The Molecular Evolution of *Hedgehog* in Stolidobranchia Ascidians

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Abstract

Ascidians are highly studied in evolution and development because they have a tiny chordate tadpole larva. As a sister clade to vertebrates, ascidian research has lead to greater understanding of the molecular roles of developmental genes in vertebrates. However, recent research on a Phlebobranchia ascidian *Ciona intestinalis* indicates that the developmental gene *hedgehog (hh)* may have undergone a duplication independent to the *hh* duplications in vertebrates (Hudson, *et al.* 2011). To investigate this hypothesis, more research is needed on the other ascidian clade, Stolibranchia. In this study we strived to use maximum-likelihood analysis to compare the evolution of *hh* in Stolidobranchia and Phleobranchia ascidians. Future research will investigate the different developmental roles of *hh* in Stolidobranchia and Phlebobranchia ascidians.

Introduction

The members of the chordate phylum—ascidians, cephalochordates, and vertebrates—share specific homologous structures including; the notochord, dorsal nerve cord, pharyngeal gill slits, and post-anal tail (Figure 1). Ascidians have a simplified chordate body plan, and thus many researchers have focused their work on characterizing ascidian development in order to better understand chordate evolution (Imai and Meinertzhagen 2007; Brown *et al*. 2008). Ascidian development is important for understanding chordate evolution and conserved chordate developmental signaling pathways (Lemaire 2009).

The tadpole larvae of ascidians have a body plan surprisingly similar to that of vertebrate embryos, which suggests a degree of conservation in the developmental mechanisms used to generate this body plan (Hudson *et al.* 2011). Approximately 3000 cells constitute the ascidian tadpole larva, which form many distinct tissues including: the dorsal central nervous system, notochord, muscle, epidermis, mesenchyme, and endoderm (Figure 1) (Takatori *et al.* 2002). Molecular analysis of solitary ascidian embryogenesis has created a blueprint for the patterning of chordate tissues, which is highly conserved between ascidians and vertebrates (Davidson *et al.* 2003).

Ascidians can be subdivided into three clades: Thaliacea, Phlebobranchia, and Stolidobranchia; in which the latter two contain the majority of ascidians (Zeng, *et al.* 2006). The major differences in development being that in Stolidobranchia the branchial sac itself is folded, and the regenerative tissue is ectodermal (Kott 1985). Differences in the development of their nervous system are unknown

Studies in evolutionary developmental biology have uncovered a 'toolkit' of developmental genes that perform similar functions in various animals (Satou *et al.* 2009). One of these genes, *hedgehog (hh)*, was discovered to affect segment number and polarity in the fruit fly *Drosophila melanogaster* (Nüsslein-Volhard and Wieschaus 1980). Mammals have three *hh* homologs that play important roles in development: *Sonic hedgehog (Shh), Indian hedgehog (Ihh)* and *Desert hedgehog (Dhh)* (Takatori *et al.* 2002). Hh proteins are autocatalyticly cleaved and function as morphogenesis, signals that elicit concentration-dependent responses from target cells (Takatori *et al.* 2002, Satou 2009). In vertebrate embryos, Shh protein expression is concentrated to the underlying notochord, the ventral midline of the neural tube, and the floor plate (Hudson *et al.* 2011). Additionally, Shh signals emanating from the notochord and floor plate induce the formation of somatic motor neurons in ventrolateral regions of the neural tube (Takatori *et al.* 2002, Hudson *et al.* 2011). Hh signaling has an essential role in humans to regulate cell fate and number in their brains and spinal cords (Jiang and Hui 2008). Aberrant expression of Hh proteins contributes to birth defects and cancer, including medulloblastoma and childhood diffuse intrinsic pontine gliomas (DIPGs) (Jiang and Hui 2008, Monje *et al.* 2011).

Patterns of developmental gene expression suggest a similarity between the vertebrate posterior neural tube and ascidian nerve cord (Takatori *et al.* 2002; Hudson *et al.* 2011). Therefore, to better understand Hh's developmental role in embryogenesis, efforts have been focused on the Phlebobranchia ascidian *Ciona intestinalis*. However, recent data suggests that the floor plate and Hedgehog signaling are not acting in the same way during vertebrate and *C. intestinalis* motor neuron formation (Hudson *et al.* 2011). To address this issue and get a better understanding of the evolution of *hh* in tunicates, examining *hh* in ascidians from the clade Stolidobranchia is important in order to reach a consensus on the developmental role of hedgehog in ancestral chordates.

Materials and Methods

Animals. *B. villosa* were collected at Friday Harbor Laboratories, WA, San Juan Island and kept under constant light in circulating seawater (12ºC). Adults were bisected longitudinally through the siphons and the gonads manually macerated through 300-mm Nitex mesh. Egg and sperm were segregated, and sperm activated by adding Tris pH 9.5 until the solution reached pH 9.0. *B. villosa* are not self-fertilizing, thus a different individual was used to fertilize eggs. After insemination, embryos were reared at 12ºC in bag-filtered seawater (FSW).

B. villosa were collected at the following stages of development for *in-situ* hybridization: F+0 (fertilized egg), F+30min (first cleavage), F+2h (4-cell stage embryo and 8-cell stage embryo), F+3.5h (16-cell stage embryo), F+5h (32-cell stage embryo), F+6h and F+8 (gastrulation), F+17h (early and middle tailbud-stage embryos), F+27h (tadpole), and F+48h (tail absorption).

B. violaceus colonies were collected off the docks in Roche Harbor, WA, San Juan Island and were maintained in plastic colanders submerged in circulating seawater (12°C) on sea tables at Friday Harbor Laboratories.

Sequence comparisons and molecular phylogenetic analysis. A BLASTp search was conducted on the NCBI database for *hedgehog* (*hh)* using *Ciona intestinalis hh2* as the query sequence. Vertebrate (*Homo sapiens, Mus musculus, Xenopus laevis,* and *Danio rerio)* and invertebrate (*Drosophila melanogaster, Saccoglossus kowalevskii, Strongylocentrotus purpuratus, Branchiostoma floridae,* and *Ciona intestinalis) hedgehog* DNA and protein sequences were aligned using MEGA5 (http://megasoftware.net) and MAFFT (http://www.ebi.ac.uk/Tools/msa/mafft/). Non-highly conserved regions were excluded from the analysis using Gblocks (http://molevol.cmima.csic.es/castresana/Gblocks_server.html) (Figure 2). Molecular phylogenetic relationships among the *hh* genes were estimated using the maximum-likelihood method on RAxML. 100 Bootstraps were used to assess the degree of support for internal branching of the tree (Figure 4).

Primer design. Primers were designed for *hh1* and *hh2* from the highly conserved 5' and 3' end regions of the above DNA alignments (Figure 3). *Sb-SHH* primers designed for *Saccoglossus kowalevskii* by Leonid Moroz and Billie Swalla were also used in this study. Each primer is between 18-21 bp and has minimal degeneracy

(Table 1).

Polymerase Chain Reaction (PCR). DNA was extracted from the gonads of *B. villosa* and the tadpoles of *B. violaceus* using a Qiagen DNeasy kit. RNA was extracted from the gonads of *B. villosa* and tadpoles of *B. violaceus* using Qiagen RNeasy Protect mini kit. cDNA was made from RNA using Ambion RETROscrip kit. PCR was ran on the genomic DNA and cDNA of *B. villosa* and *B. violaceus*, and genomic DNA from *Molgula ficus* and *Saccoglossus bromophenolosus* using the above primers at the following conditions: initial denature at 94ºC for 4 minutes; 35 cycles of denature 94ºC for 1 minute, annealing temperatures varied from 37ºC to 58ºC for 30 seconds, extension at 72ºC for 1 minute; final extension at 72ºC for 10 minutes. PCR products were ran on a 0.7% agarose gel at 110v. PCR inserts were extracted and purified using the Illustra GFX Gel Band Purification kit.

Cloning. Putative *hh* genes isolated by PCR of *B. violaceus* and *M. ficus* were ligated into the pCR II-TOPO 4.0kb vector and transformed into chemically competent TOP10 *Escherichia coli* using the heat shock method. *hh* genes purified from *B. villosa* were ligated into the pGEM-T 3kb Easy Vector and transformed into JM109 Highly Efficient Competent Cells. Both vectors have the T7 and SP6 promoter regions and EcoR1 restriction sites. TOP10 transformed cells were plated on kanamycin+X-gal LB agar plates. JM109 transformed cells were plated on ampicillin+IPTG+X-gal LB agar plates. Plates were incubated over night at 37ºC. White colonies were selected and grown up in culture. Plasmids were then extracted using 5Prime FastPlasmid mini kit. Digesting 10uL of plasmid DNA with EcoR1 restriction enzyme identified successful clones. Digestion reactions were ran on a 0.7% agarose gel at 110v.

Sequencing. Successfully cloned plasmids were sent to Genewiz for sequencing.

Dechorination and fixation. *B. villosa* and *B. violaceus* embryos were dechorinated using a dechorionation solution (4% Na thioglycolate in FSW, 2% pronase E, pH using 1M NaoH) and fixed in 4% paraformaldehyde. Embryos are stored in 80% ethanol at -20ºC.

Results

No bands were obtained by PCR using the genomic DNA of *B. villosa* or *S. bromophenolosus.* PCR products were obtained using an annealing temperature of 37ºC on genomic DNA from *M. ficus* and *B. violaceus* (Figure 5a). Successfully cloned plasmids had two bands at 4kb and 300bp (Figure 6). BLASTx was performed on the sequences of these products, however they did not correlate to *hedgehog.* PCR products were obtained using an annealing temperature of 37ºC on cDNA from *B. villosa* (Figure 5b). These genes will be sent for sequencing.

A gene tree comparing *hh* in chordates produced monophyletic clades of vertebrate *SHH, IHH,* and *DHH.* The invertebrates branch off separately, with *C. intestinalis* as the outgroup. *C. intestinalis hh* genes are not grouped in any of the vertebrate clades. In the IHH clade there is a low bootstrap value of 14 at node B. The other invertebrates *S. kowalevskii, S. purpuratus,* and *B. floridae* only have evolved one *hh* gene.

Discussion

Ascidians are highly studied in evolution and development because they have a tiny chordate tadpole larva. As such, ascidian research has lead to a greater understanding of the molecular roles of development in vertebrates. However, recent research in *C. intestinalis* on the molecular role of *hh* in motoneuron development is non-homologous to its role in vertebrates.

Figure 4 suggests that *C. intestinalis* had a *hh* duplication independent of the vertebrate *hh* duplications, resulting in *HH1* and *HH2*. The tree suggests that the ancestor at node A had one *hh* gene. The vertebrates branched off and had two gene duplications, resulting in homologous *SHH*, *IHH*, and *DHH*. This theory is supported by *hh* not being expressed in the notochord, nor playing a role in motoneuron development in *C. intestinalis* (Hudson *et al.* 2011).

In the IHH clade there is a low bootstrap value at node B. This may be caused by an independent gene duplication of *IHH* in *D. rerio.* In the DHH clade, one of the duplicated genes may have been lost, resulting in only one DHH gene in *D. rerio.*

Based on current research from *C. intestinalis*, we hypothesize that tunicates are not the ideal organism to study *hedgehog* in vertebrates (Hudson *et al.* 2011). However, *in-situ* hybridizations for *hedgehog* has not been studied in the sister clade Stolidobranchia. Therefore, further research is needed in Stolidobranchia to determine if *hedgehog* is expressed in the notochord of ascidians.

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Figures

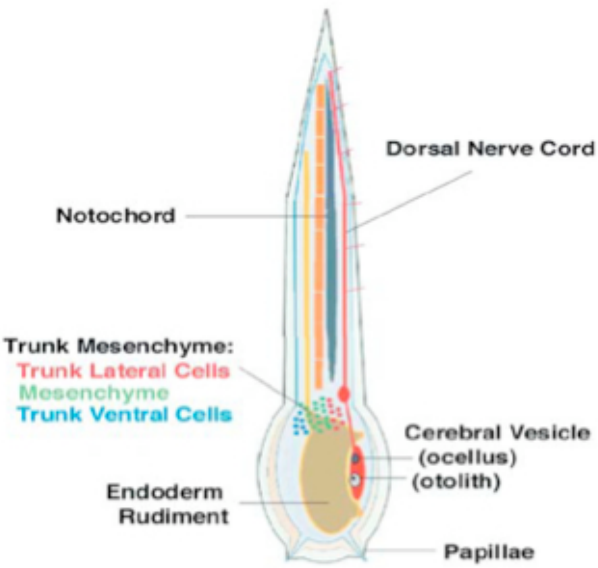

Figure 1. Diagram of a settling ascidian larva, showing the chordate dorsal nerve cord, notochord, mesenchyme, and endoderm (Davidson *et al.* 2003).

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                         10        20        30        40        50        60
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DHH_Homo_sapien  RYARKQLVPLLYK FVPGVP RTLGASGPA GRVARGS RFRDLVP Y P IIFK    S
DHHa_Xenopus_la  RRYMRKLVPLHYK FVP VP KTLGASGKS GKIHRGS RFI LVP Y P IIFK   KT
DHHb_Xenopus_la  RRYMRRLVPLLYK FVP VP KTLGASGKS GKIRRGS RFIKLVP Y P IIFK    T
DHH_Xenopus_(Si  RRYMRKLVPLRYK FVP VP KTLGASGKS GKIRRGS RFI LVP Y P IIFK    T
DHH_Mus_musculu  RYVRKQLVPLLYK FVPSMP RTLGASGPA GRVTRGS RFRDLVP Y P IIFK    S
DHH_Danio_rerio  RHRQRKLTPMSYK YVPGVS NNLGASGRA GRITRSS RFN LVC Y T IDFK   RS
SHH_Mus_musculu  RRHPKKLTPLAYK FIP VA KTLGASGRY GKITRNS RFK LTP Y P IIFK    T
SHH_Xenopus_lae  RRHPKKLTPLAYK FIP VA KTLGASGRY GKITRNSDCFK LTP Y P IMFK   ST
SHH_Homo_sapien  RRHPKKLTPLAYK FIP VA KTLGASGRY GKISRNS RFK LTP Y P IIFK    T
SHH_Danio_rerio  RRHPKKLTPLAYK FIP VA KTLGASGRY GKITRNS RFK LTP Y P IIFK    T
SHH_Xenopus_(Si  ------------------------------------------------------------
IHH_Homo_sapien  RRPPRKLVPLAYK FSP VP KTLGASGRY GKIARSS RFK LTP Y P IIFK    T
IHH_Mus_musculu  RRPPRKLVPLAYK FSP VP KTLGASGRY GKIARSS RFK LTP Y P IIFK    T
IHH_Danio_rerio  RRPPKKLTPLNYK FSP VA KTLGASGRI GKITRNS RFK LTP Y P IIFK    T
IHHb_Danio_reri  RRTPRKLTPLAYK FSP VA KTLGASGRY GKVTPSS RFK LTP Y P IIFK    T
IHH_Xenopus_lae  RRRPTKLSPLSYK FSP VP KTLGASGRY GKISRNS RFK LTP Y P IIFK   IT
HH_Drosophila_m  RHRARNLYPLVLK TIP LS YTNSASGPL GVIRRDSPKFKDLVP Y R ILFR   GT
HH_Branchiostom  RRHPRKLTPFVYK QMPAVS NTFGASGLFNGRITRDS RFHTLKQ F T IIFK   KT
HH_Saccoglossus  RRPSRELTPLLYK CIP VS NTLGASGPN KKITREDDEFKDLQTVY A IMFK   GT
HHtw_Danio_reri  RRHPKKLTPLAYK FIP VA KTLGASGKY GKITRNS RFK LIP Y P IIFK    T
HH_Strongylocen  SHRPRNRTPLQYK RVP IS DTFGASGPP GRINRND RFNTLSP N D IVFK K GT
HH1_Ciona_intes  RMPGRELVPFLKGEYVPKMS QTIGASGPVTGRIRADTPRFR LVP W T IEFR   ES
HH2_Ciona_intes  RPNQRNLRPLLRQ YVPHVS GTIGASGPS GRIYRNTPRYRKLER Y T IEFE R RD
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                           70        80        90       100       110       120
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DHH_Homo_sapien   GA RLMTRCKERV ALAIAVM MWPGVRLRVT GW   GHHAQDSLHY GRAL ITTS R
DHHa_Xenopus_la   GA RLMTRCK RV ALAISVM MWPGVKLRVT GW   GHHAHDSLHY GRAL ITTS R
DHHb_Xenopus_la   GA RLMTRCK RV ALAISVM MWPGLKLRVT GW   GHHAHDSLHY GRAL ITTS R
DHH_Xenopus_(Si   GA RLMTRCK RV ALAISVM MWPGVKLRVT GW   GHHAHDSLHY GRAL ITTS R
DHH_Mus_musculu   GA RLMTRCKERV ALAIAVM MWPGVRLRVT GW   GHHAQDSLHY GRAL ITTS R
DHH_Danio_rerio   NA RFMTRCK CL KLAIAVM QWPGVRLRVT AW   GHHPPGSLHY GRAV ITTS R
SHH_Mus_musculu   GA RLMTRCK KL ALAISVM QWPGVKLRVT GW   GHHS  SLHY GRAV ITTS R
SHH_Xenopus_lae   GA RLMTRCK KL ALAISVM QWPGVKLRVT GW   GHHL  SLHY GRAV ITTS R
SHH_Homo_sapien   GA RLMTRCK KL ALAISVM QWPGVKLRVT GW   GHHS  SLHY GRAV ITTS R
SHH_Danio_rerio   GA RLMTRCK KL SLAISVM HWPGVKLRVT GW   GHHF  SLHY GRAV ITTS R
SHH_Xenopus_(Si   --------------------------VKLRVT GW   GHHS  SLHY GRAV ITTS R
IHH_Homo_sapien   GA RLMTRCK RL SLAISVM QWPGVKLRVT GW   GHHS  SLHY GRAV ITTS R
IHH_Mus_musculu   GA RLMTRCK RL SLAISVM QWPGVKLRVT GW   GHHS  SLHY GRAV ITTS R
IHH_Danio_rerio   GA RLMTRCK KL SLAISVM MWPGVKLRVT GW   GNHF DSLHY GRAV ITTS R
IHHb_Danio_reri   GA RMMTRCK KL SLAISVM LWPGVRLRVT GW   GLHS  SLHY GRAV ITTS R
IHH_Xenopus_lae   GA RLMTRCK RL SLAISVM QWPGVKLRVT GW   GHHF  SLHY GRAV ITTS R
HH_Drosophila_m   GA GLMSRCKEKL VLAYSVM EWPGIRLLVT SW   YHHGQ SLHY GRAVTIATS R
HH_Branchiostom   GA RFMTRCK KL ALAISVM QWEGVKLRVT GW   GFHT  SLHY GRAV ITTS R
HH_Saccoglossus   GA RLMTRCK RL SLAISVM QWPGVKLRVT GW   GHHAPNSLHY GRAV ITTN R
HHtw_Danio_reri   NA RLMTRCK KL SLAISVM HWPGVKLRVT GW   GRHL  SLHY GRAV ITTS R
HH_Strongylocen   GA RLMTRCK KL TLAISVM EWPGIKLRVV AW   QPNV -PLHA GRAV ITTS R
HH1_Ciona_intes   NE RFMTICRARLDYLAILVA QWARVKLKVL AW DGNDKANDPLHY GRAV ITTD A
HH2_Ciona_intes   GS RTMTRCK KV LLSMLVK TWAGVSLKVI AW G GVHRKGSLHY GRAV IKTS N
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                          130       140       150       160       170       180
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DHH_Homo_sapien    RNKYGLLARLAV AGF WVYY SRNHVHVSVKAD SLAVRAGGCFPGNATVRLWSGERK
DHHa_Xenopus_la    RNKYGMLARLAV AGF WVYY SKAHIHVSVKAD SLGVRSGGCFPGTAMVMMGTGERK
DHHb_Xenopus_la    RNKYGMLARLAV AGF WVYY SKAHIHVSVNTD SLGVRSGGCFPGTAMVMM TGKKK
DHH_Xenopus_(Si    RSKYGMLARLAV AGF WVYY SKAHIHVSVKAD SLGVRSGGCFPGTAMVMMASGEKK
DHH_Mus_musculu    RNKYGLLARLAV AGF WVYY SRNHIHVSVKAD SLAVRAGGCFPGNATVRLRSGERK
DHH_Danio_rerio    TKKYGLLAQLAV AGF WVHY SKYHVHCSVKADHSVAVEKGGCFSASGLVTMADGVQK
SHH_Mus_musculu    RSKYGMLARLAV AGF WVYY SKAHIHCSVKA  SVAAKSGGCFPGSATVHL QGGTK
SHH_Xenopus_lae    RSKYGMLGRLAV AGF WVYY SKAHIHCSVKA  SVAAKSGGCFPAGARVMV FGGTK
SHH_Homo_sapien    RSKYGMLARLAV AGF WVYY SKAHIHCSVKA  SVAAKSGGCFPGSATVHL QGGTK
SHH_Danio_rerio    KSKYGTLSRLAV AGF WVYY SKAHIHCSVKA  SVAAKSGGCFPGSALVSLQDGGQK
SHH_Xenopus_(Si    RSKYGMLARLAV AGF WVYF SKAHIHCSVKA  SVAAKSGGCFPGSARVMV PGGTK
IHH_Homo_sapien    RNKYGLLARLAV AGF WVYY SKAHVHCSVKS HSAAAKTGGCFPAGAQVRL SGARV
IHH_Mus_musculu    RNKYGLLARLAV AGF WVYY SKAHVHCSVKS HSAAAKTGGCFPAGAQVRL NGERV
IHH_Danio_rerio    RNKYGMLARLAV AGF WVYY SKAHIHCSVKS HSVAAKTGGCFPASALVTV DGSLK
IHHb_Danio_reri    RNKYRMLARLAV AGF WVYY SKGHVHCSVKS HSVAAKTGGCFPGRALVTMKDGSHR
IHH_Xenopus_lae    RNKYGMLARLAV AGF WVYY SKAHIHCSVKS HSAAAKTGGCFPGEALATL SGEKI
HH_Drosophila_m    QSKYGMLARLAV AGF WVSYVSRRHIYCSVKSDSSISSHVHGCFTPESTALL SGVRK
HH_Branchiostom    RTKYGMLARLAV AGF WVYY SKAHIHCSVKA SDTTATQGGCFPAESWVTRDDGNRI
HH_Saccoglossus    RTKYGMLARLAX AGF WVFYQSRAHVHCSVKA SSLAAKSGSCFPGDATVTL NGLTK
HHtw_Danio_reri    KSKYGMLSRLAV AGF WVYY SKAHIHCSVKA  SVAAKSGGCFPGSGTVTLGDGTRK
HH_Strongylocen    KNKYGALARLAV AGF WVNY SKAWVHCSVKS SAAAKNSGGCFPGFSQASL NGRTI
HH1_Ciona_intes    RNKYPILARLAVVAGF WVKYDGKV-VHCSVKS ESDAAKYGGCFPGESTVIVPGEGHV
HH2_Ciona_intes    LSKNGLLARLAV SGF WVYY SKFYVHASVRAD SDAAKYGGCFDGVSEVRN SGSVI
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                          190       200       210       220       230       240
                   =========+=========+=========+=========+=========+=========+
DHH_Homo_sapien    GLRELHRG WVLAGRVVPTPVLLFL FVAV TEWPPRKLLLTPWHLVFFARRLRAGDSV
DHHa_Xenopus_la    PLSELKIG TVYTGQLITSVVLLFLHFVLI AEGHPSKLLVTPNHLLFFAYRVQIGDLV
DHHb_Xenopus_la    PLSELKLG TVFTGLLIHSVVLLFLHFVLI AEGHPTKLLVTPNHLLFFAYRVQIGDLI
DHH_Xenopus_(Si    SLSELQLG TVFTGQLIPSVVLLFLHFVLI AEGHTSKLLVTPNHLLFFAYRVQIGDLV
DHH_Mus_musculu    GLRELHRG WVLAGRVVPTPVLLFL FVAV TERPPRKLLLTPWHLVFFARRLRAGDSV
DHH_Danio_rerio    PMSCLWPGEKVLSGEVVFSRVLLFLHFFIITT-ENEKRIALTPNHLIFFARNVRIGDYI
SHH_Mus_musculu    LVKDLRPG RVLAGRLLYSDFLTFL FYVI TLEPRERLLLTAAHLLFFASRVRPGQRV
SHH_Xenopus_lae    AVKDLRPG RVLSGNLLYSDFLMFI FYVI T--SQRKIRLTAAHLLFFASNIQPGDLI
SHH_Homo_sapien    LVKDLSPG RVLAGRLLYSDFLTFL FYVI TREPRERLLLTAAHLLFFASRVRPGQRV
SHH_Danio_rerio    AVKDLNPG KVLAGNLVFSDFIMFT FYVI TQEPVEKITLTAAHLLFYASSVRAGQKV
SHH_Xenopus_(Si    AVRELRPG RVLSGNLIYSDFLLFI YYVIQT--SQTRIRMTAAHLLFFASNVRPGDVI
IHH_Homo_sapien    ALSAVRPG RVLAGSPTFSDVLIFL FQVI TQDPPRRLALTPAHLLFFASHVQPGQYVV
IHH_Mus_musculu    ALSAVKPG RVLAGTPTFSDVLIFL FQVI TQDPPRRLALTPAHLLFFASHVQPGQYVV
IHH_Danio_rerio    TLDSLQPGEKVLAGTLVYSEVIAFL FFTI T-DSGAKLSLTAAHLLFFASDVLPGQCV
IHHb_Danio_reri    QIRDLQAG LVLAGDLIYSEVLTFL FYVIRT-EDGASVSLTAAHLLFFASDAQVGQCL
IHH_Xenopus_lae    PVSQLSPGLRVLAGRPTYSDFLSFL FQVIKTQDPHRRLFLTPAHLIFFASSVRPGQYIT
HH_Drosophila_m    PLGELSIG RVLSGQAVYSEVILFM FVQLHT-DGGAVLTVTPAHLVSFAHRIEEKNQVR
HH_Branchiostom    RMRDVRPG KVLSGHPVFSEVLTFM YYTIHTDDRNITVTATPSHLVFFMSDARPGEFL
HH_Saccoglossus    SLDQLTIGEKVMAGTIVYSDVIMMM FHVI T-EDGQKLTLTPQHLVYYAADVELGHYV
HHtw_Danio_reri    PIKDLKVG RVLAGNVLISDFIMFI FIVI TSEPFTKLTLTAAHLVFFASNVKPGDTV
HH_Strongylocen    SMLDIRVG EVAVGALDYSDVIMIVHFYVI T-EDKSVVQLTPQHLIYFASEVRTNQFVL
HH1_Ciona_intes    PMSSLQPG QVLAGAVITDTFLSFM MVEITT-ENGFSVTLTRNHLIYFAAKVRQGDYIT
HH2_Ciona_intes    PMNKVNVG RILAGKPVFSEVIMFL YIEIRS--QLETIRLTANHMLFFAGDIFSGQYVT
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                          250       260       270       280       290
                   =========+=========+=========+=========+=========+=
DHH_Homo_sapien    AVGVFAPLTAHGTLLVNDVLASCYAVL SHQWAHRAFAPLRGMHWYSRLLY
DHHa_Xenopus_la    QTGVYAPMTEHGTLLV GVLTSCYATV SHTLAHVSLAPLRGVHWYCHILY
DHHb_Xenopus_la    QTGVYAPMTEHGTLLV GVLTSCYATV SHTLAHASLAPLRGVHWYCHILY
DHH_Xenopus_(Si    QTGVYAPMTEHGTLLV GVLTSCYATV SHTLAHASLAPLRGVHWYCHILY
DHH_Mus_musculu    AVGVFAPLTAHGTLLVNDVLASCYAVL SHQWAHRAFAPLRGMHWYSRLLY
DHH_Danio_rerio    RMGVYAPLTEHGNLFV GVLASNYATFQDHGLAHTVFWPFREVYWYARLLH
SHH_Mus_musculu    EAGAYAPLTAHGTILINRVLASCYAVI EHSWAHRAFAPFRGIHWYSQLLY
SHH_Xenopus_lae    DTGAYAPLTAHGTVVI QVLASCYAVI EHTWAHLAFAPLRGIHWYSQLLY
SHH_Homo_sapien    AAGAYAPLTAQGTILINRVLASCYAVI EHSWAHRAFAPFRGIHWYSQLLY
SHH_Danio_rerio    QRGSFAPVTAHGTIVV RILASCYAVI DQGLAHLAFAPARGVHWYSRLLY
SHH_Xenopus_(Si    DIGAFAPVTAQGTVVI EVLASCYAVI EHRWAHLAFAPLRGIHWYSRLLY
IHH_Homo_sapien    ALGAYAPLTKHGTLVVEDVVASCFAAVADHHLAQLAFWPLRGVHWYPQLLY
IHH_Mus_musculu    ALGSYAPLTRHGTLVVEDVVASCFAAVADHHLAQLAFWPLRGVHWYPQMLY
IHH_Danio_rerio    DRGVFAPLTSHGTVVVNGIVSSCYAAVDQHWLAHWAFGPLRGIHWYSSLLH
IHHb_Danio_reri    DQGLYPPLTAHGTVVVNDVLTSCYAAVNRQRLAHWAFAPLRGLHWYSQVLI
IHH_Xenopus_lae    NYGAYAPLTQHGTLVV DVVVSCFALVQKQRLAQIVYWPLRGIHWYSKALY
HH_Drosophila_m    SKGVVAPLTREGTIVVNSVAASCYAVINSQSLAHWGLAPMRGIHWYANALY
HH_Branchiostom    EKGAYAPLTVHGTVVV NVAMSCYALI SQALAHWVFAPFRGVHWYPSFFY
HH_Saccoglossus    NVGVYAPLTREGTIIINNIVASCYAMV-NHNIAQFAFGPIRGVHWYPYLLY
HHtw_Danio_reri    HEGSFAPVTAHGTIIV QVLASCYAVI NHKWAHWAFAPVRGIHWYSNMLF
HH_Strongylocen    GRTAVAPVTRQGSLVI DVAISSYAVMRDEWIAHASFAPVRRVHWYTQRLY
HH1_Ciona_intes    ASGAYAPLTYSGTIIVGGTAASCYAVI SDVIAHTVVSPFRGISLYSKLLH
HH2_Ciona_intes    GDGLYAPLTAHGTVVV GIVASCYGVIGSETLAHAAMLPIRGIHWYAKSLA

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Figure 2. Multiple alignment of hedgehog protein in chordates.

A.

B.

C.

D.

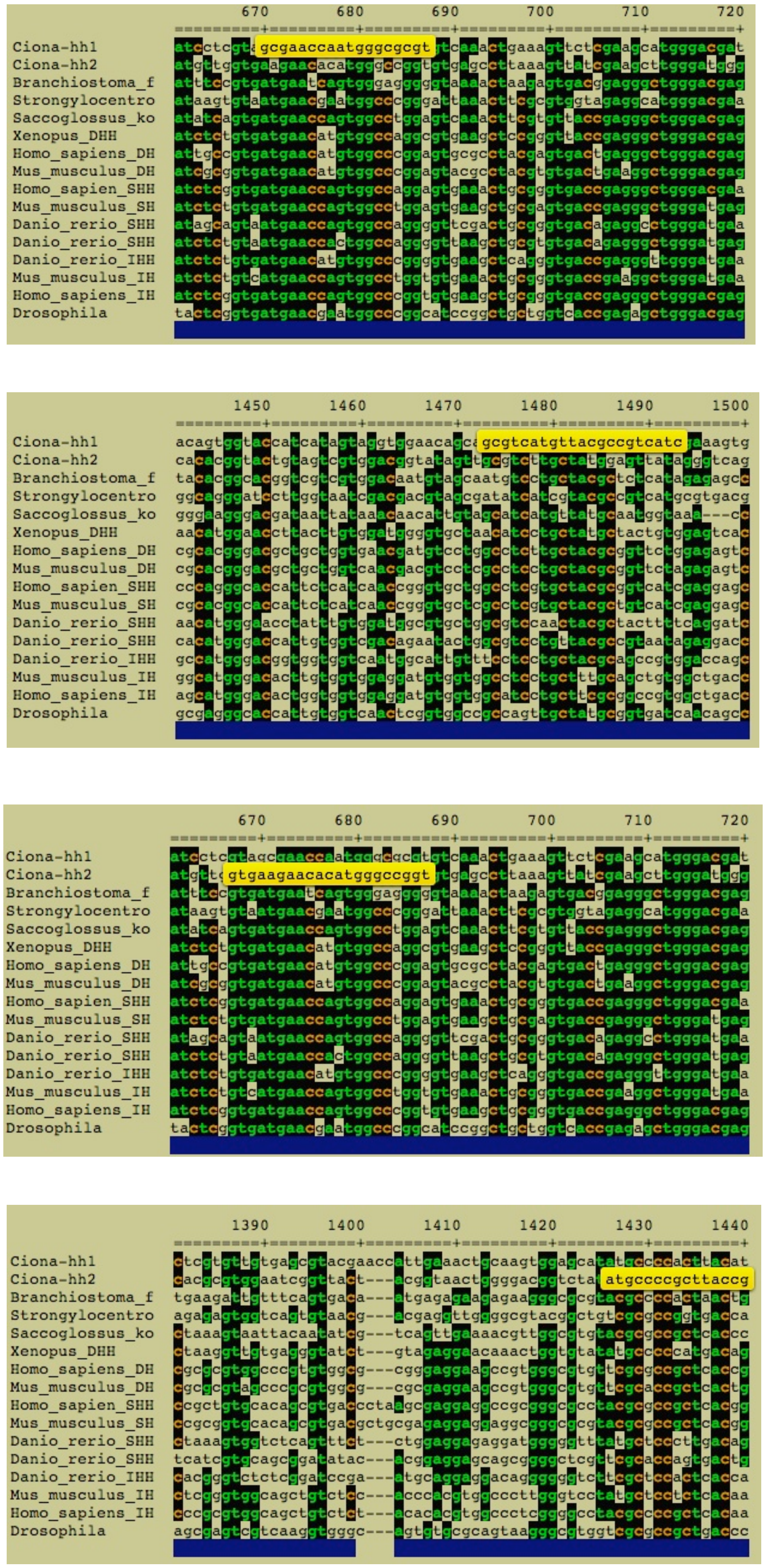


Figure 3. Alignments done using MEGA5 and Gblocks to locate conserved regions for primer design. Yellow blocks show primers. A. *bv-HH1* forward primer, B. *bv-HH1* reverse primer, C. *bv-HH2* forward primer, D. *bv-HH2* reverse primer.

Table 1. *bv-HH1, bv-HH2* forward and reverse primers showing their respective melting temperatures (Tm).

primer	Forward	Tm	Reverse	Tm
bv-HH1	GCGAACCAATGGGCGCGT	74ºC	GATGACGGCGTAACATGACGC	70ºC
bv-HH2	GTGAAGAACACATGGGCCGGT	71ºC	GTGCGGTAAGCGGGGCAT	71ºC

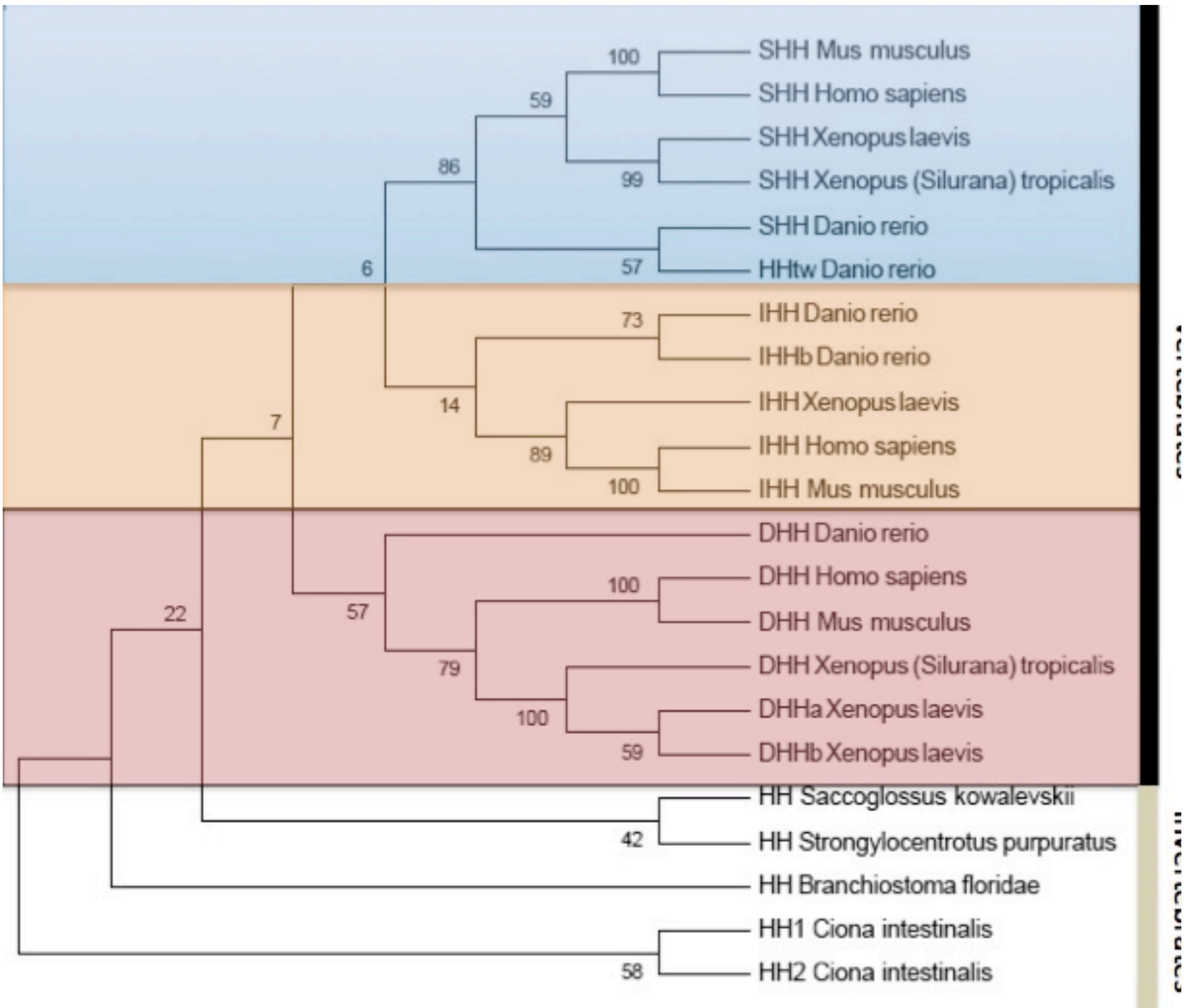
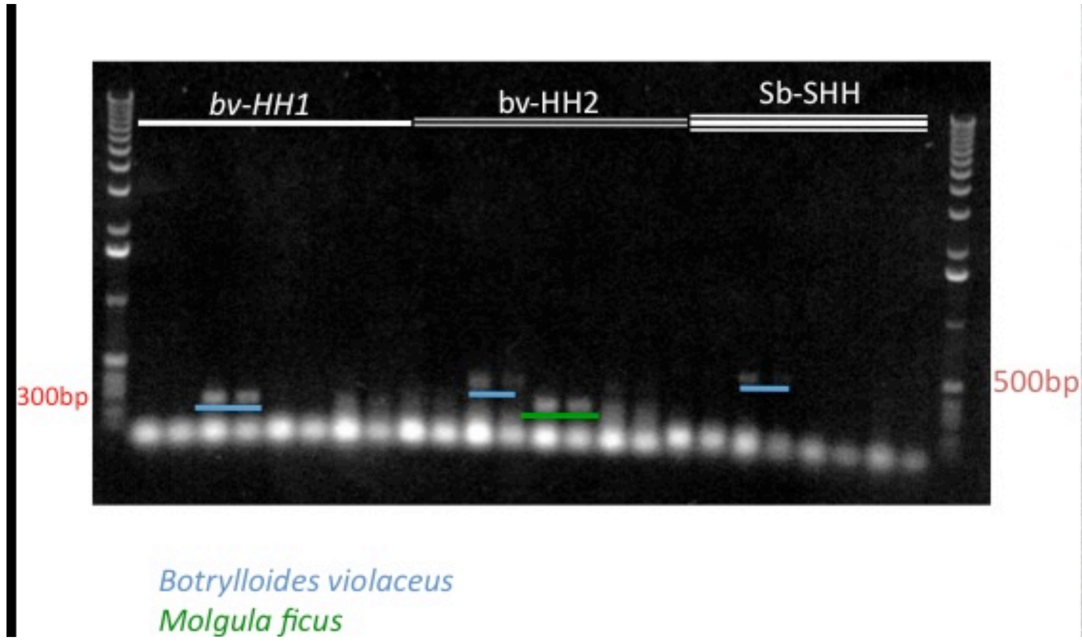


Figure 4. Maximum-likelihood gene tree showing evolution of *hedgehog* in vertebrates and invertebrates. Numbers at nodes show bootstrap values.

A.



B.

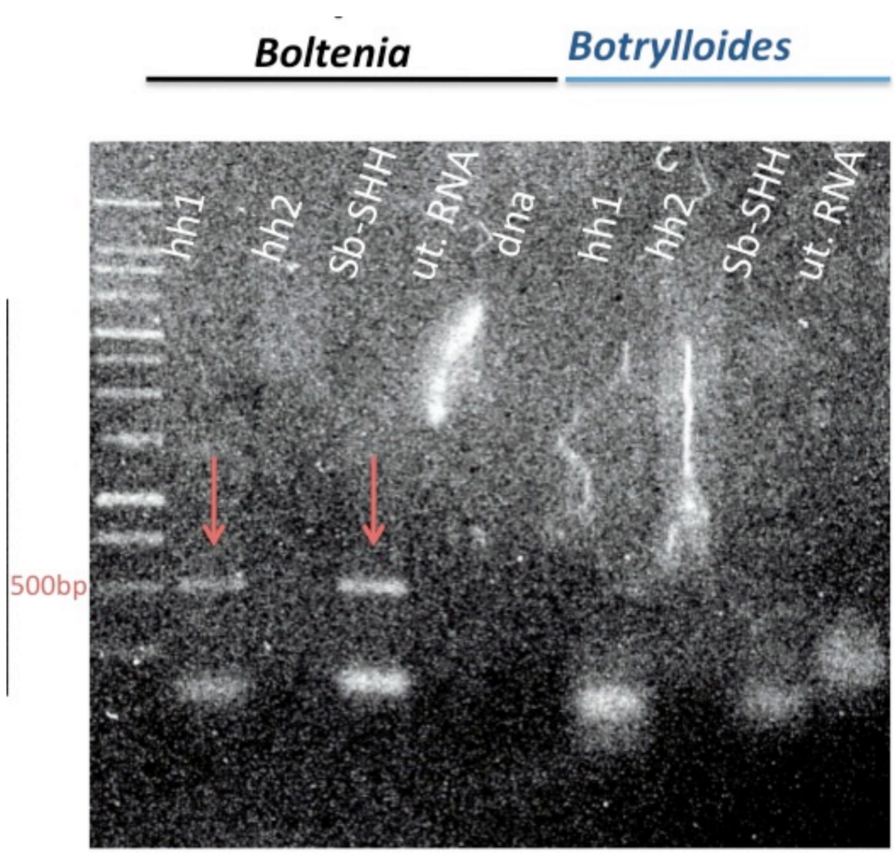


Figure 5. PCR gels. A. PCR ran on genomic DNA from *B. villosa, B. violaceus, M. ficus,* and *S. bromophenolosus.* B. PCR ran on cDNA from *B. villosa* and *B. violaceus.*

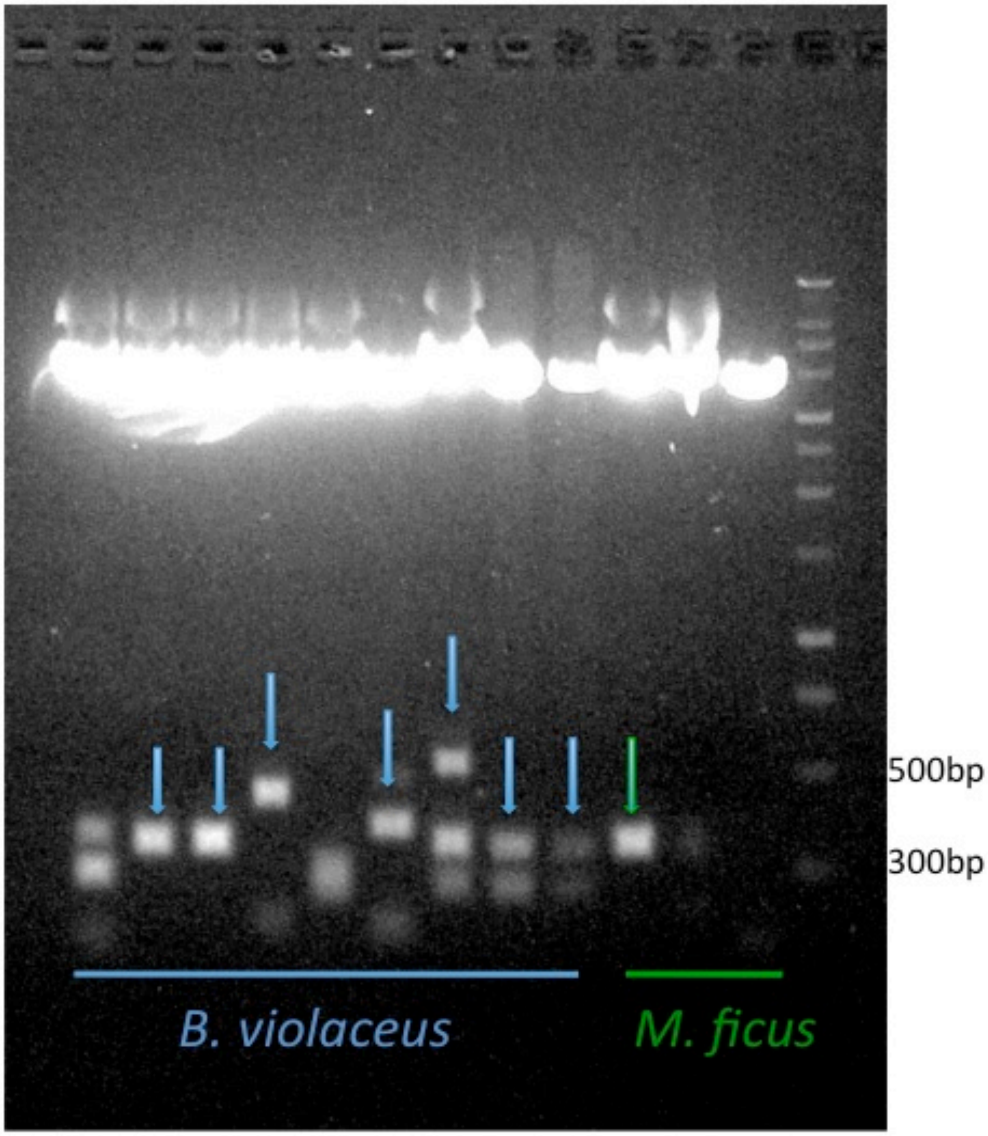


Figure 6. Gel ran on miniprep plasmid DNA from *B. violaceus* and *M. ficus* digested with EcoR1.