Investigating the molecular basis of notochord loss in *Molgula occulta* 
via transcriptome sequencing

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Introduction

Ascidian tunicates, commonly known as ‘sea squirts’, are sessile hermaphroditic filter-feeders found in marine environments (Huber et al., 2000). Tunicates are *uro*chordates (i.e. ‘tail’ chordates) because they possess the defining chordate structure, the notochord, in a larval tail that is reabsorbed upon metamorphosis. This tail is believed to benefit progeny by facilitating motility, which in turn permits a wider variety of anchoring environments and a higher probability of finding favorable settling conditions (Huber et al., 2000). Despite the utility of this adaptation the larval notochord has been independently lost several times among the ascidians (Huber et al., 2000). These anural, or tailless, tunicates occur with the highest frequency in the Molgulidae family (Huber et al., 2000).

This study uses a newly sequenced transcriptome of closely related molgulid ascidians to evaluate notochord-specific gene expression in the anural tunicate *Molgula occulta*, the tailed tunicate *Molgula oculata* and *M. occulta x M. oculata* hybrids. Highly conserved genes expressed in the notochords of the urodele species *Ciona intestinalis* and *Molgula tectiformis* were examined for expression in *M. occulta, M. oculata* and *M. occulta x M. oculata* hybrids via transcriptome analysis. The results of this study may further elucidate the expression patterns essential to notochord development and implicate the nature of the genes involved in the evolution of taillessness. This study may also facilitate creating a clearer tunicate phylogeny through phylogenetic analyses that assesses the conservation of these key developmental genes in a Molgulid genome.

One compelling gene identified in the *C. intestinalis* and *M. tectiformis* transcriptomes is *prickle*, a gene family that plays a pivotal role in the planar cell-polarity
(PCP) pathway of Wnt-signaling. Studies in mice and *Drosophila* have shown that the PCP pathway is involved in coordinating cell movements during development in both vertebrates and invertebrates (Tao *et al.*, 2009). Within *C. intestinalis* and *C. savignyi*, *prickle* is necessary for notochord intercalation and is most highly expressed in notochord cells (Kulger *et al.*, 2011). Knock-out studies have also shown that prickle is essential in mediolateral (M/L) polarization during notochord morphogenesis; *prickle* is expressed in notochord cell membranes, where cells detect interactions with neighboring tissues (Jiang *et al.*, 2005). As *C. intestinalis* contains two *prickle* genes—*prickle1* (pk1) and *prickle2* (pk2)—this study will search for expression of either *prickle* in *M. occulta* and *M. oculata*.

Other notochord-localized genes identified in the *C. intestinalis* transcriptome are *noto6* and *noto17*. These genes are enigmatic in that little is known of their products aside from their presence in urochordate notochords. Studies in the larvacean *Oikopleura dioica*, a member of another class of urochordata, showed diffuse expression of *noto17* in the notochord (Kulger *et al.*, 2011). *Noto6* has been detected in *Molgula tectiformis*, an anural ascidian, (Gyoja *et al.*, 2007) and a homologue has been isolated from human neural tissue, though its function has yet to be described (Hotta *et al.*, 2000). The implications of these results are still under investigation, but the variety of tunicates expressing these genes suggest that they may allow an interesting analysis of phylogeny.

In *C. intestinalis*, *leprecan* is a highly conserved, notochord-localized transcriptional target of Brachyury, a zinc-finger transcription factor that is essential in ascidian notochord development (Di Gregorio *et al.*, 2002). *Ci-leprecan* encodes collagen-producing prolyl-3 hydroxylases and is regulated by *Ci-Bra* via a compact *cis*-regulatory
module (CRM) (Dunn and Di Gregorio, 2009). Dunn and Di Gregorio found that *C. intestinalis* with selectively knocked-out *Ci-lep
can* develop a suboptimal notochord phenotype, which suggests *Ci-lep
can* is essential in notochord development. Another study in *C. intestinalis* found that *Ci-lep
can* is most highly expressed in the notochordal sheath (NS) (Hotta et al., 2000); as the notochord affects specification of surrounding tissue (Liem et al., 2000), this may mean *Ci-lep
can* contributes to tissue specification. While *M. occulta* has been shown to have normal early expression of *Brachyury* in the notochord precursor cells (Takada et al., 2002), Molgulid *lepre
can* expression remains unexplored. This study will then build upon past studies by assessing the extent to which the *Ci-Bra/ Ci-lep
can* association is conserved in *M. occulta* and *M. oculata*.

Finally, we will examine the expression of *merlin*, the product of the NF2 tumor suppressor gene. *Merlin* is a linker between proteins embedded in the cell membrane and the actin-cytoskeleton, making it an important contributor to the orchestration of cell-cell interactions (McClatchey and Giovannini, 2005). Probing for *merlin* in *M. oculata* will give insight into the non-autonomous expression patterns at play during the tissue development of ascidian embryos. *Merlin* is also interesting because it is closely linked with nervous tissue, as is evident by how nonfunctional *merlin* correlates with human neurofibromatosis type II, the symptoms of which are schwannomas, ependymomas and meningiomas (Stamenkovic and Yu, 2010).
Materials and Methods

Animal Samples

*M. occulta* and *M. oculata* specimens were collected on the sand flats of Pointe de Bloscon at Station Biologique in Roscoff, France by Dr. Billie Swalla in 1999. Eggs and sperm extracted from the animal gonads were fertilized to raise *M. occulta* and *M. oculata* x *M. oculata* hybrids until embryos hatched. *In situ* samples of 10 life stages (F+1, F+1.5, F+2, F+3, F+4, F+5, F+6, F+7, F+8, F+9) of the *M. occulta*, *M. oculata* and hybrids were fixed in 4% paraformaldehyde and preserved in 70% ethanol; these samples were used in this study. The procedures for insemination, embryo culture and production of hybrids are previously described (Swalla and Jeffery, 1990).

Molgulid transcriptome

Genes selected for analysis were chosen from *M. oculata*, *M. occulta* and *M. oculata* x *M. oculata* hybrid transcriptomes sequenced by Illumina sequencing at Michigan State University. Nucleotide fragments were between 200-250 bp. RNAs were extracted from several different stages of development, sequenced, then reassembled; the lengths of reassembled *prickle, noto6, noto17, leprecan* and *merlin* were 1077 bp, 1154 bp, 960 bp, 888 bp and 1121 bp respectively. Data from the gastrula (F+3), F+4, and F+6 stages were used for analysis.

Sequence Alignments

Protein and nucleotide BLAST was used to confirm the identity of genes selected and to assess protein conservation. All genes were found to be highly conserved in *Molgula tectiformis* and *Ciona intestinalis*. BLASTx was used to derive protein sequences from the *M. occulta*, *M. oculata* and hybrid DNA sequences and to check the orientation of the
DNA sequences. Reverse complements of the transcripts were obtained using Sequencher. All forward nucleotide sequences and protein sequences from the *M. occulta, M. oculata* and hybrid transcriptomes were aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). For some genes, there were sections in which one transcriptome did not have a reading; this is being attributed to mistakes inherent in DNA sequencing assembly.

**PCR and Subcloning**

Primers were designed using DNA and protein alignments. Highly conserved sections at the start and end of each protein sequence were translated using the genetic code. This DNA sequence was then compared with the actual DNA sequences derived by Dr. Titus Brown *et al.* (2011, unpublished data). Each primer is between 20-23 bp and was selected for highly conserved 5’ and 3’ end sequences and minimal degeneracy. The forward and reverse primers used are listed in Table 1:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Tm (°C)</th>
<th>Reverse</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>prickle</strong></td>
<td>5’-GATAGCGGATTTCCCTTGGGAAG-3’</td>
<td>55.4</td>
<td>5’-CGCTTCGGATGGTAGCTACAGCTC-3’</td>
<td>60.1</td>
</tr>
<tr>
<td><strong>noto6</strong></td>
<td>5’-ATGTATGAAATAAACAAGATGG-3’</td>
<td>45.7</td>
<td>5’-TCAGTCAAATAAAGAATATTTAA-3’</td>
<td>43.8</td>
</tr>
<tr>
<td><strong>noto17</strong></td>
<td>5’-GAGAGTCAAAACATGGATCCG-3’</td>
<td>53.4</td>
<td>5’-TCACTGGTTCCAGGAAGCAT-3’</td>
<td>61.2</td>
</tr>
<tr>
<td><strong>leprecan</strong></td>
<td>5’-GAACCTGAAACTGAAATTTG-3’</td>
<td>51.9</td>
<td>5’-GCGTCAGTATCAAGGAACAT-3’</td>
<td>52.4</td>
</tr>
<tr>
<td><strong>merlin</strong></td>
<td>5’-AAGAGCATTAACAGTTTGGCATTTCC-3’</td>
<td>52.6</td>
<td>5’-CTTCTTTAGTTTGGTTTTCAT-3’</td>
<td>49.4</td>
</tr>
</tbody>
</table>

Primer melting points (Tm) ranged from 43.8°C to 61.2°C (see Table 1). PCR conditions were 35 cycles of: 1. Denature at 94°C for 4 minutes, 2. Denature at 94°C for 1 minute, 3.
Anneal at 40°C for 1 minute. 4. Extend at 72°C for 2 minutes. The final PCR cycle was at 72°C for 10 minutes for extension.

PCR inserts were manually extracted from gels and isolated using the GenElute™ Plasmid Miniprep Kit. Following isolation, inserts were incorporated into 3956 bp 4-TOPO® bacterial vectors with T3 and T7 priming sites on either end. These priming sites facilitate linearization for the transcription of sense and anti-sense strands; Not I was used to linearize the T3 strand and Pst I was used for the T7 strand. On the immediate 6-10 bp following either end of the insert there are also EcoRI restriction sites useful for preliminary digests to confirm insert length. Standard transformation procedures were used to ligate plasmids and transform E. coli via temperature shock. Subclones were transformed in INVA F' strain of E. coli and grown on Kanamycin plates at 37°C. Successfully transformed and proliferated E. coli were stored in sterile glycerol and stored at -70°C.

Results

Transcriptome Alignments

BLASTp searches (Altschul et al., 1997) using protein sequences derived from the M. occulta and M. oculata transcriptomes showed that prick1e is highly conserved among the ascidians, as well as in more distantly related species like Homo sapiens (see Figure 1). The highest BLASTp scores were Mt-prick1e, Ci-prick1e2 and Ci-prick1e1. For homologues in other organisms with more than one prick1e type, prick1e2 was generally the closest match to Mo-prick1e. Three zinc-binding sites were identified: LIM1 between the 105th and 160th amino acid, LIM2 between the 170th and 220th amino acid and LIM3 between the 230th and 285th amino acid; these are all members of the LIM superfamily, a
group characterized by two highly conserved zinc-finger motifs. A fourth domain, PET_prickle from the Prickle Espinas Testin (PET) superfamily, is found between the 1\textsuperscript{st} and 100\textsuperscript{th} amino acid; this is also characterized by a zinc-finger protein-protein interaction domain. As the molgulid transcriptome sequences are approximately 400 amino acids shorter than that of \textit{M. tectiformis}, it is likely that the full molgulid sequence for prickle has not been isolated (Figure 1). Within the molgulid sequences, there are no significant gaps, meaning that \textit{Mo-prickle} has been highly conserved in the chordates (Figure 1).

\textit{Noto6} is well conserved among molgulids but not in more distantly related species like human (Figure 2). The human protein sequence is much longer because a section is absent in all the aligned ascidian sequences (Figure 2); this most likely merely indicates an evolutionary divergence between ascidians and human. Insertions were apparent in the lancelet and human \textit{noto6} homologues approximately halfway into the human protein sequence (Figure 2). No identifiable binding regions were found in BLASTp, though the full sequence was identified as Glyco-tranf-GTA-type superfamily (Marchler-Bauer et al. 2011). Domain hits include the single domain Chondroitin N-acetylgalactosaminyitransferase and multi-domain riboflavin synthase subunit alpha.

A \textit{noto17} BLASTp search yielded one nonspecific hit in the Thioredoxin_like superfamily with a CXXC motif between the 40\textsuperscript{th} and 90\textsuperscript{th} amino acid (Kikuchi et al., 2002). The molgulid \textit{noto17} sequence may be incomplete because the sequence lengths of the transcriptomes are significantly shorter than that of \textit{M. tectiformis}; more rounds of sequencing \textit{Mo-noto17} will help in resolving the full length sequence.
Leprecan was highly conserved among the molgulids, the non-molgulid ascidian *C. intestinalis* and human (Figure 4). A sizable portion of the full sequence appears to be present, but the fact that the transcriptome sequences are shorter than *M. tectiformis* suggests the ends of the sequence may be missing (Figure 4). The molgulid leprecan protein sequence had a 20G Fe(II) oxygenase superfamily match between the 110th and 296th amino acid.

Merlin is also highly conserved among the molgulids; there is a low level of conservation with human, but it is worth noting that there were no insertions or deletions present in the human protein sequence. We do not appear to have the full protein isolated, as there are large gaps of information missing between the molgulid transcriptomes themselves and between the transcriptomes and *M. tectiformis* (Figure 5). BLASTp identified an ezrin/radixin/moesin (ERM) protein domain between the first and 210th amino acid; this domain characterizes a phosphatase, which supports the previously established function of merlin (McClatchey and Giovannini, 2005). There is a strongly conserved PH-like core between the 210th and 320th amino acid within a FERM-C domain; this site is capable of both peptide and lipid binding (García-Alvarez et al., 2003, Hamada et al., 2000). Upstream there is also a FERM_N and FERM_M domain between the 20th-90th amino acid and 100th-210th amino acid respectively.

**PCR and Subcloning**

The first PCR reaction had an annealing temperature of 45°C and produced some bands, but none with a proper length. *M. occulta Mo-prickle, M. oculata Mo-prickle, M. occulta Mo-noto17, M. oculata Mo-noto17, M. occulta Mo-leprecan, M. oculata Mo-leprecan, M. occulta Mo-merlin,* and *M. oculata Mo-merlin* were amplified by PCR with
an annealing temperature of 40°C (Figure 7). PCR was repeated for *Mo-noto6* at 38°C, but this temperature also failed to amplify *Mo-noto6* (Figure 8). The failure of my *Mo-noto6* primers may be attributed to the apparent incompleteness of the *Mo-noto6* sequence; further sequencing may reveal more inclusive primer sites.

Subclones were successfully grown for *Mo-prickle, Mo-noto17, Mo-leprecan* and *Mo-merlin* for both *M. occulta* and *M. oculata*. When 100 µl of *E. coli* transformed with these genes were grown on Kanamycin plates, *Mo-prickle* for *M. oculata* produced the most colonies (31) and *Mo-leprecan* for *M. oculata* produced the least colonies (2). Minipreps digested with EcoRI revealed that the inserts for *Mo-noto17, Mo-leprecan* and *Mo-merlin* were shorter than the anticipated lengths of 960 bp, 888 bp, and 1121 bp respectively (Figure 9). *Mo-prickle* for *M. oculata* consistently appeared at approximately 1077 bp (Figure 10), as anticipated from the *Mo-prickle* sequence. *Mo-prickle* for *M. occulta*, however, appeared notably shorter than *Mo-prickle* for *M. oculata* (Figure 10).

RNA probes were successfully made for the T7 strand of *Mo-prickle* (Figure 11), but not for the T3 strand; a low concentration of DNA in the T3 transcription reaction may account for the insignificant yield of T3 probes.

Discussion

Prickle in the Molgulids

The *M. occulta* and *M. oculata prickle* sequences aligned in this study may imply the presence of more than one *prickle* in the molgulids (Figure 1). *Prickle* is well conserved between *M. occulta* and *M. oculata*, but there are several permutations in the aligned amino acid sequences that may justify a distinction between *prickle* in these species. The relative sizes of *prickle* in *M. occulta* and *M. oculata* appear to be different.
in the DNA segments seen in the miniprep *EcoRI* digestion (Figure 10). This could potentially imply a deletion in the *prickle* sequence of *M. occulta*, but only sequencing the isolated genes may give immediate conclusive results. A search for restriction sites within *prickle* using Sequencher found that *prickle* in *M. occulta* and *M. oculata* have *Hind III* and *Xba I* restriction sites in common, but otherwise have several unshared restriction sites; *prickle* in *M. occulta* has *Kpn I, BamHI*, and *Pst I* restriction sites (Figure 12) while *prickle* in *M. oculata* has *Sac I* and *Bst XI* restriction sites (Figure 13). The difference in *prickle* sequences causing these different restriction sites may also support the defining of *Mo-prickle1* and *Mo-prickle2*. The presence of *Ci-prickle1* and *Ci-prickle2* in the ascidian *Ciona intestinalis* (Hotta et al., 2000) strengthens the feasibility of more than one *prickle* being present in molgulid ascidians.

*Molecular nature of Molgulid genes*

The primers designed to isolate *prickle, noto6, noto17, leprecan* and *merlin* for *M. occulta* and *M. oculata* required exceptionally low annealing temperatures. Many A-T base-pairs translate to lower annealing temperatures because A-T hydrogen bonding is less efficient than C-G hydrogen bonding at overcoming the initial inertia of free-moving primers. It is then possible that these low annealing temperatures are due to the ubiquity of A-T bonds in the gene sequences. High A-T content in the DNA sequences of *prickle, noto6, noto17, leprecan* and *merlin* in *M. occulta* and *M. oculata* is readily noticeable at the ends of the genes, where primers were designed. It is possible that the ascidians are A-T rich due to environmental pressures in the same way thermophiles are C-G rich (Basak et al., 2010). A-T ubiquity in this sense may then be a consequence of being native to benthic, low-temperature environments.
Future Studies

The immediate next step in this project is the sequencing of the genes isolated during this study. This will confirm their identity and allow the project to move forward into an investigation of gene expression via in-situ hybridization. The genes I focused on do not contain the restriction sites used to linearize the T3 (Not I) and T7 (Pst I) strands for probe synthesis, with the exception of prickle in M. occulta. This prickle appears to have a downstream Pst I restriction site (Figure 12). Future researchers may resolve this conflict by choosing a different bacterial vector, in which, the restriction sites used to linearize sense and anti-sense strands are not found in the gene.

Once probes are synthesized for Mo-prickle, Mo-noto17, Mo-leprecan and Mo-merlin, in-situ hybridizations may be done in the neurula and tail-bud stage embryos of M. occulta, M. oculata and M. occulta x M. oculata hybrids. Dr. Billie Swalla has preserved neurula and tail-bud stage embryos of these organisms available for such a study; animals were collected in Roscoff, France in 1999. The in-situ results for M. oculata are expected to resemble those found in the ascidian Ciona intestinalis (Hotta et al., 2000); in the neurula stage, the probes will appear down the A/P axis of the embryo, while in the tail-bud stage the probes will mainly illuminate the notochord. An M. occulta x M. oculata hybrid in-situ is also expected to show an illuminated notochord; though there are fewer notochord cells present (Swalla and Jeffery, 1996), these hybrids have nonetheless completed convergence and elongation and thus produce the molecular transcripts required to orchestrate this event.

Results of the M. occulta in-situ are more elusive. No expression of these genes in the tail-bud stage of M. occulta would support the hypothesis that these genes are
required for the full development of a notochord. However, if these genes are expressed, it may mean they are not necessarily a requirement for late-stage notochord development. Knock-out studies have shown that without prickle, proper development in ascidians does not occur (Jiang et al., 2005); suboptimal development has also been described in leprecan knock-out experiments (Dunn and Di Gregorio, 2009). If this holds true for noto6, noto17 and merlin, it would mean these genes are a requirement for notochord development, but not necessarily the key genes involved in the converge-and-extend event absent in M. occulta.

This second scenario immediately raises a question of efficiency: why produce a transcriptional product that doesn’t lead to a morphological change? It is possible that expression of these genes in late stages of anural embryonic development may be vestigial signals; natural selection does not necessarily select for complete elimination of gene expression if the expression does not significantly impede the organism.

Another important question to consider while studying molgulid tail-loss is what kind of factors facilitated tail-loss. These factors may be active or passive: Active selective factors mean taillessness was selected for by pressures in the environment, while passive factors mean tail-loss simply was not selected against. Passive selection would be made possible by the genetic plasticity observed in molgulids (Iannelli et al., 2007).

In-situ hybridization is the ultimate end of this particular study, but it is not the only or even the most conclusive way to investigate the overarching question of this project: are prickle, noto6, noto17, leprecan and merlin required for the convergence and extension of notochordal cells? Aside from in-situs, a knock-out gene experiment for
each of these genes in anural and urodele molgulids would be compelling as a way to
directly determine the importance of each gene in the converge-and-extend event.

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Literature Cited


FIGURE LEGENDS

Figure 1. Protein alignment for prickle including *M. occulta* and *M. oculata* transcriptome sequences and sequences from another anural molgulid (*M. tectiformis*), a urodele non-molgulid ascidian (*Ciona intestinalis*) and human. Yellow indicates a LIM1 domain, blue indicates a LIM2 domain, green indicates a LIM3 domain and fuchsia indicates a PET_prickle domain. Amino acids conserved between all species are indicated by an asterisk (*), amino acids with the same charge are indicated by a period (.) and significant but imperfect matches are indicated by a semicolon (;).

Figure 2. Protein alignment for noto6 including *M. occulta* and *M. oculata* transcriptome sequences and sequences from another anural molgulid (*M. tectiformis*), a urodele non-molgulid ascidian (*C. intestinalis*), lancelet and human. Amino acids conserved between all species are indicated by an asterisk (*), amino acids with the same charge are indicated by a period (.) and significant but imperfect matches are indicated by a semicolon (;).

Figure 3. Protein alignment for noto17 including *M. occulta* and *M. oculata* transcriptome sequences and sequences from another anural molgulid (*M. tectiformis*) and two urodele non-molgulid ascidians (*C. intestinalis* and *Oikopleura dioica*). Yellow indicates a Thioredoxin_like domain. Amino acids conserved between all species are indicated by an asterisk (*), amino acids with the same charge are indicated by a period (.) and significant but imperfect matches are indicated by a semicolon (;).

Figure 4. Protein sequence alignment for leprecan including hybrids of *M. occulta* and *M. oculata*. In addition there are sequences for *M. tectiformis*, *C. intestinalis* and human. Yellow indicates a 20G Fe(II) oxygenase domain. Amino acids conserved between all species are indicated by an asterisk (*), amino acids with the same charge are indicated by a period (.) and significant but imperfect matches are indicated by a semicolon (;).

Figure 5. Protein sequence alignment for merlin including *M. occulta* and *M. oculata*, as well as *M. tectiformis*, *C. intestinalis*, an acorn worm (*Saccoglossus kowalevskii*) and human. Yellow indicates an ezrin/radixin/moesin (ERM) domain. White diamonds (◇) indicates a FERM_N domain, black dots (●) indicate a FERM_M domain and white pentagons (▲) indicate a FERM_C domain with a PH-like core. Amino acids conserved between all species are indicated by an asterisk (*), amino acids with the same charge are indicated by a period (.) and significant but imperfect matches are indicated by a semicolon (;).
Figure 6. Results of PCR with 45°C annealing temperature. Lanes starting from the far left are: 1 kb ladder, prickle for M. occultula, prickle for M. oculata, noto17 for M. occultula, noto17 for M. oculata, noto6 for M. occultula, noto6 for M. oculata, leprecan for M. occultula, leprecan for M. oculata, merlin for M. occultula and merlin for M. oculata. Expected bands for prickle, noto17, noto6, leprecan and merlin are 1077 bp, 960 bp, 1154 bp, 888 bp and 1121 bp respectively; the bands that appear are too small to be correct.

Figure 7. Results of PCR with 40°C annealing temperature. Lanes starting from the far left are: 1 kb ladder, prickle for M. occultula, prickle for M. oculata, noto6 for M. occultula, noto6 for M. oculata, noto17 for M. occultula, noto17 for M. oculata, leprecan for M. occultula, leprecan for M. oculata, merlin for M. occultula and merlin for M. oculata. Expected bands for prickle, noto17, noto6, leprecan and merlin are 1077 bp, 960 bp, 1154 bp, 888 bp and 1121 bp respectively.

Figure 8. Results of PCR with 38°C annealing temperature. Lanes starting from the far left are: 1 kb ladder, noto6 for M. occultula, noto6 for M. oculata, uncult merlin for M. oculata (miniprep DNA) and EcoRI digest of merlin for M. oculata.

Figure 9. Results of first miniprep. Lanes starting from the far left of Gel 1 are: 1 kb ladder, uncult noto17 for M. occultula culture 1, EcoRI digest of noto17 for M. occultula culture 1, uncult noto17 for M. occultula culture 2, EcoRI digest of noto17 for M. occultula culture 2, uncult noto17 for M. oculata culture 3, EcoRI digest of noto17 for M. oculata culture 3, uncult noto17 for M. oculata culture 4, EcoRI digest of noto17 for M. oculata culture 4, uncult noto17 for M. oculata culture 5, EcoRI digest of noto17 for M. oculata culture 5, uncult merlin for M. oculata culture 6, EcoRI digest of merlin for M. oculata culture 6, uncult merlin for M. oculata culture 7 and EcoRI digest of merlin for M. oculata culture 7.

Lanes starting from the far left of Gel 2 are: 1 kb ladder, uncult merlin for M. occultula culture 8, EcoRI digest of merlin for M. occultula culture 8, uncult merlin for M. oculata culture 9, EcoRI digest of merlin for M. oculata culture 9, uncult merlin for M. oculata culture 10, EcoRI digest of merlin for M. oculata culture 10, uncult merlin for M. oculata culture 11, EcoRI digest of merlin for M. oculata culture 11, uncult prickle for M. oculata culture 12 and EcoRI digest of prickle for M. oculata culture 12.

Figure 10. Results of second miniprep. Lanes starting from the far left of Gel 1 are: 1 kb ladder, uncult prickle for M. oculata culture 1, EcoRI digest of prickle for M. oculata culture 1, uncult prickle for M. oculata culture 2, EcoRI digest of prickle for M. oculata culture 2, uncult prickle for M. oculata culture 3, EcoRI digest of prickle for M. oculata culture 3, uncult prickle for M. oculata culture 4, EcoRI digest of prickle for M. oculata culture 4, uncult prickle for M. oculata culture 5, EcoRI digest of prickle for M. oculata culture 5, uncult prickle for M. oculata culture 6, EcoRI digest of prickle for M. oculata culture 6, uncult prickle for M. oculata culture 7 and EcoRI digest of prickle for M. oculata culture 7.
Lanes starting from the far left of Gel 2 are: 1 kb ladder, uncut *prickle* for *M. occulta* culture 8, *EcoRI* digest of *prickle* for *M. occulta* culture 8, uncut *prickle* for *M. occulta* culture 9, *EcoRI* digest of *prickle* for *M. occulta* culture 9, uncut *leprecan* for *M. occulta* culture 10, *EcoRI* digest of *leprecan* for *M. occulta* culture 10, uncut *leprecan* for *M. occulta* culture 11, *EcoRI* digest of *leprecan* for *M. occulta* culture 11, uncut *leprecan* for *M. occulta* culture 12 and *EcoRI* digest of *leprecan* for *M. occulta* culture 12.

Figure 11. Dot blot test for T3 and T7 RNA probes. Y axis shows 1/10, 1/50, 1/100 and 1/500 dilutions for the probes, while the X axis shows, from left to right, the control probe, T3 probe and T7 probe. A purple dot signifies a successful probe.

Figure 12. Sequencher representation of restriction sites in *Mo-prickle* from *Molgula occulta*.

Figure 13. Sequencher representation of restriction sites in *Mo-prickle* from *Molgula oculata*.
Figure 1

Molgula.oculata.prickle
Molgula.tectiformis.prickle
Molgula.occulta.prickle
Ciona.intestinalis.prickle
Human.prickle

Molgula.occulta.prickle
Molgula.tectiformis.prickle
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Figure 2

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Figure 3

Molgula. oculata.noto17  
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Molgulid.hybrid.67123.leprecan
LMVANSTQNLGTRFVV
DHLITENQCQDLINLELSAGVEGQYQQGKQPH
142

Molgula.tectiformis.leprecan
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545

Ciona.intestinalis.leprecan
VMLSNSTQMQGMLRFAVDGFASEQQCQDLIDLELSGGVLDGDGYGRVSFPH
218

Human.leprecan
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319

Molgulid.hybrid.67122.leprecan
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191

Molgulid.hybrid.67123.leprecan
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191

Molgula.tectiformis.leprecan
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594

Ciona.intestinalis.leprecan
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268

Human.leprecan
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369

Molgulid.hybrid.67122.leprecan
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Molgula.tectiformis.leprecan
NLYFDYTHLVCRTALPKSSSSEEDLSPVHADNCILQEGECLKKKPA
642

Ciona.intestinalis.leprecan
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316

Human.leprecan
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Ciona.intestinalis.leprecan
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Human.leprecan
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468

Molgulid.hybrid.67122.leprecan
CFHGVK
296

Molgulid.hybrid.67123.leprecan
CFHGVK
296

Molgula.tectiformis.leprecan
CFHGVK
734

Ciona.intestinalis.leprecan
CLHGVK
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Human.leprecan
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Molgulid.hybrid.67122.leprecan
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Molgulid.hybrid.67123.leprecan
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Molgula.tectiformis.leprecan
---NSKDEL 740

Ciona.intestinalis.leprecan
---LLKDEL 412

Human.leprecan
LNINPKDEL 527
Human.merlin

QQMKQAQAREEKARKQMRQLAREKQMREBAAERTDELERLLQMKKEAT 368

Molgula.occulta.merlin
Molgula.oculata.merlin
Molgula.tectiformis.merlin
Ciona.intestinalis.merlin
Acorn.worm.merlin
Human.merlin

Molgula.occulta.merlin
Molgula.oculata.merlin
Molgula.tectiformis.merlin
Ciona.intestinalis.merlin
Acorn.worm.merlin
Human.merlin

Molgula.occulta.merlin
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Molgula.tectiformis.merlin
Ciona.intestinalis.merlin
Acorn.worm.merlin
Human.merlin

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Ciona.intestinalis.merlin
Acorn.worm.merlin
Human.merlin

Molgula.occulta.merlin
Molgula.oculata.merlin
Molgula.tectiformis.merlin
Ciona.intestinalis.merlin
Acorn.worm.merlin
Human.merlin

Molgula.occulta.merlin
Molgula.oculata.merlin
Molgula.tectiformis.merlin
Ciona.intestinalis.merlin
Acorn.worm.merlin
Human.merlin


Figure 6
Figure 7
Figure 8
Figure 9

12 EcoRI prickle, *M. oculata*
12 Uncut prickle, *M. oculata*
11 EcoRI merlin, *M. oculata*
11 Uncut merlin, *M. oculata*
10 EcoRI merlin, *M. oculata*
10 Uncut merlin, *M. oculata*
9 EcoRI merlin, *M. oculata*
9 Uncut merlin, *M. oculata*
8 EcoRI merlin, *M. oculata*
8 Uncut merlin, *M. oculata*

7 EcoRI merlin, *M. occulta*
7 Uncut merlin, *M. occulta*
6 EcoRI merlin, *M. occulta*
6 Uncut merlin, *M. occulta*
5 EcoRI noto17, *M. oculata*
5 Uncut noto17, *M. oculata*
4 EcoRI noto17, *M. oculata*
4 Uncut noto17, *M. oculata*
3 EcoRI noto17, *M. oculata*
3 Uncut noto17, *M. oculata*
2 EcoRI noto17, *M. oculata*
2 Uncut noto17, *M. oculata*
1 EcoRI noto17, *M. oculata*
1 Uncut noto17, *M. oculata*
Figure 10
Figure 11

Cutters: BamHI, HindIII, KpnI, PstI & XbaI

Non-Cutters: ApaI, Bsp106I, BstXI, DmII, EcoRI, EcoRV, NotI, SacI, SacII, SalI, SmaI, SpeI, XhoI & XmaIII

Figure 12

Mapping all cutsites.

Figure 13

Cutters: BstXI, HindIII, SacI & XbaI

Non-Cutters: ApaI, BamHI, Bsp106I, DmII, EcoRI, EcoRV, KpnI, NotI, PstI, SacII, SalI, SmaI, SpeI, XhoI & XmaIII