The Molecular Evolution of Hedgehog in Stolidobranchia Ascidians

René K. Miller^{1,2}, Billie J. Swalla^{1,2,3}

Evolution and Development of Metazoans Summer 2011 Friday Harbor Laboratories

¹Friday Harbor Laboratories, University of Washington, 620 University Road, Friday Harbor, WA 98250
²Department of Biology, University of Washington, Seattle, WA 98195
³Center for Developmental Biology, University of Washington, Box 351800, Seattle, WA 98195

Contact Information: René K. Miller 29093 Gamble Pl NE Kingston, WA 98346 renekat@me.com

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 Phleobranchia 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 (12°C). 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 12°C 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/). Nonhighly 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 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 94°C for 4 minutes; 35 cycles of denature 94°C for 1 minute, annealing temperatures varied from 37°C to 58°C for 30 seconds, extension at 72°C for 1 minute; final extension at 72°C 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 37°C. 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 -20°C.

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 37°C 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 37°C 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.

Acknowledgements

I would like to take this opportunity to thank the faculty, students, and staff at Friday Harbor Laboratories. I greatly appreciate the opportunity to work alongside so many talented individuals. I would like to extend my thanks to my professors Dr. Billie Swalla and Dr. Ken Halanych, and to my TAs Joie Cannon and Kevin Kocot for all their patience and time. Thank you to Max Maliska for allowing me to use his *M. ficus* DNA. Thank you to all the students in my EvoDevo class for teaching me how to dance and putting a smile on my face.

Additionally, I'd like to thank my father Rick Miller for always believing in me and motivating me stay true to myself. Finally, I'd like to thank my mentor Hector Rincon, for his many late night talks, research knowledge, and patience.

References

- Brown FD, Prendergast A, Swalla BJ. 2008. Man is but a worm: Chordate origins. *Genesis* 46 (11) (NOV): 605-13.
- Davidson B, Wallace SES, Howsmon RA, Swalla BJ. 2003. A morphological and genetic characterization of metamorphosis in the ascidian <u>Boltenia villosa</u>. *Development Genes and Evolution* 213 (12) (DEC): 601-11.
- Hudson C, Ba M, Rouviere C, Yasuo H. 2011. Divergent mechanisms specify chordate motoneurons: Evidence from ascidians. *Development* 138 (8) (APR 15): 1643-52.
- Imai JH, Meinertzhagen IA. 2007. Neurons of the ascidian larval nervous system in <u>Ciona intestinalis</u>: I. central nervous system. *Journal of Comparative Neurology* 501 (3) (MAR 20): 316-34.
- Jiang J, Hui C. 2008. Hedgehog signaling in development and cancer. *Developmental Cell* 15 (6) (DEC 9): 801-12.

Kott, P. (1985). The Australian Ascidiacea Pt 1, Phlebobranchia and Stolidobranchia.

Mem. Queensl. Mus. 23: 1-440

- Lemaire P. 2009. Unfolding a chordate developmental program, one cell at a time: Invariant cell lineages, short-range inductions and evolutionary plasticity in ascidians. *Developmental Biology* 332 (1) (AUG 1): 48-60.
- Monje M, Mitra SS, Freret ME, Raveh TB, Kim J, Masek M, Attema JL, et al. 2011. Hedgehog-responsive candidate cell of origin for diffuse intrinsic pontine glioma. *Proceedings of the National Academy of Sciences of the United States of America* 108 (11) (MAR 15): 4453-8.
- Nüsslein-Volhard C, Wieschaus E. 1980. Mutations affecting segment number and polarity in <u>Drosophila</u>. 287 (5785) (10/30/print): 801, http://dx.doi.org/10.1038/287795a0.
- Satou Y, Kusakabe T, Araki L, Satoh N. 1995. Timing of initiation of muscle-specific gene expression in the ascidian embryo precedes that of developmental fate restriction in lineage cells. 37 (3): 327, http://dx.doi.org/10.1046/j.1440-169X.1995.t01-2-00010.x.
- Takatori N, Satou Y, Satoh N. 2002. Expression of hedgehog genes in ciona intestinalis embryos RID C-4123-2009. *Mechanisms of Development* 116 (1-2) (AUG): 235-8.
- Zeng L, Jacobs MW, Swalla BJ. 2006. Coloniality has evolved once in Stolidobranch Ascidians. *Integrative and Comparative Biology*. 46,3: 255-268.

Figures



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

	10	20) 30	0	40	50	60
	=======+====	=====+=	=======+=		+========	=+======	===+
DHH_Homo_sapien	RYARKQLVPLLYK	FVPGVP	RTLGASGPA	GRVA <mark>R</mark> GS	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	т
DHH_Xenopus_(Si	RRYMRKLVPLRYK	FVP VP	KTLGASGKS	GKIRRGS	RFI LVP Y	P IIFK	т
DHH_Mus_musculu	RYVRKQLVPLLYK	FVPSMP	RTLGASGPA	GRVT<mark>R</mark>GS	RFRDLVP Y	P IIFK	S
DHH Danio rerio	RHRQRKLTPMSYK	YV₽GVS	NNLGASGRA	GRITRSS	RFN LVC Y	T IDFK	RS
SHH Mus musculu	RRHPKKLTPLAYK	FIP VA	KTLGASGRY	GKITRNS	RFK LTP Y	P IIFK	т
SHH Xenopus lae	RRHPKKLTPLAYK	FIP VA	KTLGASGRY	GKITRNSI	CFK LTP Y	P IMFK	ST
SHH Homo sapien	RRHPKKLTPLAYK	FIP VA	KTLGASGRY	GKISRNS	RFK LTP Y	P IIFK	т
SHH Danio rerio	RRHPKKLTPLAYK	FIP VA	KTLGASGRY	GKITRNS	RFK LTP Y	P IIFK	т
SHH Xenopus (Si							
IHH Homo sapien	RRPPRKLVPLAYK	FSP VP	KTLGASGRY	GKIARSS	RFK LTP Y	P IIFK	т
IHH Mus musculu	RRPPRKLVPLAYK	FSP VP	KTLGASGRY	GKIARSS	RFK LTP Y	P IIFK	т
IHH Danio rerio	RRPPKKLTPLNYK	FSP VA	KTLGASGRI	GKITRNS	RFK LTP Y	P IIFK	т
IHHb Danio reri	RRTPRKLTPLAYK	FSP VA	KTLGASGRY	GKVTPS S	RFK LTP Y	P IIFK	т
IHH Xenopus lae	RRPTKLSPLSYK	FSP VP	KTLGASGRY	GKISRNS	RFK LTP Y	P IIFK	IT
HH Drosophila m	RHRARNLYPLVLK	TIP LS	YTNSASGPL	GVIRRDS	KFKDLVP Y	R ILFR	GT
HH Branchiostom	RRHPRKLTPFVYK	QMPAVS	NTFGASGLFN	NGRITRDS	RFHTLKQ F	T IIFK	KT
HH Saccoglossus	RRPSRELTPLLYK	CIP VS	NTLGASGPN	K <mark>K</mark> IT <mark>R</mark> EDI	EFKDLQTVY	A IMFK	GT
HHtw Danio reri	RRHPKKLTPLAYK	FIP VA	KTLGASGKY	GKITRNS	RFK LIP Y	P IIFK	т
HH Strongylocen	SHRPRNRTPLQYK	RVP IS	DTFGASGPP	GRINRND	RFNTLSP N	D IVFK	K G <mark>T</mark>
HH1 Ciona intes	RMPGRELVPFLKG	EYVPKMS	QTIGASGPV	FGRIRADTE	PRFR LVP W	T IEFR	ES
HH2 Ciona intes	RPNQRNLRPLLRQ	YV₽HVS	GTIGASGPS	GRIY RNTE	RYRKLER Y	T IEFE I	R RD
	##############	########	+#########	#########	+#########	+#######	####

		70		80	90		100	1	10	1	20
DUUL Home gapier	<u> </u>	DI MUDCKED	T7 NT 7			CM				тттс	-T
	GA	REFIRER		TCIM	MWPGVRLRVT	GW	GHHAU		GRAL	TUDE	R
DHHa_Xenopus_la	GA	REMIRCE R			MWPGVKLRVT	GW	СЦЦАЦ		CPAL		R
DHHD_Aenopus_Ia	GA	REMIRCE R			MWPGLKLRVT	GW	GHHAH		GRAL	TUDE	R
DHH_Xellopus_(SI	GA	REMIRCE R			MWPGVKLRVT	GW	GHHAH		GRAL	TUTS	R
DHH_MUS_MUSCUIU	GA	REFIRER			MWPGVRLRVT	GW	GHHAQ		GRAL	TTTS	R
DHH_Danio_rerio	NA	RFMTRCK C			QWPGVRLRVT	AW	GHHPP	GSLHI	GRAV	TTTS	R
SHH_MUS_MUSCUIU	GA	REFIRER K			QWPGVKLRVT QWPGVKLRVT	GW	GHHS	SLHI	GRAV	TTTS	R
SHH_Xellopus_lae	GA	REFIRCK K			QWPGVKLRVT	GW	GHHL	SLHI	GRAV	TTTS	R
SHH_HOMO_sapien	GA	RLMTRCK K		TCIM	QWPGVKLRVT	GW	GHHS	SLHY	GRAV	TTTS	R
SHH_Danio_rerio	GA	REPTRUK K	.ь эьғ	15VM	HWPGVKLRVT	GW	GHHF	SLHI	GRAV	TTTS	R
SHH_xenopus_(S1		DI NEDGE D			VKLRVT	GW	GHHS	SLHY	GRAV	TTTS	R
IHH_HOMO_sapien	GA	RLMTRCK R		TOW	QWPGVKLRVT	GW	GHHS	SLHY	GRAV	TTTS	R
IHH_MUS_MUSCULU	GA	RLMTRCK R		ISVM .	QWPGVKLRVT	GW	GHHS	SLHY	GRAV	TTTS	R
IHH_Danio_rerio	GA	RLMTRCK K		ISVM	MWPGVKLRVT	GW	GNHF	DSLHY	GRAV	TTTS	R
IHHD_Danio_reri	GA	RMMTRCK K	L SLA	ISVM .	LWPGVRLRVT	GW	GLHS	SLHY	GRAV	TTTS	R
IHH_Xenopus_lae	GA	RLMTRCK R	LSLA	ISVM.	QWPGVKLRVT	GW	GHHF	SLHY	GRAV	ITTS	R
HH_Drosophila_m	GA	GLMSRCKEK	L VLA	YSVM	EWPGIRLLVT	SW	YHHGQ	SLHY	GRAV	LATS	R
HH_Branchiostom	GA	RFMTRCK K	L ALA	ISVM	QWEGVKLRVT	GW	GFHT	SLHY	GRAV	ITTS	R
HH_Saccoglossus	GA	RLMTRCK R	L SLA	ISVM	QWPGVKLRVT	GW	GHHAP	NSLHY	GRAV	ITTN	R
HHtw_Danio_reri	NA	RLMTRCK K	L SLA	ISVM	HWPGVKLRVT	GW	GRHL	SLHY	GRAV	ITTS	R
HH_Strongylocen	GA	RLMTRCK K	L TLA	ISVM	EWPGIKLRVV	AW	QPNV	-PLHA	GRAV	ITTS	R
HH1_Ciona_intes	NE	RFMTICRAR	LDYLA	ILVA	QWARVKLKVL	AW	DGNDKAN	IDPLHY	GRAV	ITTD	A
HH2_Ciona_intes	GS	RTMTRCK K	V LLS	SMLVK	TWAGVSLKVI	AW	G GVHRK	GSLHY	GRAV	IKTS	N
	###	+##########	#####	*####	+##########	###7	########	#####	#####	#####	##
		130		140	150		160	1'	70	1	80
	===	130		140	150	:	160 ====+===	1'	70 =+===	1	80 =+
DHH Homo sapien	=== RN	130 =====+== \KYGLLARLA	V AGE	140 ==+==	150 =====+== SRNHVHVSV	==== KAD	160 ====+=== SLAVRAG	1 GCFPGI	70 =+==== IATVR	1 ===== LWSGE	80 =+ R <mark>K</mark>
DHH_Homo_sapien DHHa Xenopus la	=== RN RN	130 =====+== \KYGLLARLA \KYGMLARLA	V AGE	140 ==+== WVYY WVYY	150 SRNHVHVSV SKAHIHVSV	==== KAD KAD	160 ====+=== SLAVRAG SLGVRSG	1 GCFPGI	70 =+=== IATVRI FAMVMI	1 ===== LWSGEI MGTGEI	80 =+ R <mark>K</mark> R <mark>K</mark>
DHH_Homo_sapien DHHa_Xenopus_la DHHb Xenopus la	=== RN RN RN	130 =====+== VKYGLLARLA VKYGMLARLA VKYGMLARLA	V AGE V AGE V AGE	140 ==+= WVYY WVYY	150 SRNHVHVSV SKAHIHVSV SKAHIHVSV	==== KAD KAD NTD	160 SLAVRAG SLGVRSG SLGVRSG	1 GCFPGI GCFPGI	70 =+=== JATVRJ FAMVMI FAMVMI	1 ===== LWSGEI MGTGEI M TGKI	80 =+ RK RK KK
DHH_Homo_sapien DHHa_Xenopus_la DHHb_Xenopus_la DHH Xenopus (Si	=== RN RN RN RS	130 =====+== VKYGLLARLA VKYGMLARLA VKYGMLARLA SKYGMLARLA	V AGI V AGI V AGI V AGI	140 ==+== WVYY WVYY WVYY	150 SRNHVHVSV SKAHIHVSV SKAHIHVSV SKAHIHVSV	KAD KAD NTD KAD	160 SLAVRAG SLGVRSG SLGVRSG SLGVRSG	1 GCFPG GCFPG GCFPG GCFPG	70 =+=== IATVRI IATVRI IAMVMI IAMVMI	1 LWSGE MGTGE M TGK M TGK	80 =+ RK RK KK KK
DHH_Homo_sapien DHHa_Xenopus_la DHHb_Xenopus_la DHH_Xenopus_(Si DHH_Mus_musculu	=== RN RN RS RN	130 	V AGE V AGE V AGE V AGE V AGE	140 	150 SRNHVHVSV SKAHIHVSV SKAHIHVSV SKAHIHVSV SRNHIHVSV	==== KAD KAD NTD KAD KAD	160 SLAVRAG SLGVRSG SLGVRSG SLGVRSG SLAVRAG	1 GCFPG GCFPG GCFPG GCFPG	70 =+=== IATVRI IAMVMI IAMVMI IAMVMI IATVRI	1 LWSGEI MGTGEI M TGKI MASGEI LRSGEI	80 =+ RK RK KK KK RK
DHH_Homo_sapien DHHa_Xenopus_la DHHb_Xenopus_la DHH_Xenopus_(Si DHH_Mus_musculu DHH Danio rerio	=== RN RN RS RN TF	130 	V AGE V AGE V AGE V AGE V AGE V AGE	140 	150 SRNHVHVSV SKAHIHVSV SKAHIHVSV SKAHIHVSV SRNHIHVSV SKYHVHCSV	==== KAD KAD NTD KAD KAD	160 SLAVRAG SLGVRSG SLGVRSG SLGVRSG SLAVRAG	1 GCFPGI GCFPG GCFPG GCFPG GCFPGI GCFSAS	70 =+=== PATVRI PAMVMI PAMVMI PAMVMI JATVRI SGLVTI	1 LWSGEI MGTGEI M TGKI MASGEI LRSGEI MADGV(80 =+ RK RK KK KK RK OK
DHH_Homo_sapien DHHa_Xenopus_la DHHb_Xenopus_la DHH_Xenopus_(Si DHH_Mus_musculu DHH_Danio_rerio SHH Mus musculu	=== RN RN RS RN TF	130 WYGLLARLA WYGMLARLA WYGMLARLA SKYGMLARLA KYGLLARLA SKYGMLARLA	V AGE V AGE V AGE V AGE V AGE V AGE V AGE	140 	150 SRNHVHVSV SKAHIHVSV SKAHIHVSV SKAHIHVSV SRNHIVSV SKHVHCSV SKHVHCSV	==== KAD KAD KAD KAD KADI KADI	160 SLAVRAG SLGVRSG SLGVRSG SLAVRAG SLAVRAG SVAVEKG SVAAKSG	1 GCFPG GCFPG GCFPG GCFPG GCFPG GCFSAS	70 =+==== TATVRI TAMVMI TAMVMI TAMVMI JATVRI SGLVTI SATVHI	1 LWSGE MGTGE M TGK MASGE LRSGE MADGV L OGG	80 =+ RK RK KK KK RK QK TK
DHH_Homo_sapien DHHa_Xenopus_la DHHb_Xenopus_la DHH_Xenopus_(Si DHH_Mus_musculu DHH_Danio_rerio SHH_Mus_musculu SHH_Xenopus lae	=== RN RN RS RN TF RS RS	130 VKYGLLARLA VKYGMLARLA VKYGMLARLA VKYGLLARLA VKYGLLAQLA SKYGMLARLA SKYGMLGRLA	V AGF V AGF V AGF V AGF V AGF V AGF V AGF V AGF	140 	150 SRNHVHVSV SKAHIHVSV SKAHIHVSV SKAHIHVSV SRNHIVSV SKAHIHCSV SKAHIHCSV	KAD KAD NTD KAD KAD KADI KA	160 SLAVRAG SLGVRSG SLGVRSG SLAVRAG SLAVRAG SVAAKSG SVAAKSG	1 GCFPG GCFPG GCFPG GCFPG GCFPG GCFPG GCFPG	70 =+==== FAMVMI FAMVMI FAMVMI JATVRI SGLVTI SATVHI SARVMI	1 LWSGEJ MGTGEJ MSGEJ MASGEJ LRSGEJ MADGV L QGG' V FGG'	80 =+ RK RK KK KK RK QK TK
DHH_Homo_sapien DHHa_Xenopus_la DHHb_Xenopus_la DHH_Xenopus_(Si DHH_Mus_musculu DHH_Danio_rerio SHH_Mus_musculu SHH_Xenopus_lae SHH Homo sapien	=== RN RN RS RN TF RS RS	130 VKYGLLARLA VKYGMLARLA VKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA	V AGI V AGI V AGI V AGI V AGI V AGI V AGI V AGI	140 	150 SRNHVHVSV SKAHIHVSV SKAHIHVSV SKAHIHVSV SRNHIVSV SKAHIHCSV SKAHIHCSV SKAHIHCSV	===: KAD KAD KAD KAD KADI KA KA KA	160 SLAVRAG SLGVRSG SLGVRSG SLAVRAG SLAVRAG SVAAKSG SVAAKSG SVAAKSG	1 GCFPG GCFPG GCFPG GCFPG GCFPG GCFPG GCFPG GCFPG	70 JATVRI TAMVMI TAMVMI JATVRI SGLVTI SATVHI SARVMY SATVHI	1 LWSGE MGTGE M TGK MASGE LRSGE MADGV L QGG V FGG V FGG	80 =+ RK RK KK KK KK KK KK TK TK
DHH_Homo_sapien DHHa_Xenopus_la DHHb_Xenopus_la DHH_Xenopus_(Si DHH_Mus_musculu DHH_Danio_rerio SHH_Mus_musculu SHH_Xenopus_lae SHH_Homo_sapien SHH_Danio_rerio	=== RN RN RS RS RS RS RS	130 VKYGLLARLA VKYGMLARLA VKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA	V AGI V AGI V AGI V AGI V AGI V AGI V AGI V AGI V AGI	140 	150 SRNHVHVSV SKAHIHVSV SKAHIHVSV SKAHIHVSV SRNHIVSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV	KAD KAD KAD KAD KAD KAD KA KA KA	160 SLAVRAG SLGVRSG SLGVRSG SLAVRAG SLAVRAG SVAAKSG SVAAKSG SVAAKSG SVAAKSG	1 GCFPG GCFPG GCFPG GCFPG GCFPG GCFPG GCFPG GCFPG	70 =+=== FAMVMI FAMVMI FAMVMI JATVRI SGLVTI SATVHI SARVMI SATVHI SALVSI	1 LWSGE MGTGE MASGE LRSGE MADGV LQGG VFGG LQGG LQGG	80 =+ RK RK KK KK RK QK TK TK OK
DHH_Homo_sapien DHHa_Xenopus_la DHHb_Xenopus_la DHH_Xenopus_(Si DHH_Mus_musculu DHH_Danio_rerio SHH_Mus_musculu SHH_Xenopus_lae SHH_Homo_sapien SHH_Danio_rerio SHH_Xenopus (Si	=== RN RN RS RS RS RS RS RS RS RS RS	130 VKYGLLARLA VKYGMLARLA VKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA	V AGI V AGI V AGI V AGI V AGI V AGI V AGI V AGI V AGI V AGI	140 	150 SRNHVHVSV SKAHIHVSV SKAHIHVSV SKAHIHVSV SKAHIHVSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV	KAD KAD NTD KAD KAD KADI KA KA KA KA	160 SLAVRAG SLGVRSG SLGVRSG SLAVRAG SVAVEKG SVAKSG SVAAKSG SVAAKSG SVAAKSG SVAAKSG	1 GCFPG GCFPG GCFPG GCFPG GCFPG GCFPG GCFPG GCFPG GCFPG	70 =+=== FAMVMI FAMVMI FAMVMI SATVMI SATVHI SARVMI SALVSI SARVMI	1 WSGE MGTGE MASGE LRSGE MADGV UFGG VFGG QDGG VFGG VFGG VFGG	80 =+ RK RK KK KK RK QK TK QK TK
DHH_Homo_sapien DHHa_Xenopus_la DHHb_Xenopus_la DHH_Xenopus_(Si DHH_Mus_musculu DHH_Danio_rerio SHH_Mus_musculu SHH_Xenopus_lae SHH_Homo_sapien SHH_Danio_rerio SHH_Xenopus_(Si IHH Homo_sapien	=== RN RN RS RS RS RS RS RS RS RS RS RS	130 VKYGLLARLA VKYGMLARLA VKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA VKYGLLARLA	V AGI V AGI V AGI V AGI V AGI V AGI V AGI V AGI V AGI V AGI	140 	150 SRNHVHVSV SKAHIHVSV SKAHIHVSV SKAHIHVSV SKAHIHVSV SKAHIHVSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV	===: KAD KAD KAD KAD KAD KA KA KA KA KA KA	160 SLAVRAG SLGVRSG SLGVRSG SLAVRAG SVAVEKG SVAAKSG SVAAKSG SVAAKSG SVAAKSG	1 GCFPG GCFPG GCFPG GCFPG GCFPG GCFPA GCFPG GCFPG GCFPG GCFPA	70 =+=== IATVRI	1 WSGE MGTGE MASGE LRSGE MADGV J FGG J FGG L QGG J PGG J SGA	80 =+ RK RK KK KK RK QK TK TK QK TK RV
DHH_Homo_sapien DHHa_Xenopus_la DHHb_Xenopus_la DHH_Xenopus_(Si DHH_Mus_musculu DHH_Danio_rerio SHH_Mus_musculu SHH_Xenopus_lae SHH_Homo_sapien SHH_Danio_rerio SHH_Xenopus_(Si IHH_Homo_sapien IHH_Mus_musculu	=== RN RN RS RS RS RS RS RS RS RS RS RS RS RS RS	130 VKYGLLARLA VKYGMLARLA VKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA JKYGLLARLA JKYGLLARLA	V AGI V AGI	140 	150 SRNHVHVSV SKAHIHVSV SKAHIHVSV SKAHIHVSV SKAHIHVSV SKAHIHVSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHVHCSV SKAHVHCSV SKAHVHCSV	SESSIVE STREET	160 SLAVRAG SLGVRSG SLGVRSG SLAVRAG SVAKSG SVAKSG SVAKSG SVAKSG SVAKSG SVAKSG	1 GCFPG GCFPG GCFPG GCFPG GCFPA GCFPA GCFPA GCFPA GCFPA	70 =+=== IATVRI IATVRI IATVRI SGLVTI SATVHI SATVHI SATVHI SATVHI SATVHI SARVMI SARVMI SARVRI SAQVRI SAQVRI SAQVRI	1 WSGE MGTGE MASGE LRSGE MADGV JGG JGG JGG JGG JGG JGG JGG JGG JGG J	80 =+ RK RK KK KK KK CK TK TK QK TK RV RV
DHH_Homo_sapien DHHa_Xenopus_la DHHb_Xenopus_la DHH_Xenopus_(Si DHH_Mus_musculu DHH_Danio_rerio SHH_Mus_musculu SHH_Xenopus_lae SHH_Homo_sapien SHH_Danio_rerio SHH_Xenopus_(Si IHH_Homo_sapien IHH_Mus_musculu IHH_Danio_rerio	EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE	130 VKYGLLARLA VKYGMLARLA VKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA JKYGLLARLA JKYGLLARLA JKYGLLARLA	V AGI V AGI	140 	150 SRNHVHVSV SKAHIHVSV SKAHIHVSV SKAHIHVSV SKAHIHVSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHVHCSV SKAHVHCSV SKAHVHCSV SKAHVHCSV SKAHVHCSV SKAHIHCSV	STAND	160 SLAVRAG SLGVRSG SLGVRSG SLAVRAG SVAKSG SVAKSG SVAKSG SVAKSG SVAKSG SVAKSG SVAKSG SVAKSG SVAKSG SVAKSG	1 GCFPG GCFPG GCFPG GCFPG GCFPG GCFPA GCFPG GCFPA GCFPA GCFPA	70 =+=== IATVRI IATVRI IATVRI SATVRI SATVRI SATVRI SATVRI SATVRI SATVRI SALVSI SARVRI SAQVRI SAQVRI SAQVRI SALVTI	1 WSGE MGTGE MASGE LRSGE MADGV LQGG VFGG QDGG VFGG LQGG LSGA LSGA LNGE VDGS	80 =+ RK RK KK RK QK TK TK QK TK RV RV LK
DHH_Homo_sapien DHHa_Xenopus_la DHHb_Xenopus_la DHH_Xenopus_(Si DHH_Mus_musculu DHH_Danio_rerio SHH_Mus_musculu SHH_Xenopus_lae SHH_Homo_sapien SHH_Danio_rerio SHH_Xenopus_(Si IHH_Homo_sapien IHH_Mus_musculu IHH_Danio_rerio IHHb_Danio_rerio	RN RN RN RS RN TH RS RS RS RS RS RS RN RN RN RN RN RN RN RN RN RN RN RN RN	130 VKYGLLARLA VKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA JKYGLLARLA JKYGMLARLA JKYGMLARLA	V AGI V AGI	140 	150 SRNHVHVSV SKAHIHVSV SKAHIHVSV SKAHIHVSV SKAHIHVSV SKAHIHVSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHVHCSV SKAHVHCSV SKAHVHCSV SKAHVHCSV SKAHVHCSV SKAHVHCSV	STATES STREET ST	160 SLAVRAG SLGVRSG SLGVRSG SLAVRAG SVAKSG SVAKSG SVAKSG SVAKSG SVAKSG SVAAKSG SVAAKSG SVAAKSG SVAAKSG SVAAKSG SVAAKSG SVAAKSG	1 GCFPG GCFPG GCFPG GCFPG GCFPG GCFPG GCFPG GCFPG GCFPG GCFPA GCFPA GCFPA	70 =+=== IATVRI IATVRI IATVRI SGLVTI SATVHI SATVHI SATVHI SATVHI SATVHI SATVRI SATVRI SALVSI SAQVRI SAQVRI SAQVRI SAQVRI SALVTI	1 WSGE MGTGE MASGE RSGE MADGV QGG QGG QGG QDGG V FGG V FGGG V FGG V FGG	80 =+ RK RK KK RK QK TK RV RV LK HR
DHH_Homo_sapien DHHa_Xenopus_la DHHb_Xenopus_la DHH_Xenopus_(Si DHH_Mus_musculu DHH_Danio_rerio SHH_Mus_musculu SHH_Xenopus_lae SHH_Homo_sapien SHH_Danio_rerio SHH_Xenopus_(Si IHH_Homo_sapien IHH_Mus_musculu IHH_Danio_rerio IHHb_Danio_rerii IHH Xenopus lae	RN RN RS RS RS RS RS RS RS RS RS RS RS RS RS	130 VKYGLLARLA VKYGMLARLA VKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA JKYGLLARLA JKYGMLARLA JKYGMLARLA JKYGMLARLA	V AGI V AGI	140 	150 SRNHVHVSV SKAHIHVSV SKAHIHVSV SKAHIHVSV SKAHIHVSV SKAHIHVSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHVHCSV SKAHVHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV	EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE	160 SLAVRAG SLGVRSG SLGVRSG SLAVRAG SVAKSG SVA SVA SVA SVA SVA SVA SVA SVA SVA SVA	1 GCFPG GCFPG GCFPG GCFPG GCFPG GCFPG GCFPG GCFPG GCFPA GCFPA GCFPA GCFPA	70 =+=== IATVRI IATVRI IATVRI SGLVTI SATVHI SATVHI SATVHI SATVHI SALVSI SARVMI SALVSI SARVMI SALVTI SALVTI SALATI	1 WSGE MGTGE MASGE LRSGE MADGV LQGG VFGG QDGG VFGG LQGG LSGA LSGE MKDGS	80 =+ RK RK KK KK CK TK CK CK RV LK RV LK HR
DHH_Homo_sapien DHHa_Xenopus_la DHHb_Xenopus_la DHH_Xenopus_(Si DHH_Mus_musculu DHH_Danio_rerio SHH_Mus_musculu SHH_Xenopus_lae SHH_Homo_sapien SHH_Danio_rerio SHH_Xenopus_(Si IHH_Homo_sapien IHH_Mus_musculu IHH_Danio_rerio IHHb_Danio_reri IHH_Xenopus_lae HH_Drosophila m	RN RN RS RS RS RS RS RS RS RS RS RS RS RS RS	130 VKYGLLARLA VKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA JKYGLLARLA JKYGMLARLA JKYGMLARLA JKYGMLARLA	V AGI V AGI	140 	150 SRNHVHVSV SKAHIHVSV SKAHIHVSV SKAHIHVSV SKAHIHVSV SKAHIHVSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHVHCSV SKAHVHCSV SKAHVHCSV SKAHVHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV	EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE	160 SLAVRAG SLGVRSG SLGVRSG SLAVRAG SVAKSG SVAKSG SVAKSG SVAKSG SVAKSG SVAKSG SVAKSG SVAKSG SVAKSG SVAKSG SVAKSG SVAKSG SVAKSG SSAAKTG SSAAKTG SSISSHVH	1 GCFPG GCFPG GCFPG GCFPG GCFPG GCFPG GCFPG GCFPG GCFPA GCFPA GCFPA GCFPA	70 =+=== IATVRI IATVRI IATVRI SGLVTI SATVHI SATVHI SATVHI SATVHI SATVHI SALVSI SARVMI SALVSI SARVMI SALVTI SALVTI SALVTI SALATI	1 WSGE MGTGE MASGE LRSGE MADGV LQGG VFGG VFGG LQGG VFGG LSGE MKDGS LSGE SGV	80 =+ RK RK KK KK KK CK TK CV RV RV RV LK RV RV RV RV RV RV RV
DHH_Homo_sapien DHHa_Xenopus_la DHHb_Xenopus_la DHHb_Xenopus_(Si DHH_Mus_musculu DHH_Danio_rerio SHH_Mus_musculu SHH_Xenopus_lae SHH_Homo_sapien SHH_Danio_rerio SHH_Xenopus_(Si IHH_Homo_sapien IHH_Mus_musculu IHH_Danio_rerio IHHb_Danio_reri IHH_Xenopus_lae HH_Drosophila_m HH Branchiostom	RN RN RS RS RS RS RS RS RS RS RS RS RS RS RS	130 VKYGLLARLA VKYGMLARLA VKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA JKYGLLARLA JKYGMLARLA JKYGMLARLA JKYGMLARLA JKYGMLARLA	V AGI V AGI	140 	150 SRNHVHVSV SKAHIHVSV SKAHIHVSV SKAHIHVSV SKAHIHVSV SKAHIHVSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHVHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV	ESSI KAD NTD KAD KADI KA KA KA KA KA KA KA KS I KS I KS I KS	160 SLAVRAG SLGVRSG SLGVRSG SLAVRAG SVAKSG SVAKSG SVAKSG SVAKSG SVAKSG SVAKSG SVAKSG SVAKSG SVAKSG SVAKSG SSAAKTG HSAAKTG HSVAAKTG HSVAAKTG SSISHVH SSISHVH	1 GCFPG GCFPG GCFPG GCFPG GCFPG GCFPG GCFPG GCFPG GCFPG GCFPA GCFPG GCFPG GCFPG GCFPG GCFPG GCFPG GCFPG	70 =+=== IATVRI IATVRI IATVRI SGLVTI SATVHI SATVHI SATVHI SATVHI SATVHI SATVHI SATVHI SALVSI SARVMI SALVRI SALVTI SALATI SALVTI SALATI SALATI	1 WSGEJ MGTGEJ MASGEJ RSGEJ MADGVU L QGGU U FGGU L QGGGU U FGGU L SGAJ MKDGSJ MKDGSJ MKDGSJ L SGEJ L SGU	80 =+ RK KK KK KK CTK TK QK RV LK RV LK RK KI
DHH_Homo_sapien DHHa_Xenopus_la DHHb_Xenopus_la DHHb_Xenopus_(Si DHH_Mus_musculu DHH_Danio_rerio SHH_Mus_musculu SHH_Xenopus_lae SHH_Homo_sapien SHH_Danio_rerio SHH_Xenopus_(Si IHH_Homo_sapien IHH_Mus_musculu IHH_Danio_rerio IHHb_Danio_reri IHH_Xenopus_lae HH_Drosophila_m HH_Branchiostom	RN RN RS RS RS RS RS RS RS RS RS RS RS RS RS	130 VKYGLLARLA VKYGMLARLA VKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA JKYGLLARLA JKYGMLARLA JKYGMLARLA JKYGMLARLA JKYGMLARLA JKYGMLARLA	V AGI V AGI	140 	150 SRNHVHVSV SKAHIHVSV SKAHIHVSV SKAHIHVSV SKAHIHVSV SKAHIHVSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHVHCSV SKAHIHCSV	ESSI KAD NTD KAD KAD KAD KA KA KA KA KA KA KA KA KA KA KA KA KA	160 SLAVRAG SLGVRSG SLGVRSG SLAVRAG SVAKSG SVAKSG SVAKSG SVAKSG SVAKSG SVAKSG SVAKSG SVAKSG SVAKSG SVAKSG SSSSAAKTG HSVAAKTG HSVAAKTG SSISSHVH SSISSHVH SSLAAKSG	1' GCFPG' GCFPG' GCFPG' GCFPG' GCFPG' GCFPG' GCFPG' GCFPG' GCFPG' GCFPG' GCFPG' GCFPG' GCFPG' GCFPG' GCFPG' GCFPG'	70 =+=== IATVRI IATVRI IATVRI SGLVTI SATVHI SATVHI SATVHI SATVHI SALVSI SARVMI SALVSI SARVMI SALVSI SALVTI SALVTI SALATI SALVTI SALATI	1 WSGEJ MGTGEJ MASGEJ RSGEJ MADGV(L QGG' L QGG' L QGG' L QGG' L QGG' L SGEJ MKDGSJ MKDGSJ L SGEJ L SGU RDDGNJ L NGL'	80 =+ RK KK KK RK QK TK QK TK RV LK RV LK RK RK RT K
DHH_Homo_sapien DHHa_Xenopus_la DHHb_Xenopus_la DHHb_Xenopus_(Si DHH_Mus_musculu DHH_Danio_rerio SHH_Mus_musculu SHH_Xenopus_lae SHH_Homo_sapien SHH_Danio_rerio SHH_Xenopus_(Si IHH_Homo_sapien IHH_Mus_musculu IHH_Danio_rerio IHHB_Danio_reri IHH_Xenopus_lae HH_Drosophila_m HH_Branchiostom HH_Saccoglossus HHtw Danio_reri	RIN RIN RIN RSS RSS RSS RSS RSS RSS RSS RSS RSS RS	130 WKYGLLARLA WKYGMLARLA WKYGMLARLA WKYGLLARLA KYGMLARLA KYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA WKYGLLARLA WKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA	V AGI V AGI	140 • WVY • WY • WY	150 SRNHVHVSV SKAHIHVSV SKAHIHVSV SKAHIHVSV SKAHIHVSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHVHCSV SKAHIK SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIK SKAHK SKAHK SKAKK SKK S	ESSI KAD NTD KAD KADI KA KA KA KA KA KA KA KA KA KA KA KA KA	160 SLAVRAG SLGVRSG SLGVRSG SLAVRAG SVAVEKG SVAKSG SVAKSG SVAAKSG SVAAKSG SVAAKSG SVAAKSG SVAAKSG SSISAAKTG SSISSHVH SSISSHVH SSISSHVH SSISSHVH SSISSHVH SSISSHVH SSISSHVH	1' GCFPG' GCFPG' GCFPG' GCFPG' GCFPG' GCFPG' GCFPG' GCFPG' GCFPG' GCFPG' GCFPG' GCFPG' GCFPG' GCFPG' GCFPG' GCFPG' GCFPG'	70 =+=== IATVRI IATVRI IATVRI SGLVTI SATVHI SATVHI SATVHI SATVHI SATVHI SATVHI SATVHI SALVSI SALVSI SALVSI SALVTI SALVTI SALVTI SALVTI SALATI SALVTI SALVTI SALATI	1 WSGEI MGTGEI MASGEI LRSGEI MADGVU LQGGU UFGGU LQGGU VFGGU LSGAI NGEI SGEI LSGEI LSGUI LSGUI LSGDGII	80 =+ RK KK KK RK QK TK QK TK RV LK RV LK RK RTK RK
DHH_Homo_sapien DHHa_Xenopus_la DHHb_Xenopus_la DHHb_Xenopus_(Si DHH_Mus_musculu DHH_Danio_rerio SHH_Mus_musculu SHH_Xenopus_lae SHH_Homo_sapien SHH_Danio_rerio SHH_Xenopus_(Si IHH_Homo_sapien IHH_Mus_musculu IHH_Danio_rerio IHHB_Danio_reri IHH_Xenopus_lae HH_Drosophila_m HH_Branchiostom HH_Saccoglossus HHtw_Danio_reri HH Strongvlocen	RIN RIN RS RS RS RS RS RS RS RS RS RS RS RS RS	130 WKYGLLARLA WKYGMLARLA WKYGMLARLA WKYGLLARLA WKYGLLARLA XKYGMLARLA SKYGMLARLA SKYGMLARLA WKYGLLARLA WKYGLLARLA WKYGLLARLA WKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA	V AGI V AGI	140 • WVY • WY • WY	150 SRNHVHVSV SKAHIHVSV SKAHIHVSV SKAHIHVSV SKAHIHVSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHVHCSV SKAHICSV SKAHICSV SKAKICSV SKAK	STAND	160 SLAVRAG SLGVRSG SLGVRSG SLGVRSG SVAVEKG SVAAKSG SVAAKSG SVAAKSG SVAAKSG SVAAKSG SVAAKSG SSISSHVH SDTTATQG SSLAAKSG SVAAKSG	1 GCFPG	70 =+=== JATVR TAMVMI TAMVMI JATVR GGLVTI GALVTI GALVTI GALVTI GALVTI CALATI SALVTI CALATI SALVTI CALATI SALVTI CALATI SSTAL SSTAL SSTAL SSTAL	1 WSGEI MGTGEI MASGEI LRSGEI MADGVU LQGGU UFGGU LQGGU UFGGU LSGAI SGU SGU SGU LSGEI LSGU LSGDGNI LNGL LSGCT	80 =+ RKKK KK QK TK QK TK C V R V L H R K R T K R T K R T K R T K R T K R T K R T K R T K R T K R K K K K
DHH_Homo_sapien DHHa_Xenopus_la DHHb_Xenopus_la DHHb_Xenopus_(Si DHH_Mus_musculu DHH_Danio_rerio SHH_Mus_musculu SHH_Xenopus_lae SHH_Homo_sapien SHH_Danio_rerio SHH_Xenopus_(Si IHH_Homo_sapien IHH_Mus_musculu IHH_Danio_rerii IHH_Danio_rerii IHH_Danio_rerii IHH_Xenopus_lae HH_Drosophila_m HH_Branchiostom HH_Saccoglossus HHtw_Danio_rerii HH_Strongylocen HH1 Ciona intes	RIN RIN RS RS RS RS RS RS RS RS RS RS RS RS RS	130 WKYGLLARLA WKYGMLARLA WKYGMLARLA WKYGLLARLA WKYGLLARLA XKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA WKYGLLARLA WKYGLLARLA WKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA JKYGMLARLA JKYGMLARLA JKYGMLARLA JKYGMLARLA	V AGI V AGI	140 	150 SRNHVHVSV SKAHIHVSV SKAHIHVSV SKAHIHVSV SKAHIHVSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHVHCSV SKAHICSV SKAHICSV SKAHICSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHICSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHIHCSV SKAHICSV SKAKK SKAKK SKAKK SKAKK SKAKK	STAND	160 SLAVRAG SLGVRSG SLGVRSG SLGVRSG SVAVEKG SVAAKSG SVAAKSG SVAAKSG SVAAKSG SVAAKSG SVAAKSG SSISSHVH SDTTATQG SSLAAKSG SVAAKSG SVAAKSG SAAKNSG SSDAAKSG	1 GCFPG	70 =+=== JATVR TAMVMI TAMVMI JATVR SGLVTI SATVH SATVH SATVH SALVS SARVM SALVS SARVM SALVS SARVM SALVS SALVT	1 MGTGEI MGTGEI MASGEI LRSGEI MADGVU LQGG VFGG LQGG VFGG LSGAI LSGAI LSGEI LSGVI RDDGNI LSGEI LSGVI RDDGNI LSGDGTI LNGL	80 =+ RKKKRV RKK RKK RK RK RK RK RK RK RK RK RK RK R
DHH_Homo_sapien DHHa_Xenopus_la DHHb_Xenopus_la DHHb_Xenopus_(Si DHH_Mus_musculu DHH_Danio_rerio SHH_Mus_musculu SHH_Xenopus_lae SHH_Homo_sapien SHH_Danio_rerio SHH_Xenopus_(Si IHH_Homo_sapien IHH_Mus_musculu IHH_Danio_rerio IHHB_Danio_rerii IHH_Xenopus_lae HH_Drosophila_m HH_Branchiostom HH_Saccoglossus HHtw_Danio_reri HH_Strongylocen HH1_Ciona_intes HH2_Ciona_intes	RIN RIN RS RS RS RS RS RS RS RS RS RS RS RS RS	130 WKYGLLARLA WKYGMLARLA WKYGMLARLA WKYGLLARLA WYGLLARLA XYGLLAQLA SKYGMLARLA SKYGMLARLA SKYGMLARLA WKYGLLARLA WKYGLLARLA WKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA SKYGMLARLA	V AGI V AGI V V AGI V AGI V AGI V AG	140 	150 SRNHVHVSV SKAHIHVSV SKAHIHVSV SKAHIHVSV SKAHIHVSV SKAHIHVSV SKAHIHCSV SKAHICSV SKAHICSV SKAHICSV SKAHIHCSV SKAHIKAN SKAHIKAN SKAHIKAN SKAHIKAN SKAHIKAN SKAK	SAD KAD KAD KAD KAD KAD KA KA KA KA KA KS KA KA KA KA KA KA KA KA KA KA KA KA KA	160 SLAVRAG SLGVRSG SLGVRSG SLGVRSG SLAVRAG SVAAKSG SVAAKSG SVAAKSG SVAAKSG SVAAKSG SVAAKSG SSISSHVH SDTTATQG SSLAAKSG SAAKNSG SSDAAKSG SDAAKSG	1 GCFPG	70 =+=== JATVR TAMVMI TAMVMI TAMVMI JATVR SGLVTI SATVH SATVH SALVS SARVM SALVS SARVM SALVS SARVM SALVS SALVT	1 MGTGE: MGTGE: MASGE: MASGE: LRSGEI MADGVU LQGG' VFGG' LQGG' LQGG' LGGG' LSGAI LSGEI LSGU RDGNI LSGU RDDGNI LGDGTI LGGGTI LNGR' VPGEG NSGS'	80 = RK KKK KK KK KK KK KK KK KK KK KK KK KK

	190	200	210	220	230	240
DHH_Homo_sapien DHH_Xenopus_la DHHb_Xenopus_la DHH_Xenopus_(Si DHH_Mus_musculu DHH_Danio_rerio SHH_Mus_musculu SHH_Xenopus_lae SHH_Homo_sapien SHH_Danio_rerio SHH_Xenopus_(Si IHH_Homo_sapien IHH_Mus_musculu IHH_Danio_rerio IHHb_Danio_rerii IHH_Xenopus_lae HH_Drosophila_m HH_Branchiostom HH_Saccoglossus HHtw_Danio_reri HH_Strongylocen HH1_Ciona_intes	GLRELHRG WVLAG PLSELKIG TVYTG PLSELKIG TVYTG GLRELHRG WVLAG PLSELKLG TVFTG GLRELHRG WVLAG PMSCLWPGEKVLSG LVKDLRPG RVLAG AVKDLRPG RVLAG AVKDLRPG RVLAG AVKDLNPG KVLAG AVKDLNPG KVLAG ALSAVKPG RVLAG GLRDLQAG LVLAG PVSQLSPGLRVLAG PLGELSIG RVLSG SLDQLTIGEKVMAG PIKDLKVG RVLAG SMLDIRVG EVAVG PMSSLQPG QVLAG ####################################	===+=== RVVPTPVLI QLITSVVLI LLIHSVVLI QLIPSVVLI RVVPTPVLI RVVPTPVLI EVVFSRVLI RLLYSDFLM RLLYSDFLM SPTFSDVLI TPTFSDVLI TPTFSDVLI TPTFSDVLI TPTFSDVLI RPTYSDFLS QAVYSEVIA HPVFSEVLT TIVYSDVIM NVLISDFIM ALDYSDVIM AVITDTFLS KPVFSEVIM	=====+= FL FVAV FLHFVLI FLHFVLI FL FVAV FLHFFII FL FYVI FL FYVI FL FYVI FL FVVI FL FQVI FL FQVI FL FQVI FL FQVI FL FQVI FL FQVI FL FQVI FL FVVI FM FVQLF FM FVVI FI FIVI IVHFYVI FM MVEI FL YIEIF ####################################	TEWPPRKLLIT AEGHPSKLLVT AEGHPSKLLVT AEGHPSKLLVT TERPPRKLLIT TERPPRKLLIT T-ENEKRIALT TLEPRERLLIT TQEPVEKITLT TQEPVEKITLT TQDPPRRLALT TQDPPRRLALT TQDPPRRLALT TODGAVLTVT TDDGAVLTVT TDDRNITVTAT T-EDGQKLTLT TSEPFTKLTLT TSEPFTKLTLT SS-QLETIRLT	SHLVFFARR NHLLFFAYR NHLLFFAYR NHLLFFAYR WHLVFFARR NHLIFFARN AHLLFFASR AHLLFFASR AHLLFFASR AHLLFFASR AHLLFFASR AHLLFFASR AHLLFFASR AHLLFFASR AHLLFFASR AHLLFFASR AHLLFFASR AHLLFFASR AHLLFFASR AHLLFFASR AHLLFFASR AHLVFFASR QHLIYFASR NHLIYFASR AHLVFFASR	=====+ LRAGDSV VQIGDLV VQIGDLV VRIGDYI VRAGDVV VRIGDYI VRPGQRV VRAGQKV VRAGQKV VRPGDVI VQPGQYVV VQPGQYVV VQPGQYVV VLPGQCV AQVGQCL VRPGQYIT IEEKNQVR ARPGEFL VELGHYV VKPGDTV VRTNQFVL VRQGDYIT IFSGQYVT #########
DHH_Homo_sapien DHHa_Xenopus_la DHHb_Xenopus_la DHHb_Xenopus_(Si DHH_Mus_musculu DHH_Danio_rerio SHH_Mus_musculu SHH_Xenopus_lae SHH_Homo_sapien SHH_Danio_rerio SHH_Xenopus_(Si IHH_Homo_sapien IHH_Mus_musculu IHH_Danio_rerio IHHb_Danio_reri IHH_Xenopus_lae HH_Drosophila_m HH_Branchiostom HH_Saccoglossus HHtw_Danio_reri HH_Strongylocen HH1_Ciona_intes	250 AVGVFAPLTAHGTLI QTGVYAPMTEHGTLI QTGVYAPMTEHGTLI QTGVYAPMTEHGTLI AVGVFAPLTAHGTLI RMGVYAPLTEHGNLI EAGAYAPLTAHGTI DTGAYAPLTAHGTI QRGSFAPVTAHGTI DIGAFAPVTAQGTV ALGAYAPLTKHGTL DGVFAPLTSHGTV DQGLYPPLTAHGTU SKGVVAPLTREGTI EKGAYAPLTVHGTV NVGVYAPLTREGTI HEGSFAPVTAHGTI GRTAVAPVTRQGSL ASGAYAPLTYSGTI	260 	270 YAVL SHQ YATV SHJ YATV SHJ YATV SHJ YATV SHJ YAVL SHQ YAVL SHQ YAVI EHS YAVI QQ YAVI SH YAVNRQF YAVNRQF YAVNSQS YALI SQA YAMV-NHN YAVNRDEV YAVI SD YQVIGSEJ	280 WAHRAFAPLRGI LAHVSLAPLRGY LAHASLAPLRGY WAHRAFAPLRGI WAHRAFAPLRGI WAHRAFAPFRG WAHLAFAPLRGI WAHLAFAPLRGI WAHLAFAPLRGI HLAQLAFWPLRGY LAQLAFWPLRGY LAQLAFWPLRGY LAQLAFWPLRGY LAQLAFWPLRGY LAQLAFWPLRGY LAQLAFWPLRGY MAHWAFAPLRGI SLAHWGLAPMRG SLAHWGLAPMRGY MAUGFAFGPIRGY WAHWAFAPVRGY VIAHASFAPVRGY VIAHASFAPVRGY	290 HWYSRLLY /HWYCHILY /HWYCHILY /HWYCHILY /HWYCHILY /HWYSRLLY /HWYSQLLY IHWYSQLLY IHWYSQLLY /HWYSQLLY /HWYSQLLY /HWYSRLLY /HWYSRLLY /HWYSLLY /HWYSLLY /HWYSLLY IHWYSKLY IHWYSKLY /HWYPQLY IHWYSFFY /HWYPYLLY IHWYSNMLF /HWYTQRLY ISLYSKLLH IHWYAKSLA	

Figure 2. Multiple alignment of hedgehog protein in chordates.

A.

Ciona-hhl Ciona-hh2 Branchiostoma_f Strongylocentro Saccoglossus_ko Xenopus_DHH Homo_sapiens_DHH Mus_musculus_DH Homo_sapien_SHH Danio_rerio_SHH Danio_rerio_SHH Danio_rerio_IHH Mus_musculus_IH Homo_sapiens_IH Drosophila



Β.

Ciona-hh1 Ciona-hh2 Branchiostoma_f Strongylocentro Saccoglossus_ko Xenopus_DHH Homo_sapiens_DH Homo_sapien_SHH Homo_sapien_SHH Danio_rerio_SHH Danio_rerio_SHH Danio_rerio_IHH Mus_musculus_IH Homo_sapiens_IH Drosophila

1450	1460	1470	1480	1490	1500
+	+	+	+	+	+====+
acagt <mark>gg</mark> t <mark>ac</mark> catca	ta <mark>gt</mark> aggtg	gaaca <mark>g</mark> cagc	gtcatgttac	gccgtcatc	aagtg
ca <mark>cacggtac</mark> tgtag	tcgtggaeg	gta <mark>tagt</mark> tge	g <mark>te</mark> ttgetat	ggagttata	ggtcag
ta <mark>cacggcac</mark> ggtcg	tcgtggaca	at <mark>gt</mark> agcaat	gtectgetac	gctctcata	agec
gg ca g <mark>gg</mark> atccttgg	taatcgacg	acgtagcgat	atcatcgtac	gecgtcatgo	gtgacg
ggg <mark>aagg</mark> g <mark>ac</mark> gataa	tataaaca	acattgtage	atcatgttat	gcaatggtaa	acc
aacatggaaccttac	ttgtggatg	gggtgctaac	atcctgctat	gctactgtg	agtcac
cg <mark>ca</mark> cgggg <mark>ac</mark> gctgc	tg <mark>gt</mark> ga <mark>ac</mark> g	atgtcctggc	ctcttgctad	gcggttctgg	agagte
cg <mark>ca</mark> cggggacgctgc	tggtcaacg	acgtcctcgc	ctectgetac	geggttctag	agagtc
cccagggcaccattc	catcaacc	dd <mark>ar</mark> dc <mark>r</mark> dac	ctcgtgctac	geggtcatc	aggage
cgeacggeaccatte	calcaac	gg <mark>gt</mark> gctccc	ctegtgetac	getgecate	aggage
aacatgggaacctat	ttgtggatg	dcardcradae	gtecaaetae	getacttet	aggatc
cacatgggaccattg	tggtcgaca	gaacacegee	gtectgttac	gecgtaata	eggacc
gccatgggggggggggggggggggggggggggggggggg	tggtcaatg	gcattgttte	ctectgetac	geagecgeg	ccage
ggcatggggacacttg	tggtggagg	atgtggtgge	ctectgettt	gcagctgcg	ctgacc
ageatgggacactgg	cggcggagg	atgeggegge	aceccecte	geggeegeg	ctgace
gegagggcaccattg	rggreaact	cgggggggggg	cagtegetat	geggegaeea	cage

C.

Ciona-hhl Ciona-hh2 Branchicstoma_f Strongylocentro Saccoglossus_ko Xenopus_DHH Homo_sapiens_DH Homo_sapien_SHH Danio_rerio_SHH Danio_rerio_SHH Danio_rerio_SHH Danio_rerio_IHH Mus_musculus_IH Homo_sapiens_IH Drosophila

670	680	690	700	710	720
		+		+	+
atectcetage	gaaceaatgggg	gegtgtcaaa	ergaaagete	egaagcatge	gacgat
atgrei gtgaa	Igaacacatgggg				9449999
ataagtgtaat	gaaegaatgggg	coggattaaa	ettegegtggt	agagggggggggggggggggggggggggggggggggggg	gaegag
atatcagtgat	gaaccagtggc	tggagtcaaa	cttcgtgtta	egagggetg	gacgag
atetetgtgat	gaacatgtggc	aggcgtgaag	ctccgggtta	cgagggetge	gaegag
attgecgtgat	gaacat gtggc	c <mark>gg</mark> a <mark>gt</mark> gcgc	ctacgagtga	tgagggetge	gaegag
ategeggtgat	gaacatgtggco	c <mark>gg</mark> a <mark>gt</mark> acge	etaegtgtga	tgaaggetge	gaegag
ateteggtgat	gaaccagtggc	aggagtgaaa	ctgcgggtga	cgagggctgg	igaegaa
atetetgtgat	gaaccagtggco	tggagtgaag	ctgcgagtga	egagggetge	gatgag
atageageaat	gaaceagtgged	aggggttega		agaggeetge	gatgaa
atetetgtgat	gaacatgtgge	cgggggggccaag	ctcagggtga	agaggggetgg	gatgaa
atetetgtcat	gaaccagtggco	tggtgtgaaa	etgegggtgad	egaaggetge	gatgaa
ateteggtgat	gaaccagtggc	c <mark>ggtgtgaa</mark> g	etgegggtga	egaggetge	gaegag
tacteggtgat	gaacgaatggco	cggcatccgg	ctgctggtcac	egagagetge	gacgag

D.

	1390	1400	1410	1420	1430	1440
	+				+	+====+
liona-hhl	etcgtgttgtgage	gtacgaaco	cattgaaactg	caagt <mark>gg</mark> agc	atataccea	ttacat
liona-hh2	cacgcgtggaatc	g <mark>gt</mark> ta <mark>c</mark> t	-acggtaactg	gggac <mark>gg</mark> tct	al atgeccege	sttaccg
Branchiostoma f	tgaagattgtttca	a <mark>gt</mark> ga <mark>c</mark> a	atgagagaag	agaagggcgc	gtacgcccca	staactg
strongylocentro	agagagtggtcag	gtaacg	cgagettg	ggcgtacg	tgtcgcgccg	gecca
accoglossus ko	etaaa aattaca	aatateg	tcanttonaa	acgtteget	geogogogo	tcaccc
enopus DHH	staagettetga	atet	gtagaggaac	aaact ot t	atatogege	gagag
lomo saniens DH	cacacataacccat	and an	caageggaaa	contract	atterence	
us musculus DH			oggggggggggg	aagt	get a galage	
long appion CUU	egeocococococo	-geggggg	- CgCgaggaag		geogeacege	
iomo_sapien_SHH	ecgetegeacage	gegaeccta	and considered	ccgc <mark>ggg</mark> cgc	cracgegeege	accaegg
us_musculus_SH	ecgcgggggcacage	gegaegete	gegaggaggagg	aggegggege	graegegeege	accaegg
Danio_rerio_SHH	etaaagtggtctca	agtttet	-ctg <mark>gagga</mark> ga	ggat <mark>ggg</mark> ggt	ttatgeteect	acag
anio_rerio_SHH	tcatcgtgcagcg	gatatac	-acg <mark>gagga</mark> gc	ageggggete	g <mark>t</mark> t <mark>cgc</mark> a <mark>cc</mark> ag	g <mark>tgac</mark> tg
anio_rerio_IHH	cacgggtctctcg	ga <mark>t</mark> ccga	-atgcaggagg	acag <mark>ggg</mark> ggt	cttccca	stc <mark>ac</mark> ca
ius_musculus_IH	etcgggtggcaget	t <mark>gt</mark> ct <mark>c</mark> c	-acceacgtgg	cccttgggtc	c <mark>tatge</mark> tcet	et cacaa
lomo sapiens IH	eccgcgtggcaget	tgtctct	acacacatge	ccctcggggg	ctacgecccg	at cacaa
rosophila	agcgagtcgtcaad	agtagage	gtgtgcgca	gtaagegcgt	ggtcgcgccg	gaccc

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	74°C	GATGACGGCGTAACATGACGC	70°C
bv-HH2	GTGAAGAACACATGGGCCGGT	71°C	GTGCGGTAAGCGGGGCAT	71°C



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



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.