Cloning and phylogenetic analysis of RyR orthologues in primitive vertebrate species

Kelly Reimer

Supervisor: Dr. Jens Franck

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Department of Biology

The University of Winnipeg

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Abstract

Ryanodine receptors (RyRs) serve as important channels for controlled release of intracellular stores of calcium in the sarcoplasmic reticulum. This release of calcium, triggered by membrane-depolarization, is responsible for initiating muscle contraction. Three RyR isoforms have been identified in mammals, RyR1 found predominantly in skeletal muscle, RyR2 in cardiac muscle, and RyR3 which is ubiquitously distributed. RyR1 and RyR3 orthologues are co-expressed equally in fish skeletal muscle, as well as fast-twitch and slow-twitch muscle specific isoforms known respectively as RyR1-fast and RyR1-slow. Hagfish are living representatives of the primitive vertebrate condition before the divergence of jawed vertebrates including fish and tetrapods. I have cloned nine partial RyR sequences from hagfish skeletal muscle cDNA, each believed to represent an isoform more primitive than the vertebrate orthologues. Phylogenetic analysis clusters the hagfish RyR sequences in an ancestral clade apart from the derived lineages of published RyR sequences. The high sequence identity of the nine hagfish sequences suggests that only one RyR isoform is expressed in hagfish skeletal muscle.

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Abbreviations and Definitions

Ca^{2+}	Calcium
cDNA	Complementary DNA (synthesised from mRNA template)
CICR	Calcium-Induced Calcium Release
DHPR	Dihydropyridine receptor
NaOAc	Sodium Acetate
PCR	Polymerase chain reaction
RyR	Ryanodine receptor

Orthologous	**-**Two similar genes that have diverged after a speciation event
Paralogous	-Two similar genes within a species that have diverged from a gene duplication event
Neofunctionalization	-The process where a gene undertakes responsibility for a novel function unrelated to its previous function
Subfunctionalization	-The process where a gene undertakes a new function similar to its previous function

Introduction

Ryanodine Receptors: Structure and Function

Excitation-contraction coupling is the process by which an electrical nerve impulse is transformed into the actual contraction of a muscle. At the molecular level EC coupling is the process where membrane depolarization on the sarcolemma causes the release of intracellular stores of calcium ions within the sarcoplasmic reticulum, which in turn triggers the contraction of myosin and actin filaments within the cell (for review, see Fill and Copello 2002*).* The sarcoplasmic reticulum has many calcium release channels called ryanodine receptors (RyRs) that line the membrane next to the transverse tubule of the sarcolemma and are situated close to other receptors on the transverse tubule. In skeletal muscles, the receptors on the transverse tubule, called dihydropyridine receptors (DHPRs*),* respond to membrane depolarization and undergo a conformational change which mechanically induces the RyRs attached to open. Opening of the calcium channels by this method is termed depolarization-induced calcium released. RyRs can also be ligand-gated, opening in response to binding of extracellular calcium, in a process called calcium-induced calcium release (CICR). The latter mechanism is typical of the cardiac (RyR2) isoform (Fill and Copello 2002*).*

The RyR is a tetrad of four identical protein subunits, each about 500-600 kDa in size. The protein consists of two main functional domains, a regulatory region on the cytoplasmic side consisting of the amino-terminal region, and a transmembrane pore forming domain at the carboxyl region. The regulatory region has been shown to respond to regulation by calsequestrin, calmodulin, calcium, phosphorylating kinases, and other

signals (Fill and Copello 2002). The protein is found in many cell types such as neurons, but is especially abundant in striated muscle.

Ryanodine Receptor Isoforms

In mammalian organisms there are three well-characterized isoforms of ryanodine receptors named RyR1, RyR2, and RyR3 (Fill and Copello 2002). The genes encoding them are found on three separate chromosomes, and in mammals the isoforms are tissue specific (Fill and Copello 2002). RyR1 is found primarily in striated skeletal muscle and operates by a depolarization-induced mechanism, which allows for a more rapid and controlled effect than calcium-induced calcium release (Fill and Copello 2002; Morrissette *et al* 2000; Morrissette *et al* 2003; O'Brien *et al* 1993). RyR2, in contrast, operates primarily by the calcium-induced mechanism, and as such is slower to respond, but is more effectively amplified by a small signal since the release of calcium triggers the opening of neighbouring RyR2s. This form is found primarily in cardiac muscle (Fill and Copello 2002). RyR3 is expressed ubiquitously and is found in mammalian skeletal muscle in small amounts, <5% of the RyRs present in mammalian skeletal muscle are of RyR3 form (Fill and Copello 2002; Morrissette *et al* 2000; Sorrentino 2003).

Homologues to the mammalian RyRs found in fish, amphibian and bird skeletal muscle were initially termed RyRα and RyRβ but were later found to be orthologous to RyR1 and RyR3 respectively (Fill and Copello 2002; Franck *et al* 1998; Morrissette *et al* 2000; Morrissette *et al* 2003). In these organisms both of these isoforms are present in approximately equal amounts in contrast to the specificity for RyR1 seen in mammals. Additionally fish express two distinct forms of RyR1 in fast and slow-twitch muscle

fibers termed RyR1-fast and RyR1-slow respectively (Franck *et al* 1998*).* Only one RyR gene has been identified in invertebrates including *Drosophila* and *C. elegans* and is believed to represent the ancestral form of RyRs. This invertebrate RyR is responsible for contraction of skeletal, cardiac, and visceral muscles (Finn and Kristoffersen 2007; Kao and Lee 2002*).*

Whole Genome Duplication

During the evolution of the vertebrate lineage there is ample evidence that at least two rounds of whole genome duplication have taken place. Supporting evidence comes from studies of gene clusters such as *Hox* genes as well as the numbers of orthologues of various genes present (Amores *et al* 2004; Blomme *et al* 2006; Escriva *et al* 2002; Finn and Kristoffersen 2007; Inoue, Tsutsui, Bone 2002; Meyer and Van de Peer 2005*).* Following whole genome duplication one would expect to see double the number of isoforms of a gene and the other neighbouring genes in a duplicated cluster would maintain the same order or synteny. The first genome duplication may predate the Cambrian explosion in diversity in early chordate evolution coinciding with the divergence of cephalochordates (Meyer and Van de Peer 2005; Xian-guang *et al* 2002), and the second duplication during the early Devonian in the evolution of vertebrates. Evidence exists for a third round of whole genome duplication in the evolution of teleost fish, differing from that of sarcopterigians (Finn and Kristoffersen 2007; Meyer and Schartl 1999; Meyer and Van de Peer 2005*).*

The duplication of genomes leads to one of several outcomes. The extra copy could subsequently become lost through selection if it is redundant, or may be retained as a

backup with no change in function. The additional copy may also diversify to take on completely new roles by neofunctionalization, or modifications of its previous role in a process termed subfunctionalization. One copy of the functional gene would be retained and the new copy would be able to specialize and diverge from its original function (Blomme *et al* 2006*).* It is believed this is how the different isoforms of RyR were derived (Sorrentino, Barone, Rossi 2000*).* The ancestral RyR gave rise to the RyR2 and RyR3, followed by the split of RyR3 into RyR1. The third genome duplication in fish could possibly account for the divergence of the fast and slow twitch isoforms of RyR1.

Hagfish as Model Organisms

Hagfish are a living representative of the earliest vertebrate condition. They arose after the divergence of the cephalochordates but before the divergence of the jawed vertebrates and as such offer a look at the condition between the two major rounds of genome duplication (Escriva *et al* 2002; Inoue, Tsutsui, Bone 2002*).* At present, no work has been done at the molecular level in the study of ryanodine receptors in hagfish and other primitive organisms such as lamprey and shark.

Objectives:

The objective of this study is to isolate and sequence ryanodine receptor isoforms in the skeletal muscle of hagfish and putatively annotate them through online searching of the Genbank database as well as phylogenetic analysis.

Materials and Methods:

RNA Extraction and First Strand cDNA Synthesis

A frozen sample of Pacific hagfish (*Epatretus stouti*) skeletal muscle tissue was obtained and placed in 1 ml of TRIzol reagent for every 100 mg of tissue. Samples were homogenized using a motorized Teflon pestle and incubated at room temperature for 5 minutes. Addition of chloroform allows separation of RNA from other cellular components such as proteins and DNA which remain in the phenol-based TRIzol (Chomczynski and Sacchi 1987*).* Total RNA was then precipitated with isopropanol and pelleted by centrifugation. The RNA pellet was washed once with 75% ethanol, air-dried and resuspended in RNase-free water. The RNA was quantified by UV_{260} absorbance readings (Bird 2005*).*

First-strand cDNA was synthesized from 10 µg of each sample of total RNA. The RNA was used to make a 20 µl mixture containing 0.5 µg/µl total RNA, and 0.005 µg/µl Oligo dT_{15}. The mixture was heated to 95° C and then quickly chilled on ice. Once chilled, 12 µl of 5x Oligo dT_{15} reaction buffer, 2 µl of 10 mM dNTPs, 24 µl of RNase-free water, 1 µl of RNasin (an RNase inhibitor) and 1 µl of reverse transcriptase were added to the mixture and were mixed by pipetting. The mixture was incubated for 15 minutes at room temperature, then for 30 minutes at 37° C. 40 µl of 10X TE (pH 8.0) was added to stop the reaction and the first strand cDNA was precipitated by addition of 10 µl of 3M NaOAc and 250 µl of 95% Ethanol. Each sample was precipitated for several hours at -20° C, centrifuged at 16 000g for 15 min, washed with 75% ethanol, and then centrifuged again at 8000g for 5 min. The final precipitated cDNA was resuspended in 100 µl of RNase-free water.

Polymerase Chain Reaction (PCR) Amplification of Target Gene Products

Degenerate primers were designed with the assistance of the CODEHOP program based on alignments from conserved regions of RyR message from frog, rabbit and fish (see Appendix A). The primers were designed to target conserved regions of the amino acid sequence so that any expressed isoform of RyR would be amplified (Rose *et al* 1998*)*. The primers used were:

HFF1 5′-TGGCTTTCAACTTCTTCCGTAARTTYTAYAA-3′

HFF2 5′-GGAGGAGGACGAGCCAGAYATGAARTG-3′

HFR1 5′-CAGCAACGCTCCTGGTACATYTTCCA-3′

HFR2 5′-CCCAGCAACGCTCCTGRTACATYTT-3′

Where R is either G or A, and Y is either C or T. The calculated melting points under standard conditions for HFR1 and HFR2 were between 58°C and 61°C, whereas HFF1 was between 55°C and 59°C, and HFF2 was between 61°C and 64°C. These primers were then used to amplify RyR messages from the first strand cDNA.

For each PCR reaction 45 µl of master mix along with 5 µl of template first strand cDNA was used. The master mix consisted of 1x PCR buffer (Invitrogen Life Technologies), 1.5 mM $MgCl_2$ (Invitrogen Life Technologies), 0.2 mM dNTPs, 1.0 µM forward primer, 1.0 µM reverse primer, and 2.5 units of Taq DNA polymerase (Invitrogen Life Technologies). The PCR reaction was set up with an initial denaturation step at 95°C for nine minutes, followed by subsequent steps of denaturation at 95°C for one minute, annealing step at 60°C for one minute and thirty seconds, and an extension step at 72°C for one minute. The annealing step was chosen to be around the melting

points of the primer pairs. These steps were cycled 35 times, followed by a final extension step at 72°C for seven minutes. Samples were held at 4°C until needed.

The PCR products were separated and identified on a 1% agarose gel loaded with 10 µl of each sample per well (See Figure 1*).* The remaining amplified product was then separated and extracted from a 1% low melting point agarose gel and purified by gel purification (Wizard PCR Preps DNA Purification System, Promega).

Cloning of PCR Products

The purified PCR products were ligated with pGEM-T Easy vectors (Promega Biotech, see Appendix B), transformed into competent *E. coli* cells (JM109 strain) and plated onto LB plates containing 50 µg/ml ampicillin (Invitrogen Life Technologies). This allows for selection for cells containing the ampicillin resistance-inducing vector. Plates were then incubated at 37°C overnight.

PCR Detection and Amplification of Recombinants

Colonies from the LB + ampicillin plates were randomly selected for PCR. PCR detection used M13 Forward (5'-GTTTTCCCAGTCACGAC-3') and M13 Reverse (5'-CAGGAAACAGCTATGAC-3') primer pairs. Recognition sites for the primers flanking the insert allow amplification of the cloned partial RyR message if present. For each PCR reaction a sterile pipette tip was touched to a bacterial colony and was allowed to sit in a PCR tube containing 15 µl of PCR mix for approximately one minute. The PCR reaction consisted of four steps, of which steps two through four were repeated 35 times. The initial denaturation step consisted of 95°C for five minutes, followed by the repeated cycle of 95°C for one minute, annealing at 55°C for thirty seconds, and extension at 72°C

for one minute. Reactions were then run on a 1% agarose gel and recombinants containing inserts were detected based on migration distance (See Figure 2*).* Recombinant colonies were grown in LB media containing 0.05 mg/ml ampicillin overnight.

Plasmids were purified using the Spinbind kit (FMC) which consists of a system using minicolumns to isolate plasmid DNA using a desktop microfuge. Purified plasmids were sequenced at the Center for Applied Genomics at the University of Toronto.

Phylogenetic Analysis

The sequences were aligned with other published RyR isoforms using ClustalW (Thompson *et al* 1997*).* Amino acids at variable positions were determined to be ancestral or derived in nature based on their identity to published invertebrate and vertebrate sequences. Bootstrapping was performed on the alignment to generate pseudo replicates for analysis using Seqboot, a program in the PHYLIP package (Efron *et al* 1996; Felsenstein 1992*).* Five hundred bootstrap pseudo replicates were analyzed using the Protpars program of PHYLIP. A majority rule consensus tree was created using the Consense program from the Phylip package.

Results

PCR amplification and purification

The first strand cDNA was amplified using hagfish degenerate primer combinations F1/R1, F1/R2, F2/R1, and F2/R2, (Figure 1*).* Negative lanes containing no template for all primer combinations except F1/R1 showed a band appearing at the expected distance of an amplified message. The F1/R1 negative lane showed no such DNA contamination or PCR product, and the amplified cDNA in the F1/R1 experimental lane was purified.

Figure 1: PCR Product amplified using F1/R1, F1/R2, F2/R1, and F2/R2 primers on hagfish skeletal muscle cDNA in lanes 2, 3, 4, and 5 respectively. Amplification was seen with F1/R1, F2/R1, and F2/R2 primers, negative lanes 7-10 for the above primers show contamination from DNA at the expected distance for the PCR product. Negative lane 7 corresponding to F1/R1 was clean and the product from lane 2 was purified. A 1 kbp ladder was loaded into lane 1 and lane 6 was left empty.

The PCR products were gel-purified and cloned into pGEM-T vectors and recombinants were detected by PCR amplification and running on a gel. Figure 2 shows the results of the gel, with colonies labeled HFS-02, HFS-03, HFS-05, HFS-07, HFS-08, HFS-13, HFS-14, HFS-16, and HFS-19 showing an insert in the vector indicating successful recombination.

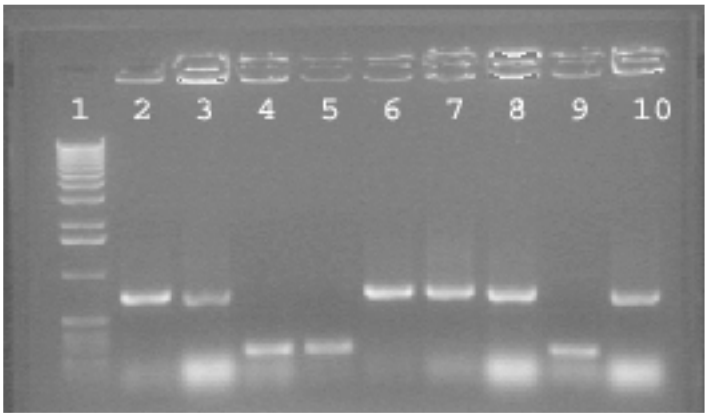

Figure 2: Agarose gel showing PCR products of randomly selected colonies using M13 forward and reverse primers. Lanes 2, 3, 6-8, and 10 show the expected size of a vector containing an insert from the amplified PCR Product. Primer dimers resulting from amplification of the primers themselves can be seen as a broad smear at the bottom of the gel.

Sequencing

Nine of the randomly selected clones from the HF degenerate F1/R1 primer pair were sequenced (clones HFS-02, HFS-03, HFS-05, HFS-07, HFS-08, HFS-13, HFS-14, HFS-16, and HFS-19). The sequences show high sequence identity with some differences in nucleotides at 10 out of the 497 positions (Figure 3*).* HFS-08, HFS-13, and HFS-19 were only able to be determined accurately to 489, 495, and 496 nucleotides from the original sequence data. The deduced amino acid sequences and were also aligned (Figure 4*).* The amino acid sequences of HFS-08, HFS-14, and HFS-19 each differed from the rest by one amino acid at different positions, but the 161 other remaining positions of the 164

amino acids were identical. A tBLASTn search of the GenBank database was performed using each of the amino acid sequences, in which the amino acid sequence is compared to the translated nucleotide database (Table 1). This search revealed opossum RyR3 and RyR2 as the top 3 hits for each of the sequences with similar E values for each except in the case of HFS-08, where the third top hit was zebrafish RyR1. The consensus sequence was used to determine sequence similarities with published isoforms via a pairwise comparison (Table 2*).* A multiple sequence alignment of the consensus and selected published RyR forms was generated (Figure 4) and where the amino acids in the consensus varied from the published sequences, it was determined whether that particular amino acid represented an ancestral or derived condition (Table 3*).*

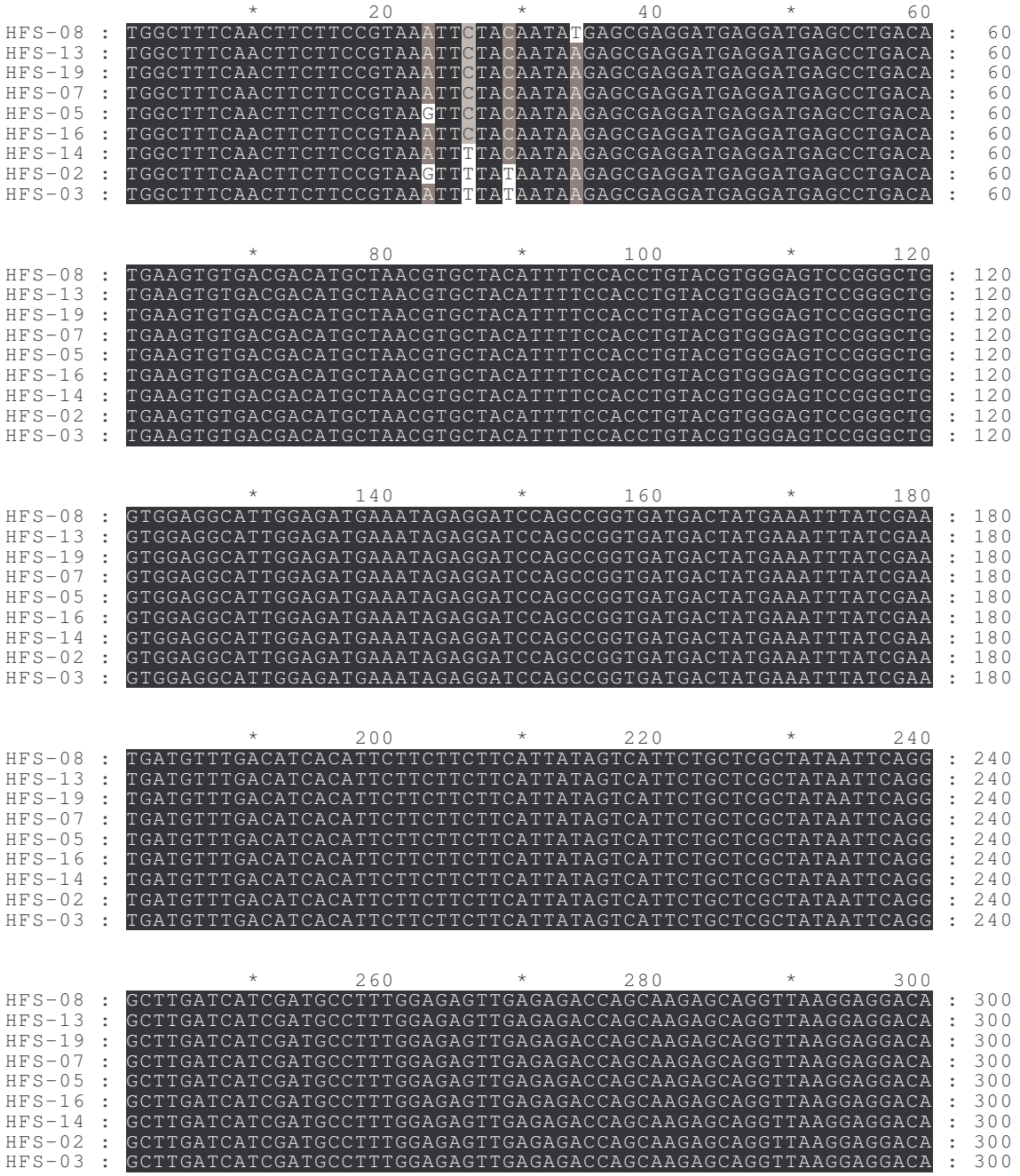

Figure 3: Alignment of hagfish nucleotide sequences with identical nucleotides in black, and less conserved nucleotides in shades of grey. HFS-08, HFS-13, and HFS-19 were unable to be accurately determined to 497 nucleotides and are truncated in the alignment.

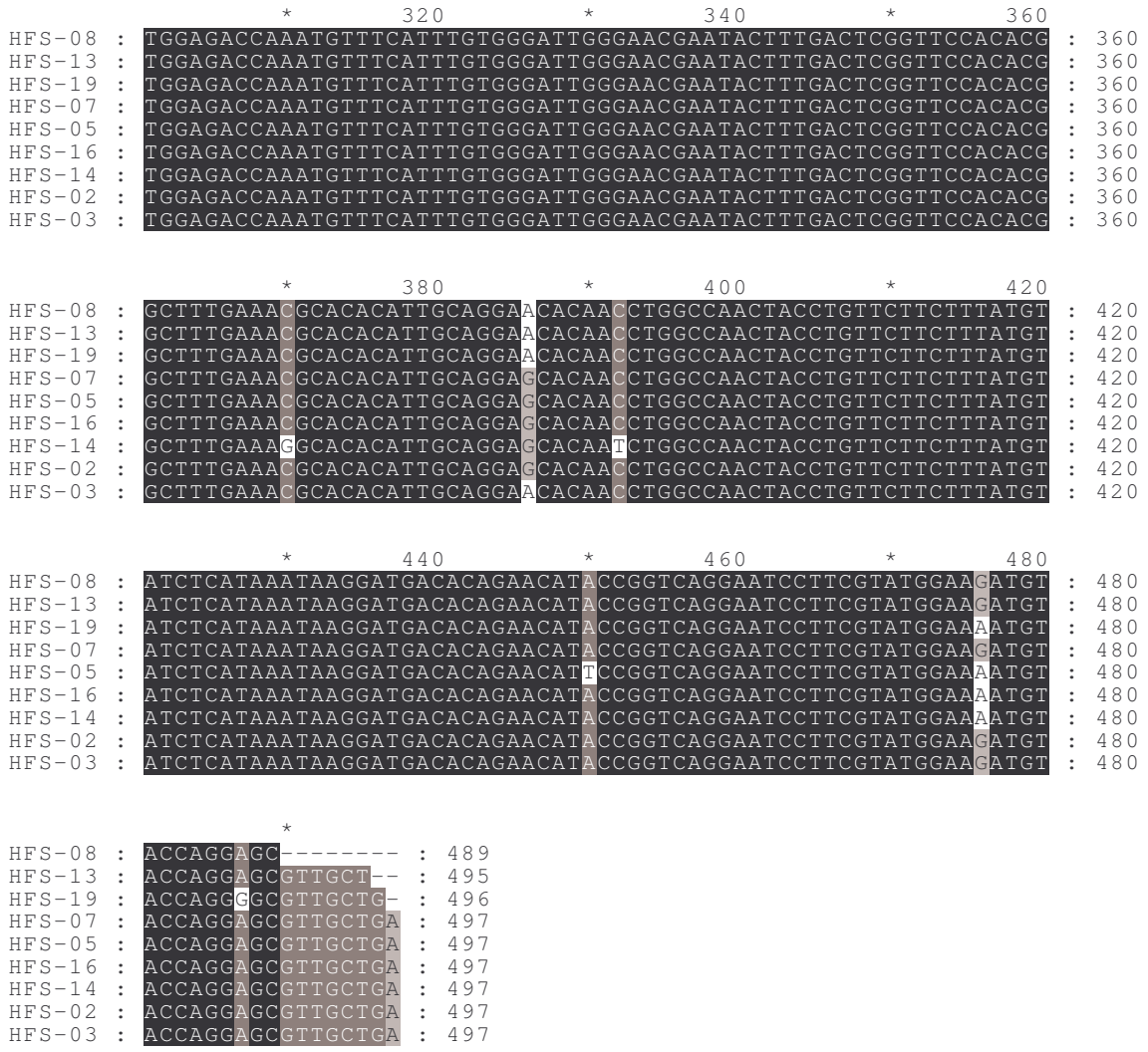

Figure 3: Continued

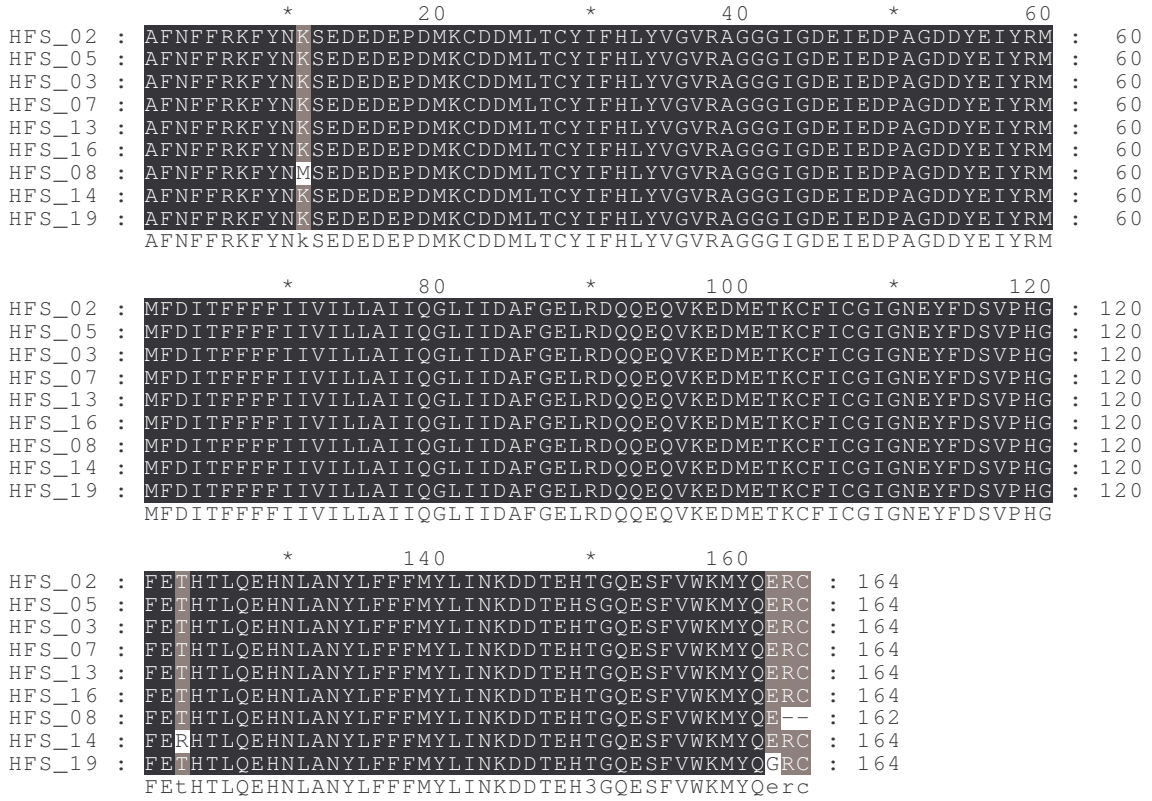

Figure 4: Alignment of hagfish deduced amino acid sequences with identical amino acids in black and conservative amino acid substitutions in shades of grey. The consensus sequence is at the bottom of the alignment.

Table 1: Top 3 matches from a tBLASTn search of Genbank database for the hagfish amino acid sequences. Accession numbers of the matching hypothetical protein sequences are shown, followed by E values in parentheses. E values (Expect values) are a statistical measure of how likely you would expect to find that particular result by chance. Thus a lower E value indicates a more significant similarity.

Sequence	Match 1 (E Value)	Match 2 (E Value)	Match 3 (E Value)
HFS 02	Opossum RyR3, XM_001380766 (1e-74)	Opossum RyR2, XM_001378263 (2e-74)	Mouse RyR2, NM_023868 (2e-74)
HFS 03	Opossum RyR3, XM_001380766 (1e-74)	Opossum RyR2, XM_001378263 (2e-74)	Mouse RyR2, NM_023868 (2e-74)
HFS 05	Opossum RyR3, XM_001380766 (3e-74)	Opossum RyR2, XM_001378263 (5e-74)	Mouse RyR2, NM_023868 (5e-74)
HFS 07	Opossum RyR3, XM_001380766 (1e-74)	Opossum RyR2, XM_001378263 (2e-74)	Mouse RyR2, NM_023868 (2e-74)
HFS 08	Opossum RyR3, XM_001380766 (2e-72)	Opossum RyR2, XM_001378263 (3e-72)	Zebrafish, RyR1, XM_686930 (3e-72)
HFS 13	Opossum RyR3, XM_001380766 (1e-74)	Opossum RyR2, XM_001378263 (2e-74)	Mouse RyR2, NM_023868 (2e-74)
HFS 14	Opossum RyR3, XM_001380766 (5e-74)	Opossum RyR2, XM_001378263 (8e-74)	Mouse RyR2, NM_023868 (8e-74)
HFS 16	Opossum RyR3, XM_001380766 (1e-74)	Opossum RyR2, XM_001378263 (2e-74)	Mouse RyR2, NM_023868 (2e-74)
HFS 19	Opossum RyR3, XM_001380766 (6e-74)	Opossum RyR2, XM_001378263 (1e-73)	Mouse RyR2, NM_023868 (1e-73)

Table 2: Percent sequence similarity of the hagfish consensus sequence with selected published sequences based on pairwise analysis. Percentages for identities indicate the proportion of amino acids that match exactly with the published sequence. Percentages for positives indicate the number of amino acids that score a positive value based on the scoring method used, but is not necessarily identical to the query.

Sequence	Opossum RyR3	Opossum RyR2	Mouse RyR2	Zebrafish RyR1	Human RyR1	Human RyR2	Human RyR3
Hagfish Consensus Identities	91%	92%	92%	90%	89%	92%	90%
Hagfish Consensus Positives	98%	98%	98%	96%	98%	98%	98%

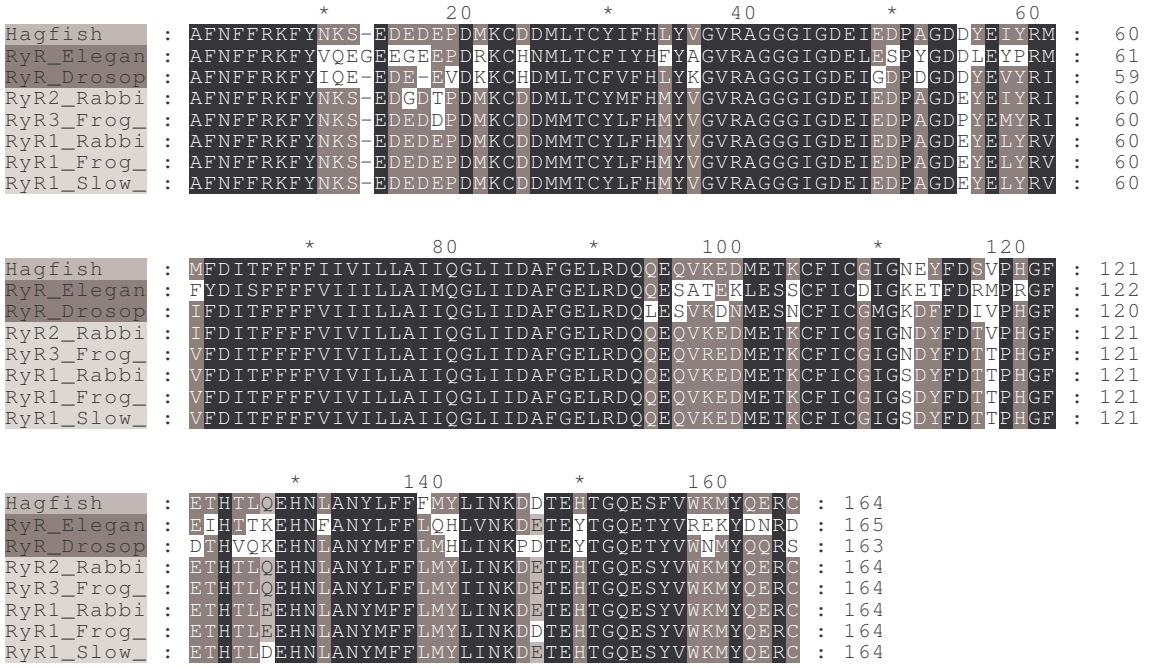

Figure 5: Multiple sequence alignment of hagfish consensus sequence with selected published RyR expressed protein sequences from nematode (BAA08309), *Drosophila* (NM_057646), fish RyR1-slow (U97329), frog RyR3 (D21071), and rabbit RyR1 (X15209) and RyR2 (U50465). Identical amino acids are in black, and functionally conserved amino acids are in shades of grey.

Table 3: Ancestral or derived characteristics of varied amino acid characteristics from multiple sequence alignment of hagfish consensus sequence and selected published RyR sequences. Gaps in amino acid sequences were eliminated for this analysis. Similarity to *Drosophila* or nematode was used to indicate ancestral characteristics whereas similarity to rabbit, frog, and fish was used to indicate derived characteristics for the varied amino acid locations.

Variable Amino Acid Position	Ancestral or Derived
P19	Ancestral (Shared with C. elegans)
M21	Derived
D24	Derived
V36	Derived
A52	Derived
D55	Ancestral
Q96	Derived
K104	Derived
N112	Derived
E113	Ancestral
V118	Ancestral (Shared with RyR2)
L127	Derived
Y141	Derived
D147	Ancestral (Shared with RyR1)
H150	Derived
K159	Derived

Phylogenetic Analysis

A multiple sequence alignment of the hagfish amino acid sequences and selected published RyR sequences was used to determine the phylogenetic position of the hagfish RyR. The *Drosophila* RyR sequence was used to root the tree due to the ancestral nature of invertebrate RyR genes (Figure 6*)*. The parsimony analysis was performed using the software Protpars program from the PHYLIP package. The parsimony analysis was performed using multiple bootstrapped datasets generated by the Seqboot program of PHYLIP. To generate the consensus tree the Consense program from PHYLIP was used. All of the cloned sequences cluster apart from the rest of the published RyR sequences with a bootstrap value of 99.

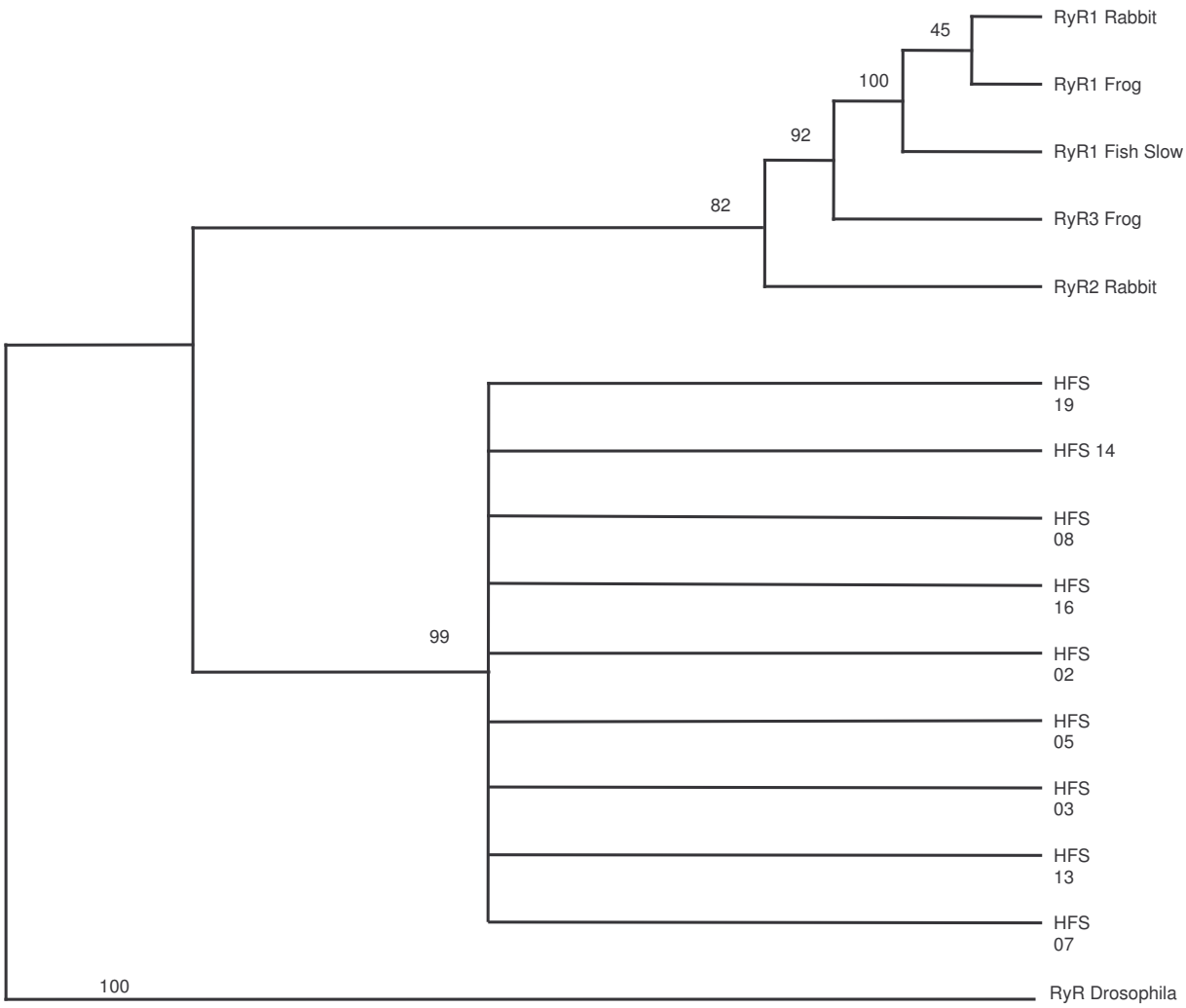

Fig 6: Protein parsimony tree based on multiple sequence alignments of cloned RyR sequences and published RyR sequences from GenBank with accession numbers in parentheses including frog RyR1 (D21070), frog RyR3 (D21071), rabbit RyR2 (U50465), rabbit RyR1 (X15209), and fish RyR1-slow (U97329*). Drosophila* RyR (NM_057646) was designated as the outgroup. The programs used to create the tree include Seqboot, Protpars, and Consense from the PHYLIP software package. Bootstrap values are given as a percentage of 500 replicates.

Discussion

Sequencing

The results of the sequencing showed nine highly similar nucleotide sequences. The deduced amino acid sequences are identical at 161 of the 164 positions, with only 3 sequences showing 1 amino acid change. The top hits from a tBLASTn searches against the Genbank database are RyR3 for all sequences, and RyR2 as the top 2nd and 3rd result in all but HFS-08 clone (Table 1). A consensus sequence was generated for the 9 Hagfish RyR deduced RyR amino acid sequences and aligned with published representative RyR sequences.

The high sequence similarity of the nine hagfish RyR sequences suggests that only one RyR isoform is expressed in Hagfish skeletal muscle. If more than one isoform was expressed in hagfish skeletal muscle, as is the case for derived teleost fish, one would expect to amplify messages representative of both isoforms. The results of the PCR survey suggest that hagfish do not co-express both the RyR1 and RyR3 isoforms in their skeletal muscle. An alternative explanation could be that the extra isoforms are indeed present, but are more like mammalian skeletal muscle in that RyR3 or some primitive orthologue may be present but in very low amounts, lower than 5% of total RyR present (Fill and Copello 2002). If this were the case one would only expect there to be one representative of the second isoform in every 20 or so clones. In order to determine more accurately whether a second isoform is present many more clones would need to be surveyed.

There are several positions in the consensus sequence in which the amino acid differs from published RyR sequences. These locations have been identified in Table 3 as

variable amino acid locations and were classified as being either ancestral or derived in nature. Ancestral positions shared amino acids with those found in *Drosophila* or nematode, whereas derived positions shared amino acids with fish, rabbit, or frog. At many locations the varied amino acids are ancestral in nature, while others are derived. The remaining positions are found to be conserved throughout all forms and represent functionally conserved regions. Because the consensus sequence contained many ancestral and derived amino acids it was suggested that the hagfish sequence represents an evolutionary intermediate between the ancestral RyR and more recent specialized forms. This is consistent with the divergence in function from invertebrates as discussed in the next section (Di Biase and Franzini-Armstrong 2005; Fill and Copello 2002; Sorrentino, Barone, Rossi 2000; Xian-guang *et al* 2002*).*

Phylogenetic Analysis

Parsimony analysis places the hagfish RyR sequences in an ancestral clade to the other RyR isoforms with a robust 99% bootstrap value. The tree was rooted using the *Drosophila* gene and indicates that the hagfish RyR represents a separate lineage from the other vertebrates, consistent with the placement of hagfish in the vertebrate tree (See Appendix C) (Escriva *et al* 2002; Vazquez-Martinez *et al* 2003; Xian-guang *et al* 2002*).*

Hagfish represent the lowest known vertebrate and are even more primitive than lamprey. The divergence of these vertebrates from invertebrates and *Amphioxus* was accompanied by a physiological change in excitation contraction coupling in muscle tissue. Invertebrates and *Amphioxus* require extracellular Ca^{2+} to enter skeletal muscle cells before contraction takes place via the calcium induced calcium release mechanism,

whereas more derived skeletal muscles of vertebrates do not. It is also during this period where direct physical coupling of DHPRs and RyRs appear, as indicated from electron micrographs of muscle cells of these organisms. (Inoue, Tsutsui, Bone 2002) Because of this one would expect the RyR message to be different from the ancestral RyR to correspond with the changes in functionality. There is also some evidence based on the organization and arrangement of the tetrads formed from the association of DHPRs and RyRs that there may be two isoforms of RyR present in hagfish skeletal muscle, (Inoue, Tsutsui, Bone 2002) but my molecular investigation does not confirm this. It can be proposed that the divergence into the other isoforms occurred at a later point than the hagfish, corresponding with the second genome duplication event in the vertebrate line. A phylogenetic tree for vertebrates for reference can be found in Appendix C.

Future Studies

In order to more accurately confirm the presence of only one isoform of ryanodine receptor in hagfish skeletal muscle with a higher degree of statistical confidence, many more clones need to be obtained and new primers need to be designed to extend the length of sequences obtained. Furthermore, more studies on the identity of RyRs in other primitive vertebrates such as *Amphioxus,* lamprey, shark, and non-teleost fish such as bichir need to be done to confirm that the duplication of RyR into the separate skeletal isoforms occurred along with the predicted whole genome duplication events in vertebrates and teleost fish. *Amphioxus* represents an ancestral form of EC-coupling which is similar to that of invertebrates in which extracellular calcium is required, and so one would expect to find an RyR orthologue similar to ancestral forms (Escriva *et al*

2002; Kao and Lee 2002; Meyer and Schartl 1999*).* Bichir are representatives of older fish that have not undergone the third round of genome duplication seen in other fish, and as such one would not expect to find the divergence of slow and fast twitch specific isoforms of RyR (Chiu *et al* 2004; Ledje, Kim, Ruddle 2002; Meyer and Van de Peer 2005*).*

Conclusions:

1. A single isoform of ryanodine receptor was isolated from hagfish skeletal tissue and identified using tBLASTn searches of Genbank and parsimony analysis
2. The partial sequence was found to cluster apart and in an ancestral position to more derived published RyR1, RyR2, and RyR3 isoforms.

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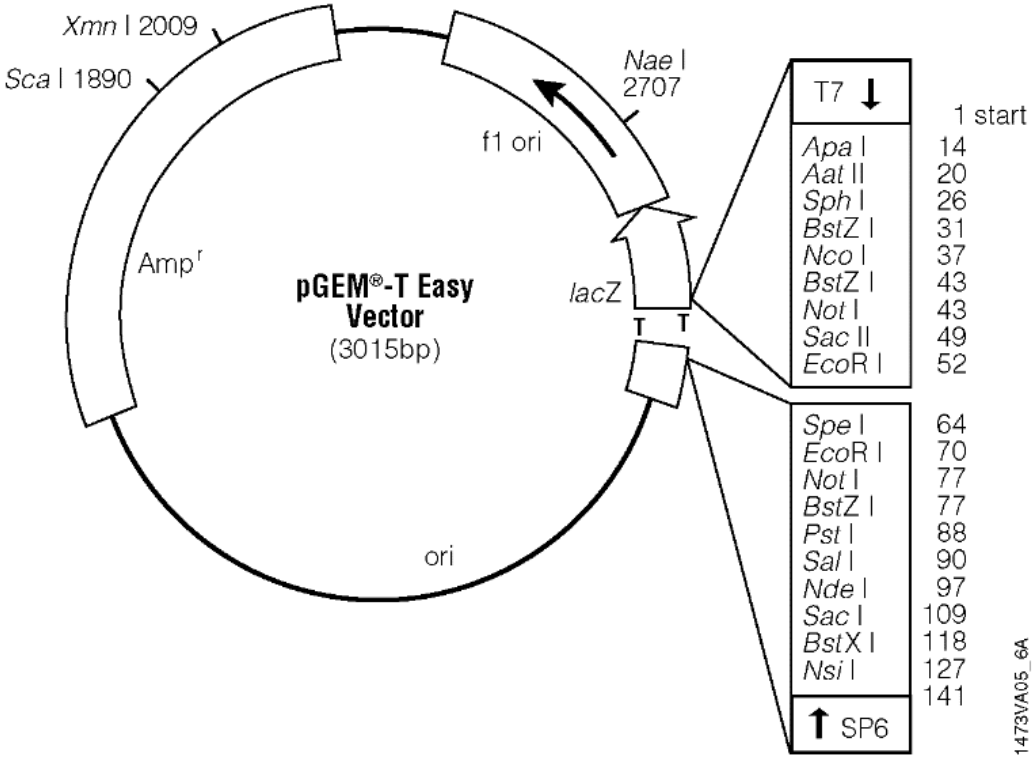
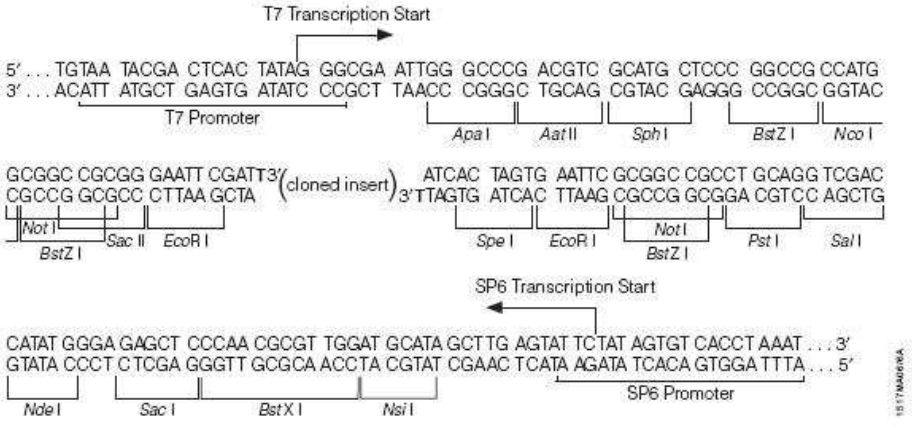
Appendix A: Amino Acid alignment for hagfish degenerate primer design.

```
FrogRyR1          ILSSVTHNGKQLMMTVGLLAVVVYLYTVVAFNFFRKFYNKSEDEDEPDMKCDDMMTCYLF
FishRyR1slow      ILSSVTHNGKQLMMTVGLLAVVVYLYTVVAFNFFRKFYNKSEDEDEPDMKCDDMMTCYLF
RabRyR1           ILSSVTHNGKQLVMTVGLLAVVVYLYTVVAFNFFRKFYNKSEDEDEPDMKCDDMMTCYLF
FrogRyR3          ILSSVTHNGKQLVLTVGLLAVVVYLYTVVAFNFFRKFYNKSEDEDDPDMKCDDMMTCYLF
RabRyR2           ILSSVTHNGKQLVLTVGLLAVVVYLYTVVAFNFFRKFYNKSEDGDTPDMKCDDMLTCYMF
                  ************::***************************** * ********:***:*

FrogRyR1          HMYVGVRAGGGIGDEIEDPAGDEYELYRVVFDITFFFFVIVILLAIIQGLIIDAFGELRD
FishRyR1slow      HMYVGVRAGGGIGDEIEDPAGDEYELYRVVFDITFFFFVIVILLAIIQGLIIDAFGELRD
RabRyR1           HMYVGVRAGGGIGDEIEDPAGDEYELYRVVFDITFFFFVIVILLAIIQGLIIDAFGELRD
FrogRyR3          HMYVGVRAGGGIGDEIEDPAGDPYEMYRIVFDITFFFFVIVILLAIIQGLIIDAFGELRD
RabRyR2           HMYVGVRAGGGIGDEIEDPAGDEYEIYRIIFDITFFFFVIVILLAIIQGLIIDAFGELRD
                  ********************** **:**::*****************************

FrogRyR1          QQEQVKEDMETKCFICGIGSDYFDTTPHGFETHTLEEHNLANYMFFLMYLINKDDTEHTG
FishRyR1slow      QQEQVKEDMETKCFICGIGSDYFDTTPHGFETHTLDEHNLANYMFFLMYLINKDETEHTG
RabRyR1           QQEQVKEDMETKCFICGIGSDYFDTTPHGFETHTLEEHNLANYMFFLMYLINKDETEHTG
FrogRyR3          QQEQVREDMETKCFICGIGNDYFDTTPHGFETHTLQEHNLANYLFFLMYIINKDETEHTG
RabRyR2           QQEQVKEDMETKCFICGIGNDYFDTVPHGFETHTLQEHNLANYLFFLMYLINKDETEHTG
                  *****:*************.*****.*********:*******:*****:****:*****

FrogRyR1          QESYVWKMYQERCWDFFPAGDCFRKTYEDQLG
FishRyR1slow      QESYVWKMYQERCWDFFPAGDCFRKQYEDQLS
RabRyR1           QESYVWKMYQERCWDFFPAGDCFRKQYEDQLS
FrogRyR3          QESYVWKMYQERCWDFFPAGDCFRKQYEDQLG
RabRyR2           QESYVWKMYQERCWEFFPAGDCFRKQYEDQLN
                  **************:********** *****.
```

Appendix B: pGEM®-T Easy Vector Maps



pGEM®-T Easy Vector



Appendix C: Phylogenetic Tree of Vertebrate Lineages

Appendix D: Composition of Media

10X Ficoll Buffer (tracking dye)
20% (w/v) Ficoll 400
0.1 M Na_2EDTA - pH 8.0
1% (w/v) SDS
0.25% (w/v) Bromphenol Blue

10X TAE Buffer
Tris (0.4 M)
Na_2EDTA (0.01 M)
Acetic acid (0.2 M)

10X TE Buffer (per litre)

1M Tris-Cl, pH 7.5	100 mL
0.5M EDTA pH 8.0	20 mL

LB Media with ampicillin (per litre)
10 g tryptone
5 g yeast extract
5 g NaCl
1 ml 1 N NaOH
Ampicillin Final Concentration 50µg/ml
15 g agarose (for pouring into plates)