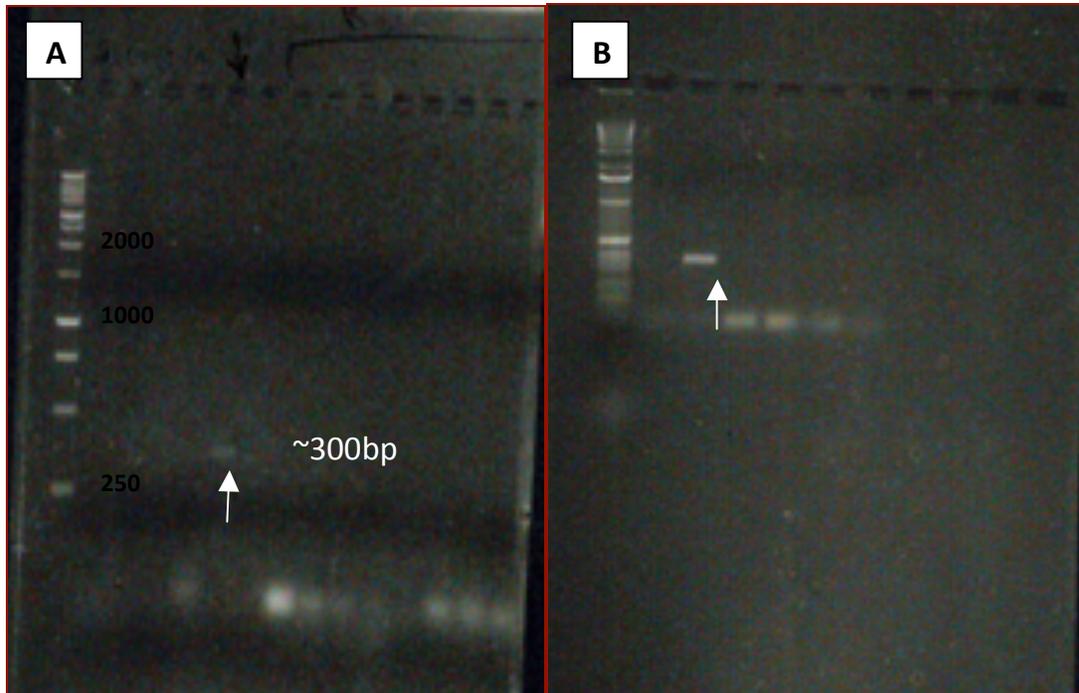
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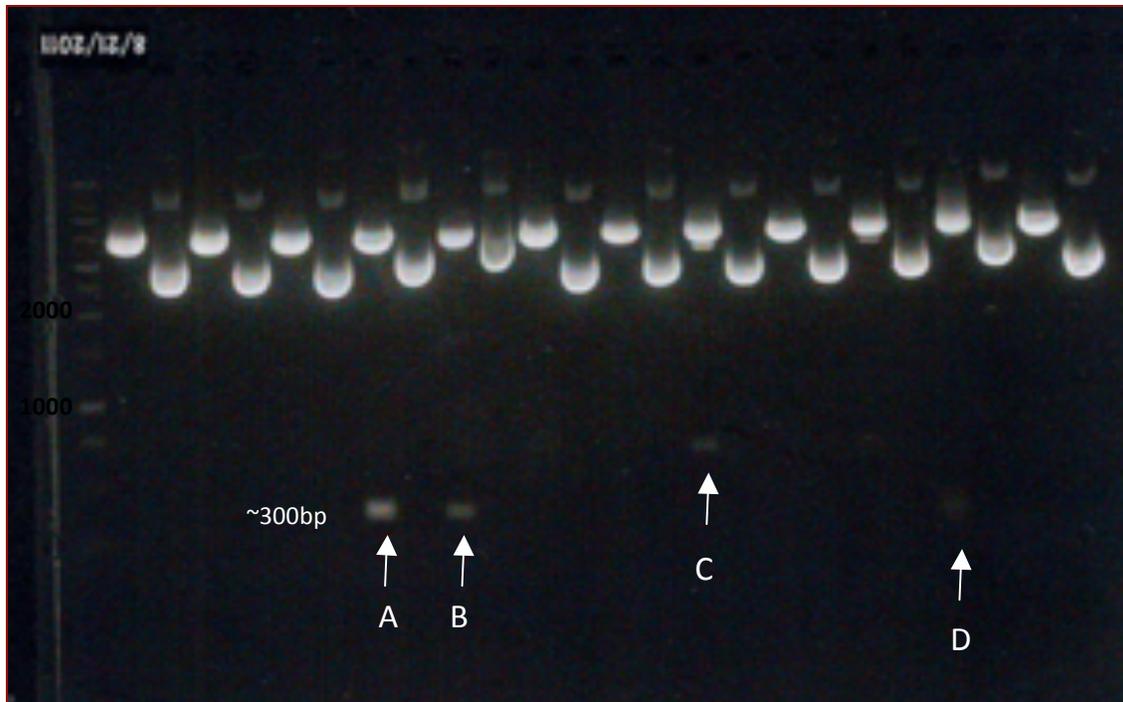
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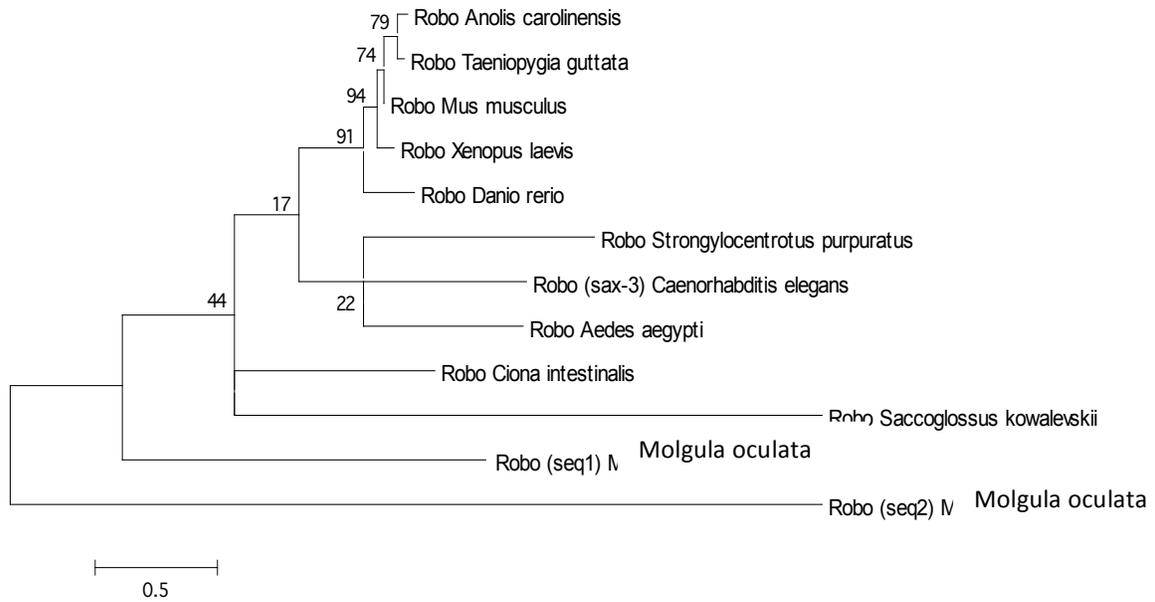
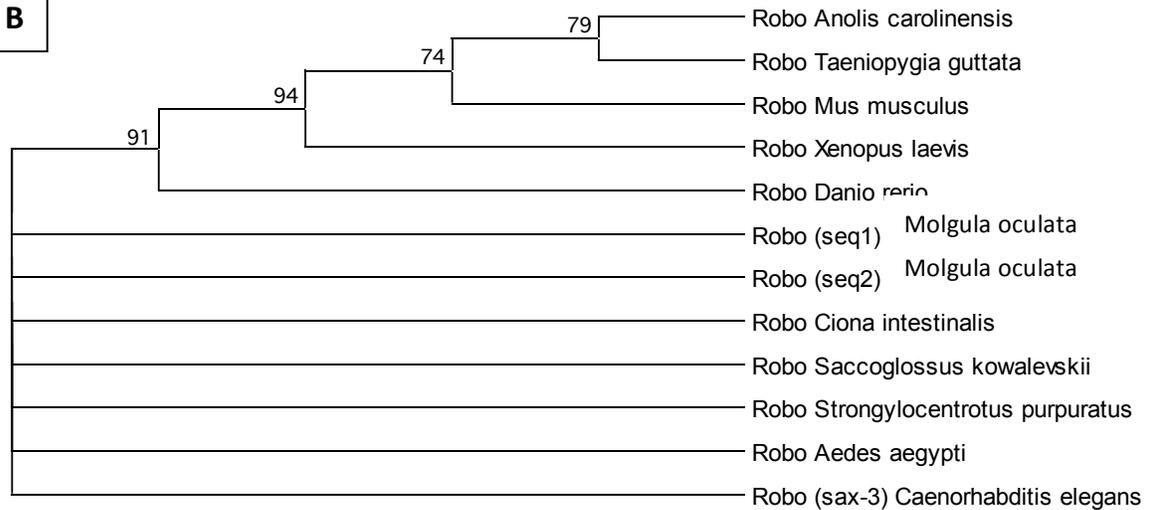
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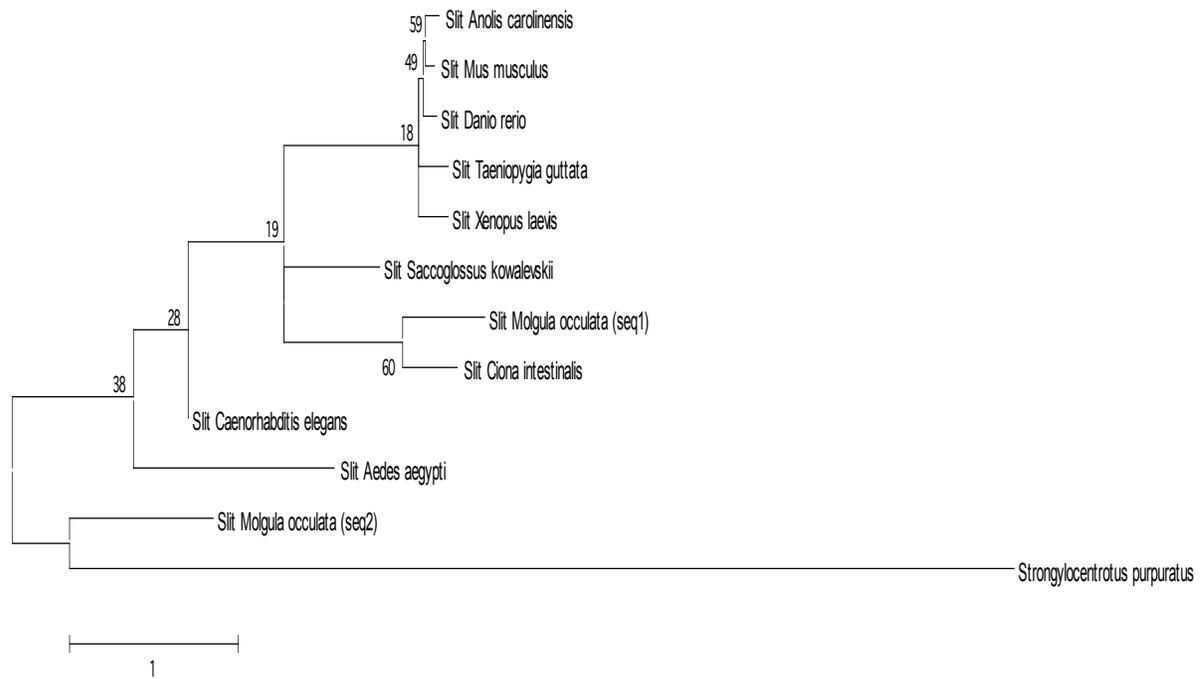
**Figure 1.** PCR Product initially thought to be slit. **(A)** A faint band is seen when the slit forward and #2 reverse primer were used (white arrow). The band was extracted and purified. **(B)** Reamplification of the band in gel A. Other bands are primer dimers.



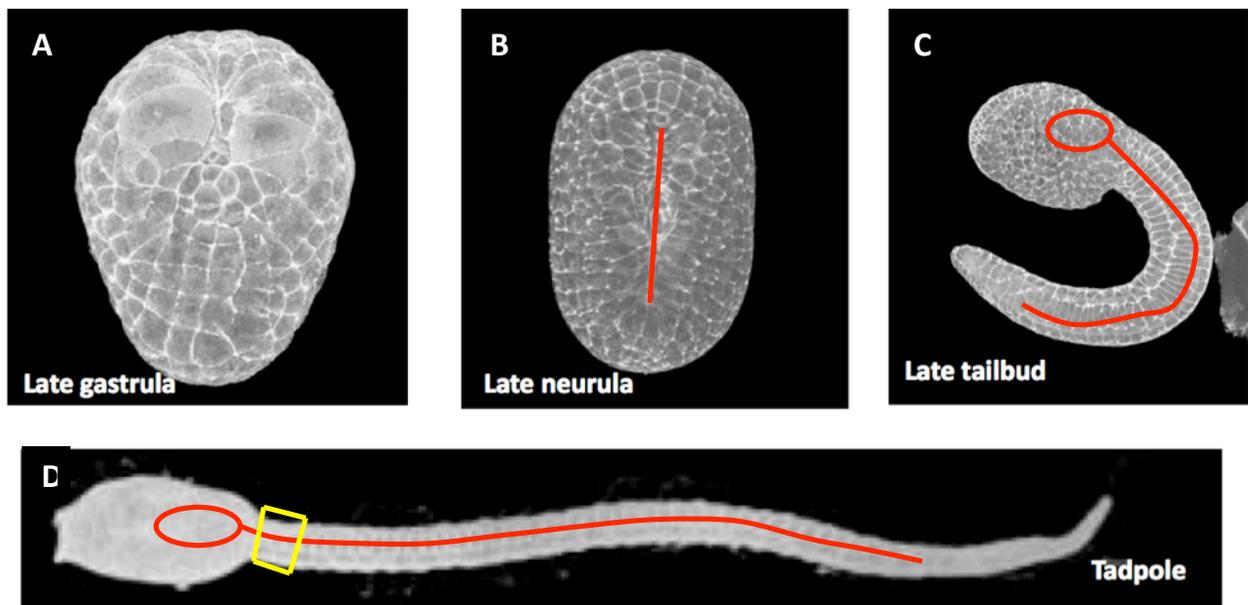
**Figure 2.** Restriction digest of TOPO TA vector with PCR product insert. Lanes alternate between uncut plasmid DNA (lanes 1, 3, 5ff) and digested plasmid DNA (lanes 2, 4, 6ff). Arrows indicate bands which were sequenced. Band 3 is clearly larger than the others. Each was identified as a zinc finger domain containing protein. Band sequences (A-D) are found in Appendix 1.

**A****B**

**Figure 3.** Gene tree for Robo (*Mega* software) (see Methods for details). **(A)** Maximum likelihood gene tree. **(B)** Collapsed robo gene tree (bootstrap values >70%)



**Figure 4.** Maximum likelihood gene tree for slit. (See Methods for details.)



**Figure 5.** Hypothesized regions of *slit/robo* expression. Expression may begin in the late neurula stage as the neural plate folds. Neurons forming the sensory vesicle in the head and the nerve cord require developmental signals, making these regions likely areas of expression (assuming *slit/robo* is expressed). Expression may also be seen around the ganglia located in the neck (yellow box). Images from [www.aniseed.cnrs.fr/](http://www.aniseed.cnrs.fr/)