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CLONING OF *Astacus leptodactylus* RYANODINE RECEPTOR GENE

Nazlı COŞKUN JIHAD

Program of Biophysics

MASTER THESIS

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Supervisor: Prof. Dr. Nuhan PURALI

This thesis study has been approved and accepted as a Master dissertation in "Biophysics Program" by the assessment committee, whose members are listed below, on 03.01.2022

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This dissertation has been approved by the above committee in conformity to the related issues of Hacettepe University Graduate Education and Examination Regulation.

Prof. Müge YEMİŞCİ ÖZKAN, MD, PhD

Director

YAYIMLAMA VE FİKRİ MÜLKİYET HAKLARI BEYANI

Enstitü tarafından onaylanan lisansüstü tezimin/raporumun tamamını veya herhangi bir kısmını, basılı (kağıt) ve elektronik formatta arşivleme ve aşağıda verilen koşullarla kullanıma açma iznini Hacettepe Üniversitesine verdiğimi bildiririm. Bu izinle Üniversiteye verilen kullanım hakları dışındaki tüm fikri mülkiyet haklarım bende kalacak, tezimin tamamının ya da bir bölümünün gelecekteki çalışmalarda (makale, kitap, lisans ve patent vb.) kullanım hakları bana ait olacaktır.

Tezin kendi orijinal çalışmam olduğunu, başkalarının haklarını ihlal etmediğimi ve tezimin tek yetkili sahibi olduğumu beyan ve taahhüt ederim. Tezimde yer alan telif hakkı bulunan ve sahiplerinden yazılı izin alınarak kullanılması zorunlu metinlerin yazılı izin alınarak kullandığımı ve istenildiğinde suretlerini Üniversiteye teslim etmeyi taahhüt ederim.

Yükseköğretim Kurulu tarafından yayınlanan ***"Lisansüstü Tezlerin Elektronik Ortamda Toplanması, Düzenlenmesi ve Erişime Açılmasına İlişkin Yönerge"*** kapsamında tezim aşağıda belirtilen koşullar haricince YÖK Ulusal Tez Merkezi / H.Ü. Kütüphaneleri Açık Erişim Sisteminde erişime açılır.

- Enstitü / Fakülte yönetim kurulu kararı ile tezimin erişime açılması mezuniyet tarihimden itibaren 2 yıl ertelenmiştir. [1]
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- Tezimle ilgili gizlilik kararı verilmiştir. [3]

03 / 01 /2022

Nazlı COŞKUN JIHAD

_____

1"Lisansüstü Tezlerin Elektronik Ortamda Toplanması, Düzenlenmesi ve Erişime Açılmasına İlişkin Yönerge"

*(1) Madde 6. 1. Lisansüstü tezle ilgili patent başvurusu yapılması veya patent alma sürecinin devam etmesi durumunda, tez **danışmanının** önerisi ve **enstitü anabilim dalının** uygun görüşü üzerine **enstitü** veya **fakülte yönetim kurulu** iki yıl süre ile tezin erişime açılmasının ertelenmesine karar verebilir.*

*(2) Madde 6. 2. Yeni teknik, materyal ve metotların kullanıldığı, henüz makaleye dönüşmemiş veya patent gibi yöntemlerle korunmamış ve internetten paylaşılması durumunda 3. şahıslara veya kurumlara haksız kazanç imkanı oluşturabilecek bilgi ve bulguları içeren tezler hakkında tez **danışmanının** önerisi ve **enstitü anabilim dalının** uygun görüşü üzerine **enstitü** veya **fakülte yönetim kurulunun** gerekçeli kararı ile altı ayı aşmamak üzere tezin erişime açılması engellenebilir.*

*(3) Madde 7. 1. Ulusal çıkarları veya güvenliği ilgilendiren, emniyet, istihbarat, savunma ve güvenlik, sağlık vb. konulara ilişkin lisansüstü tezlerle ilgili gizlilik kararı, **tezin yapıldığı kurum** tarafından verilir *. Kurum ve kuruluşlarla yapılan işbirliği protokolü çerçevesinde hazırlanan lisansüstü tezlere ilişkin gizlilik kararı ise, **ilgili kurum ve kuruluşun önerisi** ile **enstitü** veya **fakültenin** uygun görüşü üzerine **üniversite yönetim kurulu** tarafından verilir. Gizlilik kararı verilen tezler Yükseköğretim Kuruluna bildirilir.*

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****** Tez **danışmanının** önerisi ve **enstitü anabilim dalının** uygun görüşü üzerine **enstitü** veya **fakülte yönetim kurulu tarafından karar verilir***

ETHICAL DECLARATION

In this thesis study, I declare that all the information and documents have been obtained in the base of the academic rules and all audio-visual and written information and results have been presented according to the rules of scientific ethics. I did not do any distortion in data set. In case of using other works, related studies have been fully cited in accordance with the scientific standards. I also declare that my thesis study is original except cited references. It was produced by myself in consultation with supervisor (Prof. Dr. Nuhan PURALI) and written according to the rules of thesis writing of Hacettepe University Institute of Health Sciences.

Nazlı COŞKUN JIHAD

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ABSTRACT

COŞKUN JIHAD, N., Cloning of *Astacus Leptodactylus* Ryanodine Receptor Gene, Hacettepe University Graduate School of Health Sciences, Master Thesis in Biophysics, Ankara, 2022. Cytoplasmic Ca2+ concentration plays an essential role in many types of cellular function including electro-mechanical coupling in striated muscle fibers. Ryanodine receptor channels (RyR), mediating Ca2+ release from sarcoplasmic reticulum (SR), has a homotetrameric structure. It is the largest ion channel with a size of 2.2 MDa. Vertebrate and invertebrate RyR channels are structurally and functionally similar. Although *Astacus leptodactylus,* narrow-clawed crayfish, is a widely used model animal in neuroscience, information about genetic properties of the animal is rather limited. The present study is focused onto *de novo* cloning of the mRNA of the crayfish RyR channel which encodes the largest ion channel. A hybrid cloning method has been used, referring to the homology between RyR mRNA molecules and the computational assembly of the next generation sequencing data. A mRNA molecule of 15236 bp in size has originally been cloned. The putative RyR protein, with 5042 amino acids, has a significant similarity to the sequences reported in other species. Furthermore, the putative sequence possessed many of the conserved domains specific to the RyR channel. Thus, it has been proposed that a mRNA coding RyR channel has originally been cloned in the present study. The 3D protein structure can also be determined by the help of this revealed genetic information, or future mutation studies can be designed.

Keywords: Ryanodine receptor, crayfish, cloning, Sanger sequencing, *de novo* assembly

ÖZET

COŞKUN JIHAD, N., Cloning of *Astacus leptodactylus* Ryanodine Receptor Gene. Hacettepe Üniversitesi Sağlık Bilimleri Enstitüsü Biyofizik Yüksek lisans Tezi, Ankara, 2022. Sitoplazmik Ca2+ konsantrasyonu, çizgili kas liflerinde elektro-mekanik bağlantı gibi birçok hücresel fonksiyonda önemli bir rol oynamaktadır. Ryanodin reseptörü (RyR), sarkoplasmik retikulumdan (SR) Ca2+ salınımına aracılık eden, 4 eş alt birimli yapıya sahiptir. 2.2MDa büyüklüğüyle en büyük iyon kanalıdır. Omurgalı ve omurgasız RyR kanalları yapısal ve fonksiyonel olarak benzerdir. *Astacus leptodactylus,* dar pençeli kerevit, nörobilim gibi birçok çalışmada kullanılan yaygın bir model hayvan olmasına karşın genetik bilgileri oldukça kısıtlıdır. Bu çalışma, en büyük kanalı kodlayan kerevitRyR mRNA molekülünün *de novo* klonlaması üzerine odaklanmaktadır. RyR mRNA molekülleri arasındaki homoloji ve yeni nesil sekanslama verisinin hesaplamalı birleştirilmesiyle ifade edilen hibrid klonlama metodu kullanılmıştır. 15236 bp uzunluğunda mRNA molekülü klonlandı. 5042 amino asitlik varsayılan RyR proteini, diğer türlerden elde edilen sekanslarla önemli bir benzerliğe sahiptir. Ayrıca, varsayılan RyR sekansı, RyR kanalına spesifik birçok korunmuş alanlara sahiptir. Böylece, bu çalışmada, RyR kanalını kodlayan mRNA molekülünün özgün biçimde klonlandığı ileri sürülmektedir. Ortaya çıkarılan bu genetik bilginin yardımıyla üç boyutlu protein yapısı da belirlenebilir ya da ilerideki mutasyon çalışmaları tasarlanabilir.

Anahtar Sözcükler: Ryanodin reseptörü, kerevit, gen klonlama, Sanger sekanslama, *de novo* dizileme

TABLE OF CONTENTS

ABBREVIATIONS

^{o}C	Degree Celsius
Å	Angstrom
aa	amino acid
bp	Base pair
BLAST	Basil local alignment search tool
Ca	Calcium
CCD	Central core disease
cDNA	Complementary deoxyribonucleic acid
CICR	Ca^{2+}-induced Ca^{2+} release
DHPR	Dihydropyridine receptors
DICR	depolarization induced Ca2+ release
DNA	Deoxyribonucleic acid
dNTP	Deoxy nucleoside triphosphate
kb	Kilobase pair
MH	Malignant hyperthermia
mRNA	Messenger ribonucleic acid
NGS	Next generation sequencing
ORF	Open reading frame
PCR	Polymerase chain reaction
RACE	Rapid amplification of cDNA ends
RNA	Ribonucleic acid
RyR	Ryanodine receptor
Tm	Melting temperature
TMS	Transmembrane segment
UPM	Universal Primer Mix

FIGURES

TABLES

1. INTRODUCTION

Ryanodine receptor (RyR) is the largest ion channel with about 2.2MDa size and has a homotetrameric structure (1,2). It releases Ca2+ from sarcoplasmic reticulum (SR), which rapidly increases the cytoplasmic Ca2+ concentration to trigger several cellular functions. In mammalians, RyR1, RyR2 and Ryr3 are mostly expressed in skeletal muscle fibers, myocytes, and smooth muscle and non-muscle cell types, respectively (3). In reference to previous studies, there is no difference between morphology and function of vertebrate and invertebrate RyR channels (4,5). Mutations in RyR are associated with several genetic diseases such as, central core disease (CCD) and malignant hyperthermia (MH) (6-9).

Astacus leptodactylus, narrow-clawed crayfish, is widely used as a model animal in several studies including neuroscience and viral infections (10-15). However, information about its genetic properties is limited.

The primary focus of the present stud is to originally explore mRNA sequence for the putative crayfish ryanodine receptor channel from cDNA samples constructed from crayfish muscle tissue. By using molecular biology methods and bioinformatics a complete mRNA has originally been cloned and the related amino acid sequence of the RyR channel protein has been calculated. The compiled data may lead to further studies of crayfish RyR channels, e.g., investigating functional regions of the channel protein.

2. LITERATURE REVIEW

Ca^{2+} ion is a second messenger molecule which plays a key role in many cellular functions including fertilization, development, secretion, muscle contraction and apoptosis (1,16). At resting state, the intracellular Ca^{2+} concentration is about 10^{-7} M, which is extremely low as compared to the extracellular concentration which is about 10^{-3} M (17). In response to an appropriate stimulus, cytosolic Ca^{2+} concentration can rapidly increase 10-100 fold in a fraction of a second and generates a huge chemical signal to trigger downstreaming cellular events (18,19). The rapid rise in calcium concentration is mediated mainly by two pathways, Ca^{2+} channels in the cell membrane and those in the endoplasmic/sarcoplasmic reticulum (ER/SR). Depending on the cell types the relative contribution the pathways may differ. However, sarcoplasmic reticulum calcium release channel is the dominant mechanism in genesis of the rapid rise in the cytosolic calcium signal in the skeletal muscle fibers. Further, SR is the major intracellular Ca^{2+} storage in the skeletal muscle fibers. Cytosolic calcium concentration is strictly regulated by some cellular mechanisms. Thus, Ca^{2+} homeostasis is crucial for a cell as many diseases, e.g., cardiac disease, are associated with its dysregulation (20).

Ryanodine receptor Ca^{2+} release channels (RyR), located in the ER/sarcoplasmic reticulum (SR) (1), are the largest in size among the known intracellular ion channels, ~2.2 MDa (2). The name of the receptor is originated from ryanodine, the plant alkaloid, as it binds and blocks the channel (1). RyR channel has a homotetrameric structure, consists of monomers with ~560 kDa (2).

In mammalians, three isoforms of RyR genes, located on different chromosomes, have been identified (21). Although all three types of RyR genes are co-expressed in many mammalian cells, the isoforms can be categorized according to tissues where they are mostly expressed. It has been reported that RyR1 is predominantly expressed in skeletal muscle; RyR2 is expressed mostly in heart muscle; and RyR3 is present in brain and smooth muscle (3). However, terminology differs in non-mammalian skeletal muscle types, e.g., chicken and frog. α-RyR and β-

RyR isoforms in non-mammalians are homologs of RyR1 and RyR3 genes, respectively (4,22). Quinn *et al.* reported that the invertebrate RyR is structurally and functionally similar to the vertebrate isoforms of the channel (4,5). The modulators, binding to the mammalian RyR, has been shown to be active on the invertebrate channels. Thus, the pharmacological properties of the channels are similar. However, biophysical properties differs so that, the conductance of invertebrate channels are lower than that of the vertebrate RyR (4).

RyR channels have a crucial role in Excitation-Contraction (E-C) coupling in which muscle contraction is initiated by the electrical impulse (5). In this process, the L-type voltage-gated Ca^{2+} channels, also known as the dihydropyridine receptors (DHPR), are activated (23,24). As a result, Ca^{+2} are released rapidly from the SR to the sarcoplasm by activated RyR channels (23,25,26). The DHPR interacts directly to RyR1 in skeletal muscle (Figure 2.1). However, the mechanism of the electro-mechanical coupling, also known as depolarization induced Ca^{2+} release (DICR), is poorly understood (26-28). Free Ca^{2+} can also activate RyR channels to release a large amount of Ca2+ from the storage site in SR (Figure 2.1), the phenomenon is termed as Ca^{2+}-induced Ca^{2+} release (CICR) (29). By this way, CaV coupled RyR1 channels can stimulate other uncoupled RyR channels in close proximity (30,31).

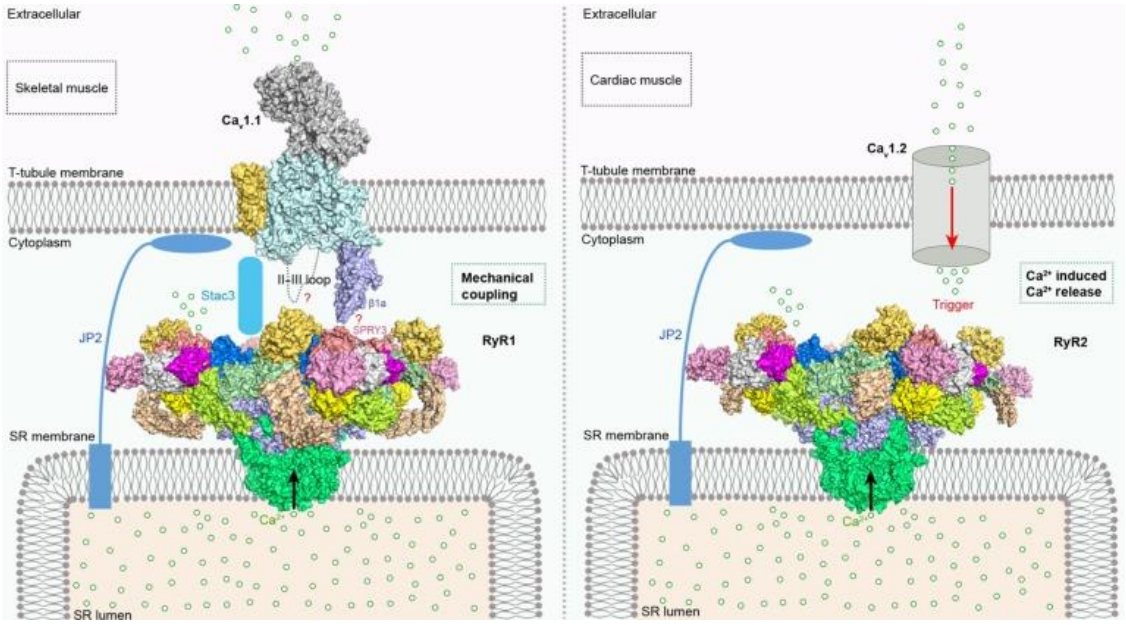


Figure 2.1. Activation mechanisms of RyR1 in skeletal muscle and RyR2 in cardiac muscle, respectively (32).

RyR channel subunits have a huge hydrophilic N-terminal domain, known as the foot region (Figure 2.2), with dimensions of 275 Å × 275 Å × 120 Å (33). This cytoplasmic part of the channel provides the binding sites for regulators and modulators including Ca2+ (primarily), FKBPs (FK506-binding proteins), ATP (adenosine triphosphate), caffeine, PCB95 (2,2′,3,5′,6-pentachlorobiphenyl) and ryanodine (34-39). The subunits also contain a hydrophobic C-terminal domain with several transmembrane segments whose number varies between 4-10. The dimensions of the transmembrane region of the channel are 120 Å × 120 Å × 60 Å (5). Pore forming region of ryanodine receptor is formed by the last two transmembrane segments present at the hydrophobic C-terminal region of the channel (Figure 2.2) (40,41).

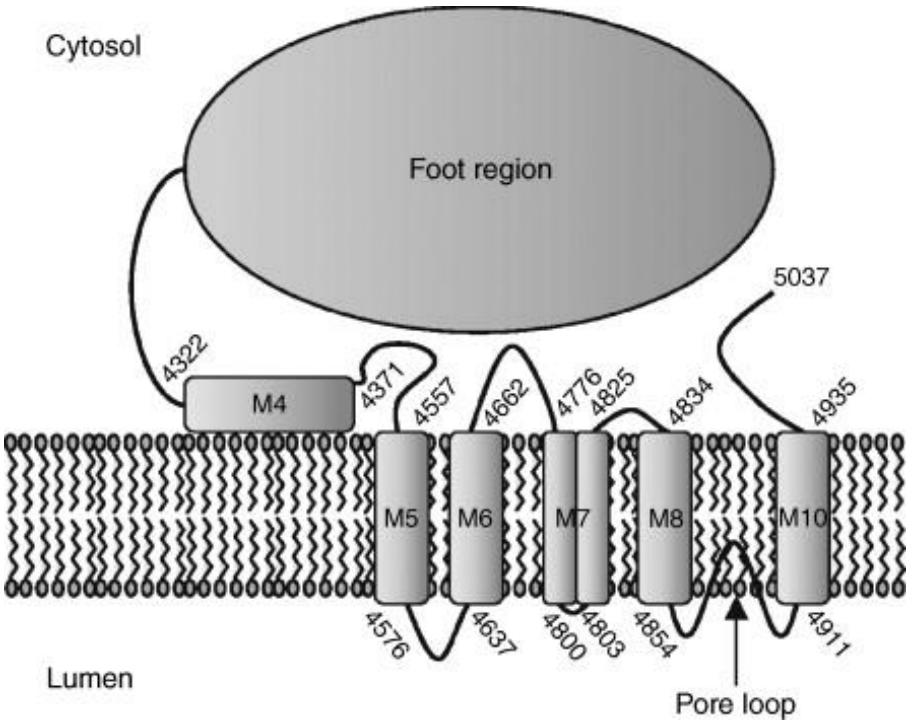


Figure 2.2. Ryanodine receptor (RyR) membrane topology. Each subunit contains foot region, transmembrane domains and pore forming region. Coordinates are for rabbit RyR1 (33).

Bai *et al.* reported that the calculated pore diameter of the closed RyR1 is approximately 1.6 Å, which would block Ca^{2+} passage (42). However, in the presence of modulators, the diameter of a dilated pore is calculated to be near 5 Å, which is large enough to allow passage of hydrated Ca^{2+}. It was reported that the conformational change of the cytoplasmic extension of S6 segment leads to channel opening while the channel domain and its central domain remain almost the same (42).

The mutations of the RyR1, located in the cytoplasmic domains, are associated mostly with malignant hyperthermia (MH) and central core disease (CCD) (6,7) while the mutations of RyR2 are associated with catecholaminergic polymorphic ventricular tachycardia (CPVT) and arrhythmogenic right ventricular dysplasia type 2 (ARVD2) (7-9). As the pore-forming region is directly affect Ca^{2+} passage, mutations in the pore region may lead to some structural changes, which may directly block or reduce the ion conduction through the channel (43,44). For example, the Ile4898Thr point mutation in the pore region of RyR1 causes CCD in the phenotype (45).

Astacus leptodactylus, narrow-clawed crayfish, is also known as Galicia, swamp, or pond crayfish (46). This native freshwater crayfish is widely distributed in the lakes in Turkey and can be easily recognized by its long chelae (47-49). Although crayfish is widely used as a model animal for different experimental purposes such as neuroscience, animal behavior and viral infections (10-15), information about its genetic properties is rather limited.

3. MATERIALS AND METHODS

3.1. Animals

Fresh samples from *Astacus leptodactylus* (crayfish) muscle has been used in the cloning experiments. Animals were collected from the lakes in Central Turkey. They were kept in an aerated freshwater aquarium at 18-20 ^{o}C and fed with an alternating carrot and fish diet once a week.

In the use of experimental animals, the guidelines by Hacettepe University have been followed and ethics committee approval has been obtained.

3.2. Decapitation of the Crayfish and Tissue Excision

Intermolt crayfishes were taken from the aquarium and surrounded by ice for 10-15 minutes to be anesthetized and then, decapitated quickly. Abdominal flexor muscle was dissected rapidly by using sterile surgical equipment and scissors. Collected tissue samples were placed in a beaker and kept on ice until the start of total RNA isolation procedure.

3.3. Total RNA Isolation from The Muscle Tissue

Qiazol Lysis Reagent (Qiagen) has been used to extract the total RNA content of the samples as described in the manual. Firstly, a randomly excised chunk of tissue, weighing about 30 mg, has been placed in 1mL of Qiazol Lysis Reagent in a 1.5 ml microcentrifuge tube. The sample was homogenized by using a single-use plastic pestle and incubated at room temperature for 5 minutes. After the incubation, 0.2 mL of chloroform was added to the homogenate, and the tube was vortexed for 20 seconds. The tube was shaken vigorously by hand for 15 seconds and incubated for 2-3 min at room temperature. The sample was centrifuged at 12000 g for 15 minutes at 4 ^{o}C. The upper aqueous phase of the sample was replaced carefully into a new tube. 0.5 mL isopropanol was added, and the solution was mixed vigorously by vortexing. The sample was allowed to rest at room temperature for 10 minutes then,

centrifuged at 12000 g for 10 minutes at 4 °C. The supernatant was discarded carefully, and the gel-like RNA pellet was washed by adding 1 mL of 75% ethanol. The tube was centrifuged at 7500g for 5 min at 4 °C and then, the supernatant was discarded. Remaining ethanol was let to vaporize near to the flame to briefly air-dry the RNA pellet. Finally, the RNA pellet was dissolved in 50 µL RNase-free water. The concentration of isolated total RNA was measured by Qubit dsDNA HS assay kit (Thermo Fisher). The product was aliquoted into 10 ul tubes and stored at -80 °C for the down streaming experiments.

3.4. cDNA Synthesis

cDNA synthesis has been performed immediately after the total RNA isolation, as RNA is less stable than dsDNA. A thermal cycler *(Applied Biosystems Veriti)* was used for both cDNA synthesis and following PCR experiments.

In the present study, either REPLI-g WTA Single Cell Kit (Qiagen) or *SMARTer RACE 5' / 3' Kit* (Clontech) were used to reverse transcribe the isolated RNA and synthesize cDNA library.

3.4.1. cDNA synthesis by using REPLI-g WTA Single Cell Kit

This kit contains novel REPLI-g SensiPhi DNA polymerase which displaces the generated strand from cDNA strand, thus, it becomes a template itself for amplification. As utilizing this property, Multiple Displacement Amplification (MDA), the kit allows uniform amplification of whole transcriptome with negligible sequence bias. In other words, it provides sensitive detection of even low-abundance transcripts successively.

Table 3.1. Step I of cDNA synthesis by using REPLI-g WTA Single Cell Kit.

	Volume (µl)
Total RNA (> 10pg- 100ng)	**X**
dH2O	**8-x**
NA Denaturation Buffer	**3**

Samples has been kept on ice throughout the synthesis procedure. The component shown in Table 3.1 was prepared and incubated at 95 ^{o}C for 3 minutes. 2 µl of *Genomic DNA Wipeout Buffer* was added and mixed by vortexing. While Quantiscript RT mix was being prepared fresh (Table 3.2), the sample was incubated at 42 ^{o}C for 10 minutes.

Table 3.2. Quantiscript RT mix**.**

	Volume (µl)
RT/Polymerase Buffer	**4**
Random Primer	**1**
Oligo dT Primer	**1**
Quantiscript RT Enzyme Mix	**1**
Total volume	**7**

7 µl of Quantiscript RT mix has been mixed with the sample from Step I and incubated at 42 ^{o}C for 1 hour. The reaction was stop by incubating at 95 ^{o}C for 3 minutes and then the tube was cooled down on ice. Towards the end of the incubation, a ligation mix has been prepared freshly by adding the components in the order given in the Table 3.3.

Table 3.3. Ligation mix**.**

	Volume (µl)
Ligase Buffer	**8**
Ligase Mix	**2**
Total volume	**10**

10 µl of ligation mix has been added and the tube was incubated at 24 ^{o}C for 30 minutes and then at 95 ^{o}C for 5 minutes. As the tube was let cool down on ice, REPLI-g SensiPhi amplification mix has been prepared (Table 3.4).

Table 3.4. REPLI-g SensiPhi amplification mix**.**

	Volume (µl)
REPLI-g sc Reaction Buffer	**29**
REPLI-g SensiPhi DNA Polymerase	**1**
Total volume	**30**

30 µl of REPLI-g SensiPhi amplification mix was added to the tube and incubated at 30 ^{o}C for 2 hours and at 65 ^{o}C for 5 minutes. Amplified cDNA was hundred times diluted for downstream PCR experiments. Amplified and diluted cDNA products were aliquoted and stored at – 20 ^{o}C.

3.4.2. cDNA Synthesis by using SMARTer RACE 5’ / 3’ Kit.

Rapid amplification of cDNA ends (RACE), a technique to obtain the full-length sequences of transcripts, has been used to reveal 5' and 3' end sequences of the target gene. The SMARTer RACE 5'/3' Kit provides efficient cDNA synthesis of long and GC-rich transcripts.

Buffer Mix was prepared as given in Table 3.5 for both the 5’- and 3’-RACE-Ready cDNA synthesis reactions. The mixture was let set aside at room temperature.

Table 3.5. Buffer Mix.

	Volume (µl)
5X First Strand Buffer	4
DTT (100 mM)	0.5
dNTPs (20 mM)	1
Total volume	5.5

In separate tubes, 5'-RACE-Ready and 3'-RACE-Ready cDNA preparation mixtures were prepared (Table 3.6).

Table 3.6. Mixtures for preparation of 5'-RACE-Ready cDNA and 3'-RACE-Ready cDNA.

5'-RACE-Ready cDNA		**3'-RACE-Ready cDNA**	
	Volume (µl)		**Volume**
Total RNA (*10 ng–1 µg*)	1-10	***Total RNA (*10 ng–1 µg*)***	1-11
5'-CDS Primer A	1	**3'-CDS Primer A**	1
Sterile H2O	0-9	**Sterile H2O**	0-10
Total volume	11	**Total volume**	12

The mixture was incubated at 72 ^{o}C for 3 minutes and cooled down at 42 ^{o}C for 2 minutes. The tubes were span briefly to collect contents at the bottom. 1 µl of *SMARTer II A Oligonucleotide* was added only for 5'-RACE cDNA synthesis reaction. During incubation, Master mixes for both 5'- and 3'-RACE-Ready cDNA synthesis reactions were prepared at room temperature in the following order as given in Table 3.7.

Table 3.7. Master mixes for both 5'- and 3'-RACE-Ready cDNA synthesis reactions.

	Volume (µl)
Buffer Mix	5.5
RNase inhibitor (40 U / µl)	0.5
SMARTScribe Reverse Transcriptase (100 U)	2
Total volume	8

8 µl of master mixes were added onto the denatured RNA mixtures by gently pipetting. The tubes were incubated at 42 ^{o}C for 1.5 hours and then at 70 ^{o}C for 10 minutes. The first-strand cDNA synthesis reaction products were diluted by addition of 10 µl of Tricine-EDTA buffer. Both 5'- and 3'-RACE-Ready cDNA samples were aliquoted and stored at – 20 ^{o}C.

3.5. Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR), a method developed by Kary Mullis in the 1980s, is used to generate new copies of the target sequence exponentially by the activity of a DNA polymerase enzyme (50). Primer design is the first and the most crucial step in amplification of the amplicon specifically.

In the present study, different DNA polymerases have been used for amplification of different amplicon sizes. For target amplification less than 3 kilobase pairs (kbp), OneTaq DNA Polymerase (NEB) was used while Platinum SuperFi II DNA Polymerase (Thermo Fisher) was used to amplifiy longer amplicons, between 3-12 kbp. While performing the 5'-RACE and 3'-RACE PCR reactions from the 5'- and 3'-RACE-Ready cDNA samples to reveal the 5' and 3' ends of the gene, SeqAmp DNA Polymerase (Takara) was used.

3.5.1. Primer Design

Primers, short synthetic oligonucleotides, are used in PCR, sequencing reactions and hybridization studies as a probe.

There are some guidelines for the primer design to obtain optimal performance in PCR (51):

1) Primer length should be between 16 – 28 nucleotides and the length difference between primer pairs should not be more than 3 nucleotides.

2) GC content of a primer should be between 40 – 60 %.

3) 3’ end of primer should contain G/C bases instead of A/T to bind more tightly to the template. However, presence of more than 3 G/C nucleotides at last 5 bases of 3’end may lead to nonspecific priming of 3’-ends of primers.

4) Tm of the primers should be in the range of 50 – 64 ^{o}C. In addition, Tm values of primer pairs should not differ by more than 5°C as they should bind simultaneously.

5) Primer dimers (the annealing of two primers; cross-dimers and self-dimers) and hairpins (self-annealing) should be avoided.

6) Runs of 3 or more of one base, or dinucleotide repeats should be avoided.

If the target region is completely known, gene specific primers can be designed. However, if the target sequence is unknown or partly known, as in this study, the primers should be designed by analyzing the homology between related species. Degenerate primers can be designed in these situations. Degenerate primers include a set of alternative oligonucleotides to cover the ambiguous nucleotides of the homologous sequence.

According to manufacturer, gene specific primers (GSPs) used in RACE reaction have different criteria:

1) Their length should be between 23 and 28 nucleotides.

2) GC content should be between 50-70 %.

3) Their Tm values should be higher than 65 °C but for best result, Tm can be higher than 70 °C.

4) They should not be complementary to the 3’-end of the UPM and Short primer which are provided by the manufacturer.

3.5.2. PCR Procedures

In the present study, different PCR kits were used for amplification of different amplicon sizes depending on the experimental conditions. All steps of the procedures were carried out on ice.

OneTaq DNA Polymerase Kit (NEB) was used to amplify target sequences less than 3 kbp.

Table 3.8. Reaction mix for *OneTaq DNA Polymerase*.

	Volume (µl)
Nuclease-free dH2O	14.2
5x OneTaq Standard Reaction Buffer	5
dNTPs (10 mM each)	0.5
Forward Primer (10 µM)	2
Reverse Primer (10 µM)	2
Template	1
OneTaq DNA Polymerase (5 Units / µl)	0.3
Total volume	25

Table 3.9. Thermal Cycling protocol for *OneTaq DNA Polymerase.*

Steps		**Temperature**	**Duration**
Initial denaturation		95 °C	2 minutes
40 cycles	Denaturation	95 °C	20 seconds
	Annealing	55 – 65 °C	20 seconds
	Extension	68 °C	1 minute/kbp
Final extension		68 °C	5 minutes

Platinum SuperFi II green PCR master mix (Thermo Fisher) was used to amplify long amplicons up to 12 kbp. Platinum SuperFi II DNA Polymerase is a proofreading DNA polymerase with high fidelity and universal primer annealing. In addition, this mix is useful as the PCR products are directly loaded to agarose gel.

Table 3.10. Reaction mix for Platinum SuperFi II *DNA Polymerase*.

	Volume (µl)
Nuclease-free dH2O	19
2X Platinum SuperFi II PCR Master Mix	25
Forward Primer (10 µM)	2.5
Reverse Primer (10 µM)	2.5
Template	1
Total volume	50

Table 3.11. Thermal Cycling protocol for Platinum SuperFi II *DNA Polymerase.*

Steps		**Temperature**	**Duration**
Initial Denaturation		98 °C	30 seconds
30 cycles	Denaturation	98 °C	20 seconds
	Annealing	60 °C	10 seconds
	Extension	72 °C	1 minute/kbp
Final extension		72 °C	5 minutes
Hold		4 °C	

RACE (Rapid amplification of cDNA ends) reactions for both 5'- and 3'-ends of the gene was performed, SeqAmp DNA Polymerase (Takara) was used**.**

*SeqAmp PCR Master M*ix was prepared. The same master mix can be used for both 5'- and 3'-RACE reactions. The components were mixed by pipetting.

Table 3.12. *SeqAmp PCR Master M*ix for *SeqAmp DNA Polymerase*.

	Volume (µl)
PCR-Grade dH2O	15.5
2X SeqAmp Buffer	25
SeqAmp DNA Polymerase	1
Total volume	41.5

The reaction mix was prepared as in the order given in Table 3.13 and mixed gently.

Table 3.13. Reaction mix for 5'- and 3'-RACE reactions.

	Volume (µl)
5'-/3'-RACE-Ready cDNA	2.5
10X UPM	5
5'/3' Gene Specific *Primer*	1
***SeqAmp* PCR Master Mix**	41.5
Total volume	50

As the primers that were designed for RACE reactions have Tm between 60-70 ^{o}C, PCR program shown in Table 3.14 was used.

Table 3.14. Thermal Cycling protocol for *SeqAmp DNA Polymerase.*

Steps	**Temperature**	**Duration**
Initial Denaturation	94 ^{o}C	2 minutes
25 cycles	94 ^{o}C	30 seconds
	68 ^{o}C	30 seconds
	72 ^{o}C	1 minute/kbp
Hold	4 ^{o}C	

3.6. Agarose Gel Electrophoresis

Agarose gel electrophoresis, the easiest and most popular way of separation of DNA fragments differing in sizes. Separation of DNA molecules is based on force acting on the molecular charges in the electric field of the electrophoresis apparatus. To observe the success of each PCR experiment, the PCR products were loaded to the agarose gel.

1% w/v agarose gel was prepared by dissolving 1 gram of Agarose (Sigma A9539) in 100 ml of 1X TBE Buffer. The mixture was heated in a microwave oven to provide a complete dissolution of the agar in the buffer. Meanwhile, gel casting tray was prepared by sealing ends of gel chamber and placing the combs in it. While being let cool down for about 5 minutes at room temperature, the solution was randomly mixed. 3 µl of ethidium bromide (10 mg / ml, SNP Biyoteknoloji) was added to the molten agarose and poured into the prepared gel casting tray. It was allowed to solidify for about 30 minutes at room temperature. The solid agarose gel was placed in electrophoresis tank as submerged in 1X TBE buffer. After being mixed by 6X *Gel Loading* Dye (NEB), both a molecular weight marker (100 bp or 1kb DNA Ladders, NEB) and the PCR products were loaded into wells. The samples were run at 100V for about 45 minutes. The separated bands of PCR products were visualized under UV light by using AlphaImager EC (Protein Simple).

3.7. Purification of PCR Product and Gel Extraction.

After agarose gel electrophoresis, products with expected size were purified to continue downstream applications, i.e., sequencing.

To achieve this process, NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel), making use of silica membrane-based column purification method, was used. If the PCR product has an apparent single band, manufacturer's PCR clean-up kit procedure was followed to remove unincorporated primers, primer dimers, dNTPs and other components of PCR reaction mix from the product. However, if there are non-specific bands, the one with expected size was cut out of the agarose gel under

UV light by using AlphaImager EC (Protein Simple). While cutting the gel band, long-wavelength UV for as short time as possible was used to minimize the risk of DNA damage. Then, manufacturer's gel isolation kit was used. 15 µl of Elution Buffer was used to elute the products for both procedures. Finally, concentrations of the purified products were measured by Qubit dsDNA HS assay kit (Thermo Fisher).

3.8. Sequencing and Data Analysis.

PCR products up to 400bp were sequenced by Sanger sequencing method with BigDye Terminator Cycle Sequencing Kit (Thermo Fisher Scientific). After kit reaction, the samples were loaded to a capillary electrophoresis system (ABI 3130 Applied Biosystems). The sequence electropherogram was analyzed by SnapGene Wiever (Insightful Science; available at snapgene.com).

To sequence large and complex samples a next generation sequencing technology (NGS) was used. depending on the size of the sequence Illumina Miseq or Novaseq Platforms were employed. The samples were prepared for sequencing by fragmenting the sample to appropriate short read sizes and ligation of tags to the ends by using Nextera XT DNA Library Kit (Illumina).

The short-read data has been processed by both DNASTAR Software (SeqMan NGen®. Version 17.2. DNASTAR. Madison, WI, USA) and SPAdes Tool (St. Petersburg genome assembler) for a *de novo* assembly of the contigs and scaffolds.

The generated scaffold sequences were submitted into BLASTn platform (52) to identify the scaffolds related to RyR mRNA while conserved domains were predicted using the Conserved Domains Database (53).

The theoretical molecular weight was predicted using ExPASy Proteomics Server (54). Transmembrane segments were predicted using the TMHMM server 2.0 (55).

65 characterized vertebrates and invertebrates RyR homologue protein sequences used in the phylogenetic analysis were retrieved from GenBank databases (56). GenBank accession numbers of all sequences are listed in the Table 8.1. The

phylogenetic tree has been constructed in Matlab environment where distances were calculated by using Jukes-Cantor method.

4. RESULTS

Ryanodine receptor mRNA sequences of closely related species were examined as the genome and transcriptome of *Astacus leptodactylus* are unknown. In Table 4.1, some of ryanodine receptor mRNA sequences are listed according to proximity in the taxonomic classification, from superfamily (Astacoidea) to clade (Pancrustacea). Primers were designed by the help of these mRNA sequences and were tested on cDNA samples synthesized from crayfish muscle tissue.

Table 4.1. Ryanodine receptor mRNA sequences of closely related species used in homology studies.

ACCESSION	DESCRIPTION
JQ350826.1	*Procambarus clarkii* ryanodine receptor mRNA, partial cds
AF051936.1	*Homarus americanus* ryanodine receptor (RyR) mRNA, partial cds
HM367069.1	*Litopenaeus vannamei* ryanodine receptor gene, partial cds
NM_001321659.1	*Tribolium castaneum* ryanodine receptor (LOC655265), mRNA
NM_001309073.1	*Plutella xylostella* ryanodine receptor (Ryr), mRNA
KJ082086.1	*Bactrocera dorsalis* ryanodine receptor (RyR) mRNA, complete cds

Homarus americanus RyR partial mRNA (AF051936.1) was firstly considered for the primer design. However, this sequence is 4916 bp in length and contains mostly 3' part of the RyR gene (Figure 4.1).

By focusing onto the homologous regions between the selected RyR sequences, designed primers were expected to align on RIH associated domain and RR_TM4-6 region (Figure 4.2).

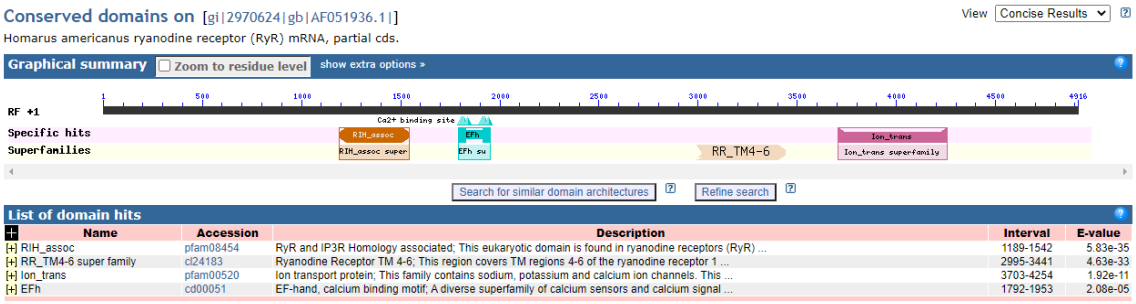


Figure 4.1. Conserved Domains Analysis of *Homarus americanus* ryanodine receptor (RyR) mRNA, partial cds (AF051936.1).

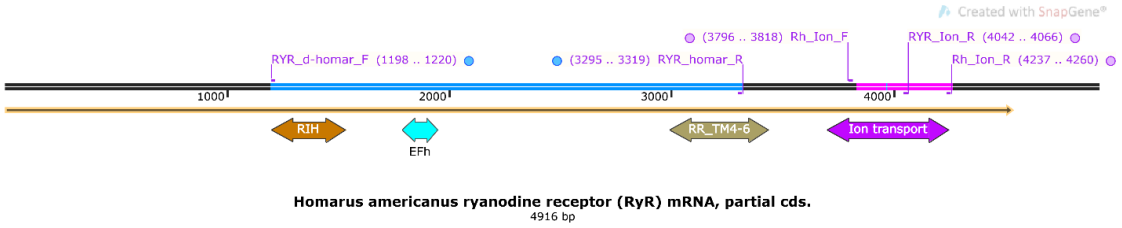


Figure 4.2. Conserved Domains Analysis of Homarus americanus ryanodine receptor (RyR) mRNA, partial cds (AF051936.1) and alignment of primers that were used in the experiments.

Table 4.2. Primers used in the experiment in which the first successful amplicon was amplified.

Primer Name	Sequence (5'->3')
RYR_d-homar_F	GAGTTCACTTGTGCGCTCTTCAG
RYR_homar_R	TCCATTCTTCAGCCTCTTCGTCCTC

First successful PCR product of crayfish RyR gene has been obtained by using primer pair shown in Table 4.2. The size of the product was approximately 1.5kb (Figure 4.3) although the expected size was about 2kb (Figure 4.2). The band, with expected size, was extracted from the gel and sequenced by Illumina Miseq Platform. The short read data was assembled by SPAdes algorithm into multiple scaffolds. The generated scaffold sequences were submitted into BLASTn platform to identify the scaffold(s) related to RyR mRNA.

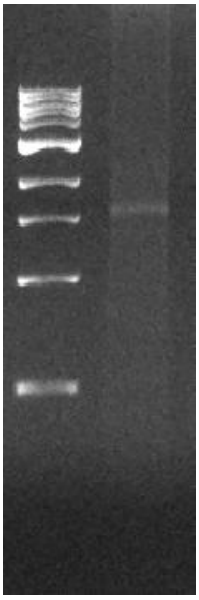


Figure 4.3. Gel photo of the first successful PCR product of crayfish RyR gene. Lane 1: 1kb DNA Ladder (NEB). Lane 2: product of RYR_d-homar_F and RYR_homar_R reaction. Distinct band is approximately 1.5kb.

PREDICTED: Homarus americanus ryanodine receptor-like (LOC121879297), mRNA	Homarus americanus	1471	1682	61%	0.0	84.94%	17771	XM_042385894.1
Homarus americanus ryanodine receptor (RyR) mRNA, partial cds	Homarus americanus	1471	1682	61%	0.0	84.94%	4916	AF051936.1
PREDICTED: Penaeus japonicus ryanodine receptor-like (LOC122266745), transcript variant X3, mRNA	Penaeus japonicus	1230	1230	54%	0.0	82.15%	16970	XM_043036607.1
PREDICTED: Penaeus monodon ryanodine receptor-like (LOC119592110), mRNA	Penaeus monodon	1177	1177	54%	0.0	81.41%	3329	XM_037940930.1
PREDICTED: Penaeus vannamei ryanodine receptor-like (LOC113815056), mRNA	Penaeus vannamei	1168	1168	54%	0.0	81.38%	17394	XM_027367149.1
PREDICTED: Penaeus japonicus ryanodine receptor-like (LOC122266745), transcript variant X8, mRNA	Penaeus japonicus	1158	1158	54%	0.0	81.26%	16898	XM_043036612.1
PREDICTED: Penaeus japonicus ryanodine receptor-like (LOC122266745), transcript variant X7, mRNA	Penaeus japonicus	1158	1158	54%	0.0	81.26%	16892	XM_043036611.1
PREDICTED: Penaeus japonicus ryanodine receptor-like (LOC122266745), transcript variant X6, mRNA	Penaeus japonicus	1158	1158	54%	0.0	81.26%	16889	XM_043036610.1
PREDICTED: Penaeus japonicus ryanodine receptor-like (LOC122266745), transcript variant X5, mRNA	Penaeus japonicus	1158	1158	54%	0.0	81.26%	16898	XM_043036609.1
PREDICTED: Penaeus japonicus ryanodine receptor-like (LOC122266745), transcript variant X4, mRNA	Penaeus japonicus	1158	1158	54%	0.0	81.26%	16964	XM_043036608.1
PREDICTED: Penaeus japonicus ryanodine receptor-like (LOC122266745), transcript variant X2, mRNA	Penaeus japonicus	1158	1158	54%	0.0	81.26%	16970	XM_043036606.1
PREDICTED: Penaeus japonicus ryanodine receptor-like (LOC122266745), transcript variant X1, mRNA	Penaeus japonicus	1158	1158	54%	0.0	81.26%	16970	XM_043036605.1
Procambarus clarkii ryanodine receptor mRNA, partial cds	Procambarus clarkii	1099	1099	29%	0.0	92.06%	801	JQ350826.1

Figure 4.4. Nucleotide BLAST results for the primary scaffold.

Similarity observed in sequence analysis indicated that a part of putative ryanodine receptor sequence, 1.6kb, has been revealed. When it was analyzed in BLASTn, an apparent similarity to the other known ryanodine receptors could be observed (Figure 4.4). Further, presence of *Conserved Domains*, both RIH associated domain and EFh motif, supported this idea (Figure 4.5). However, it should also contain RR_TM4-6 region (Figure 4.2) which has been explored by the following experiments.

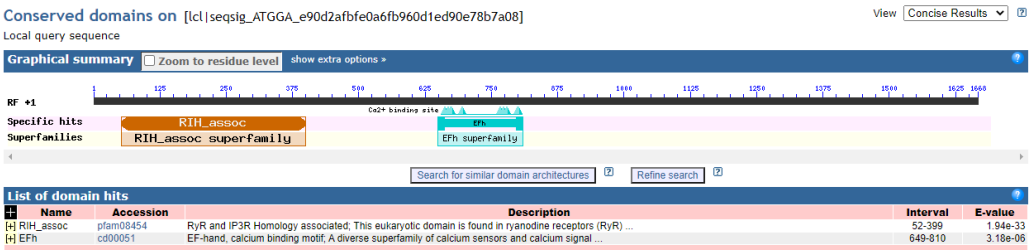


Figure 4.5. Graphic of Conserved Domains Analysis of the primary scaffold.

To determine the sequence of ion transport domain of crayfish RYR mRNA, primers listed in Table 4.3 were designed and, their annealing sites can be observed in Figure 4.2. Two PCR experiments were done by pairing Rh_Ion_F with both RYR_Ion_R and Rh_Ion_R. The expected amplicon sizes were 270bp and 460bp, respectively (Figure 4.6)

Table 4.3. Primers designed for amplification of ion transport domain sequence of crayfish RyR mRNA.

Primer Name	Sequence (5'->3')
Rh_Ion_F	TACTTGACCTTCTCTGTGCTGGG
RYR_Ion_R	GGAACACGAAGCACGTAAGCATGTC
Rh_Ion_R	TACAGACTCCAATTGATCTCTCAG

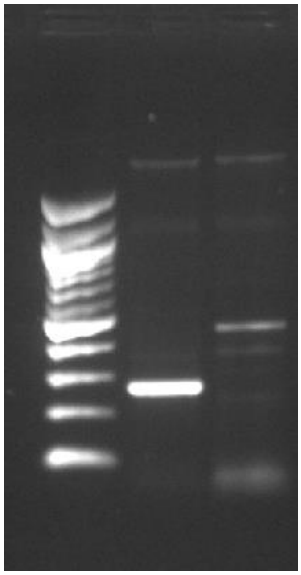


Figure 4.6. Gel photo of amplification of ion transport domain of crayfish RyR mRNA. Lane 1: 100bp DNA Ladder. Lane2: Product of Rh_Ion_F and RYR_Ion_R reaction. Distinct band is approximately 270 bp. Lane3: Product of Rh_Ion_F and Rh_Ion_R reaction. A band with approximate size of 460bp is visible.

Both of the obtained amplicons were Sanger sequenced and sequencing data was examined by SnapGene Wiever (Figure 4.7).

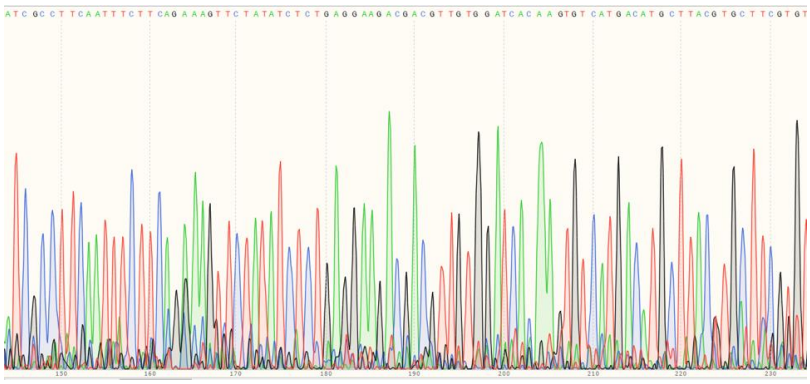


Figure 4.7. A sample of Sanger Sequencing Electropherogram of a RyR amplicon.

Sanger data obtained by forward and reverse primers has been used to improve fidelity of the sequence information.

The sequence was submitted into BLASTn (Figure 4.8) and Conserved Domains platforms (Figure 4.9). The apparent similarity to other RyR mRNAs and presence of estimated domains indicated that the sequence with ion transport domain sequence of crayfish RyR mRNA was revealed.

PREDICTED: Homarus americanus ryanodine receptor-like (LOC121879297), mRNA	Homarus americ...	488	488	100%	2e-133	87.50%	17771	XM_042385894.1
Homarus americanus ryanodine receptor (RyR) mRNA, partial cds	Homarus americ...	488	488	100%	2e-133	87.50%	4916	AF051936.1
PREDICTED: Penaeus japonicus ryanodine receptor-like (LOC122266745), transcript variant X8, mRNA	Penaeus japonic...	379	379	98%	1e-100	83.05%	16898	XM_043036612.1
PREDICTED: Penaeus japonicus ryanodine receptor-like (LOC122266745), transcript variant X7, mRNA	Penaeus japonic...	379	379	98%	1e-100	83.05%	16892	XM_043036611.1
PREDICTED: Penaeus japonicus ryanodine receptor-like (LOC122266745), transcript variant X6, mRNA	Penaeus japonic...	379	379	98%	1e-100	83.05%	16889	XM_043036610.1
PREDICTED: Penaeus japonicus ryanodine receptor-like (LOC122266745), transcript variant X5, mRNA	Penaeus japonic...	379	379	98%	1e-100	83.05%	16898	XM_043036609.1
PREDICTED: Penaeus japonicus ryanodine receptor-like (LOC122266745), transcript variant X4, mRNA	Penaeus japonic...	379	379	98%	1e-100	83.05%	16964	XM_043036608.1
PREDICTED: Penaeus japonicus ryanodine receptor-like (LOC122266745), transcript variant X3, mRNA	Penaeus japonic...	379	379	98%	1e-100	83.05%	16970	XM_043036607.1
PREDICTED: Penaeus japonicus ryanodine receptor-like (LOC122266745), transcript variant X2, mRNA	Penaeus japonic...	379	379	98%	1e-100	83.05%	16970	XM_043036606.1
PREDICTED: Penaeus japonicus ryanodine receptor-like (LOC122266745), transcript variant X1, mRNA	Penaeus japonic...	379	379	98%	1e-100	83.05%	16970	XM_043036605.1
PREDICTED: Penaeus vannamei ryanodine receptor-like (LOC113815056), mRNA	Penaeus vanna...	374	374	98%	5e-99	82.82%	17394	XM_027367149.1
PREDICTED: Penaeus monodon ryanodine receptor-like (LOC119592110), mRNA	Penaeus monodon	292	292	98%	2e-74	79.95%	3329	XM_037940930.1

Figure 4.8. Nucleotide BLAST results for the sequence contains ion transport domain of crayfish RyR mRNA.

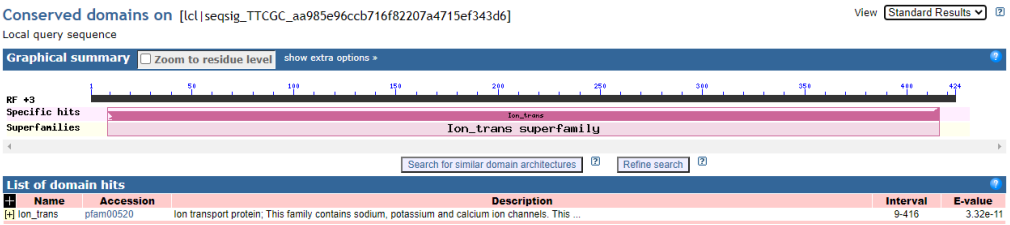


Figure 4.9. Graphic of Conserved Domains Analysis of the sequence contains ion transport domain of crayfish RyR mRNA.

To fill the gap between two identified fragments of the putative crayfish RyR mRNA, a PCR experiment was done by using the primers shown in Table 4.4. The expected PCR product size was about 2.8kb and a single band was observed on the gel photo (Figure 4.10).

Table 4.4. Primers used to fill the gap between two obtained sequences of crayfish RyR mRNA.

Amplicon name	Primer Name	Sequence (5'->3')
RYR 5	RYR_d-homar_F	GAGTTCACTTGTGCGCTCTTCAG
	RYR_Ion_R	GGAACACGAAGCACGTAAGCATGTC

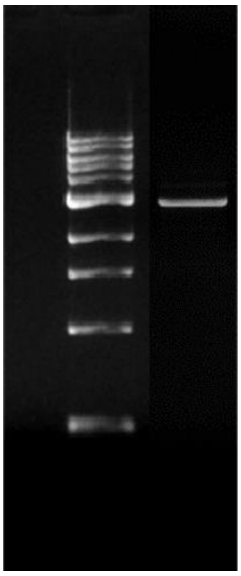


Figure 4.10. Gel photo of the PCR product of RYR5 amplicon. Lane 1: 1kb DNA Ladder (NEB). Lane2: Product of RYR_d-homar_F and RYR_Ion_R reaction. Distinct band is approximately 2.8 kb.

The sequencing data of the amplified sequence was assembled by SPAdes algorithm, and the resulted scaffold sequences were analyzed in BLASTn.

According to assembly results, the 3' partial sequence of putative crayfish RyR mRNA with a size of 2850bp, was revealed. It was also analyzed in both BLASTn (Figure 4.11) and Conserved Domains (Figure 4.12) Algorithms.

Homarus americanus ryanodine receptor (RyR) mRNA, partial cds	Homarus americanus	2667	2667	99%	0.0	83.60%	4916	AF051936.1
PREDICTED: Penaeus japonicus ryanodine receptor-like (LOC122266745), transcript variant X3, mRNA	Penaeus japonicus	2002	2002	99%	0.0	79.47%	16970	XM_043036607.1
PREDICTED: Penaeus japonicus ryanodine receptor-like (LOC122266745), transcript variant X8, mRNA	Penaeus japonicus	1930	1930	99%	0.0	79.02%	16898	XM_043036612.1
PREDICTED: Penaeus japonicus ryanodine receptor-like (LOC122266745), transcript variant X7, mRNA	Penaeus japonicus	1930	1930	99%	0.0	79.02%	16892	XM_043036611.1
PREDICTED: Penaeus japonicus ryanodine receptor-like (LOC122266745), transcript variant X5, mRNA	Penaeus japonicus	1930	1930	99%	0.0	79.02%	16898	XM_043036609.1
PREDICTED: Penaeus japonicus ryanodine receptor-like (LOC122266745), transcript variant X4, mRNA	Penaeus japonicus	1930	1930	99%	0.0	79.02%	16964	XM_043036608.1
PREDICTED: Penaeus japonicus ryanodine receptor-like (LOC122266745), transcript variant X2, mRNA	Penaeus japonicus	1930	1930	99%	0.0	79.02%	16970	XM_043036606.1
PREDICTED: Penaeus japonicus ryanodine receptor-like (LOC122266745), transcript variant X1, mRNA	Penaeus japonicus	1930	1930	99%	0.0	79.02%	16970	XM_043036605.1
PREDICTED: Penaeus vannamei ryanodine receptor-like (LOC113815056), mRNA	Penaeus vannamei	1882	1882	99%	0.0	78.75%	17394	XM_027367149.1
PREDICTED: Homarus americanus ryanodine receptor-like (LOC121879297), mRNA	Homarus americanus	1578	1578	59%	0.0	83.61%	17771	XM_042385894.1
PREDICTED: Penaeus monodon ryanodine receptor-like (LOC119592110), mRNA	Penaeus monodon	1184	1184	51%	0.0	81.19%	3329	XM_037940930.1
PREDICTED: Penaeus japonicus ryanodine receptor-like (LOC122266745), transcript variant X6, mRNA	Penaeus japonicus	1179	1179	52%	0.0	80.86%	16889	XM_043036610.1
Procambarus clarkii ryanodine receptor mRNA, partial cds	Procambarus clarkii	1099	1099	27%	0.0	92.06%	801	JQ350826.1

Figure 4.11. BLASTn results for RYR5 amplicon sequence of putative crayfish RyR mRNA.

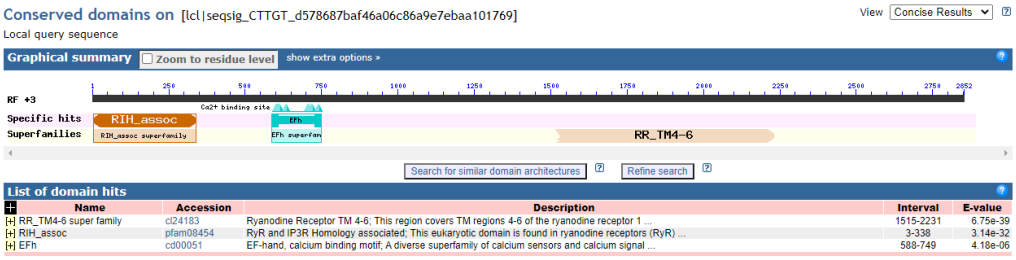


Figure 4.12. Graphic of Conserved Domains Analysis of RYR5 Amplicon sequence of crayfish RyR mRNA.

By using conventional cloning methods, it would be extremely difficult to reveal such a long sequence, estimated as 15 kb in size. We have used a recent data for *H. americanus* RyR sequence as a template and aligned ready to use 100M of short reads constructed for muscle total RNA sample. *De novo* and ref-based combined modality of DNAStar platform has been used. The analysis ended the top with 388 contigs and 12 scaffolds. 9 of the scaffolds successively aligned along the *H. americanus* RyR sequence. Details of the alignment of each of the scaffolds have been given in the Figure 4.13. Thus, calculated scaffolds revealed majority of the crayfish RYR mRNA sequence. Those segments have been further analyzed and used for design and synthesis of primer pairs.

The length of the estimated target mRNA was one of the major challenges in the present study. A complete amplification PCR was beyond the capacity of the SuperFi II DNA Polymerase, which is the best option for long amplicons. Thus, we have segmented the target sequence into 5 overlapping parts (Figure 4.14) and designed specific primer pairs for each one of them (Table 4.1).

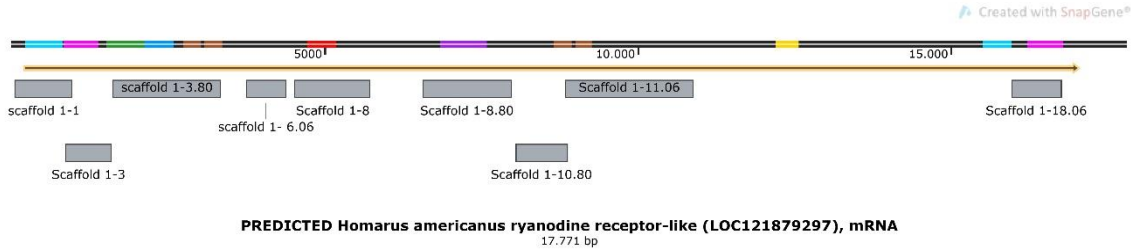


Figure 4.13. Alignment of obtained scaffolds with the predicted *Homarus americanus* ryanodine receptor-like mRNA (XM_042385894.1).

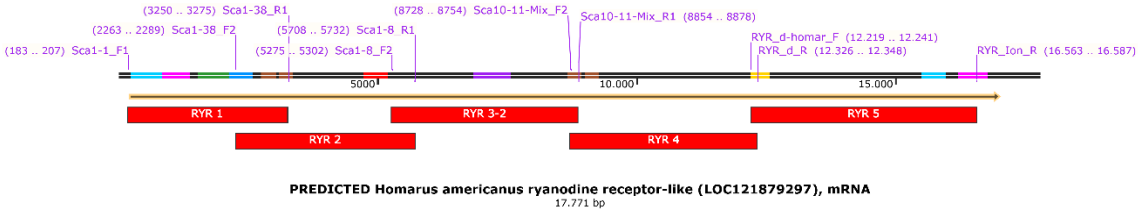


Figure 4.14. Arrangement of overlapping regions on the reference sequence and annealing sites of the primers.

Table 4.5. Primers designed for amplification of 5’ part of crayfish RyR mRNA.

Amplicon Name & Expected size	Primer Name	Sequence (5’->3’)
RYR 1 (3 kb)	Sca1-1_F1	CAGTGTGAATCAAGCGTCATTATGG
	Sca1-38_R1	GTTGCCGTAGCTGACGAGGTGAGGTG
RYR 2 (3.4 kb)	Sca1-38_F2	CCAATACCAAGGGCTACGTTAGCTACC
	Sca1-8_R1	TTCCTCTGCATAGATCTCCTTCAGC
RYR 3 (3.6 kb)	Sca1-8_F2	TCCAGATACTTAAGCCTTACCAGTGGTC
	Sca10-11-Mix_R1	CTGTATCGCTCCCGCTCATAGTCAG
RYR 4 (3.6 kb)	Sca10-11-Mix_F2	AGAAGTTCAGTGAGCATTACCACGACG
	RYR_d_R	AGTCCACGGTGCAGTTGATGACG

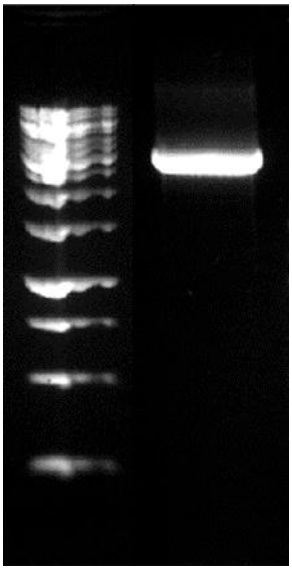


Figure 4.15. Gel photo of amplification of RYR 1 amplicon. Lane 1: 1kb DNA Ladder (Thermo Fisher). Lane2: Product of Sca1-1_F1 and Sca1-38_R1 reaction. Distinct band is approximately 3 kb.

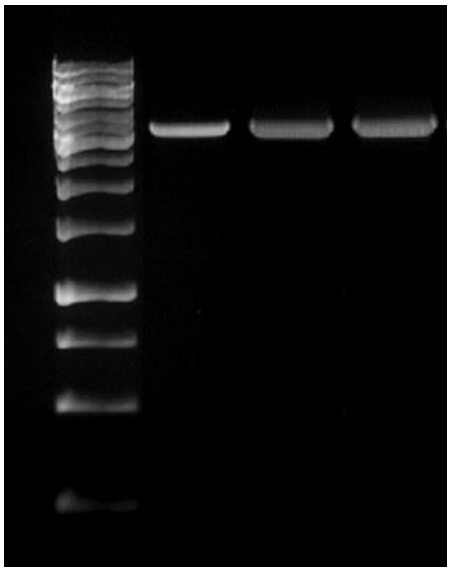
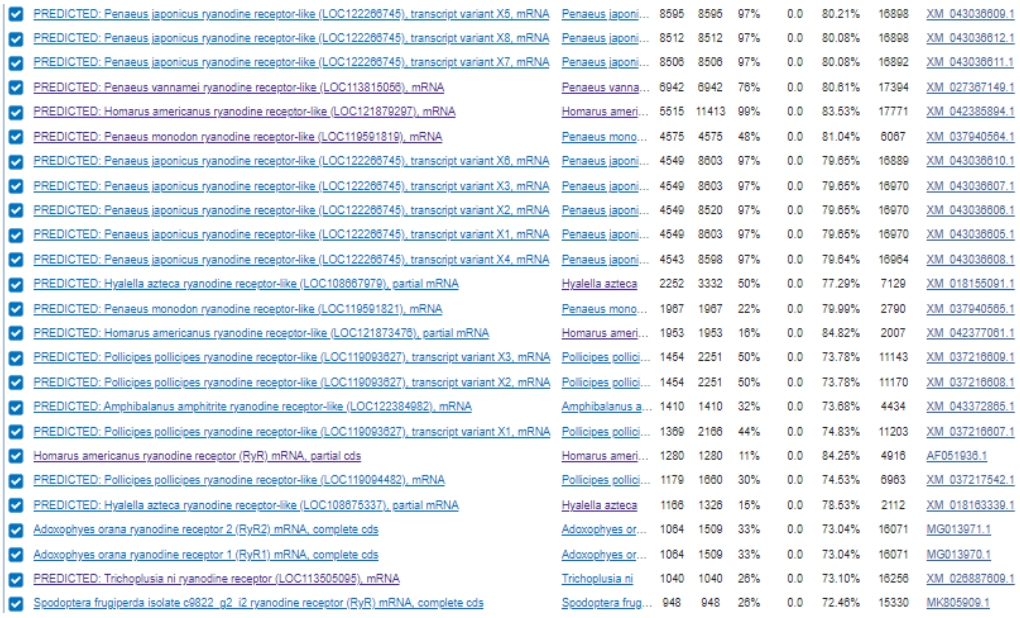
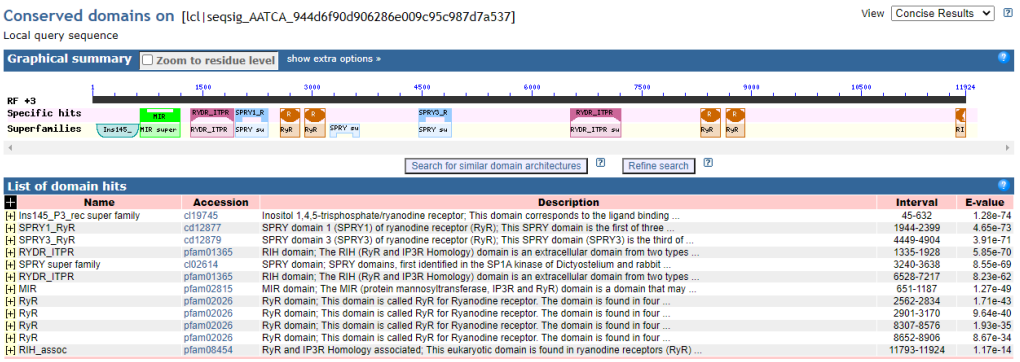


Figure 4.16. Gel photo of amplification of RYR 2, 3 and 4 amplicons. Lane 1: 1kb DNA Ladder (Thermo Fisher). Left to right are amplicons of RYR 2, 3 and 4, respectively. Bands are approximately 3.4 kb.



PREDICTED: Penaeus japonicus ryanodine receptor-like (LOC122266745), transcript variant X5, mRNA	Penaeus japoni...	8595	8595	97%	0.0	80.21%	16898	XM_043036609.1
PREDICTED: Penaeus japonicus ryanodine receptor-like (LOC122266745), transcript variant X8, mRNA	Penaeus japoni...	8512	8512	97%	0.0	80.08%	16898	XM_043036612.1
PREDICTED: Penaeus japonicus ryanodine receptor-like (LOC122266745), transcript variant X7, mRNA	Penaeus japoni...	8506	8506	97%	0.0	80.08%	16892	XM_043036611.1
PREDICTED: Penaeus vannamei ryanodine receptor-like (LOC113815056), mRNA	Penaeus vanna...	6942	6942	76%	0.0	80.61%	17394	XM_027367149.1
PREDICTED: Homarus americanus ryanodine receptor-like (LOC121879297), mRNA	Homarus ameri...	5515	11413	99%	0.0	83.53%	17771	XM_042386894.1
PREDICTED: Penaeus monodon ryanodine receptor-like (LOC119591819), mRNA	Penaeus mono...	4575	4575	48%	0.0	81.04%	6067	XM_037940564.1
PREDICTED: Penaeus japonicus ryanodine receptor-like (LOC122266745), transcript variant X6, mRNA	Penaeus japoni...	4549	8603	97%	0.0	79.65%	16889	XM_043036610.1
PREDICTED: Penaeus japonicus ryanodine receptor-like (LOC122266745), transcript variant X3, mRNA	Penaeus japoni...	4549	8603	97%	0.0	79.65%	16970	XM_043036607.1
PREDICTED: Penaeus japonicus ryanodine receptor-like (LOC122266745), transcript variant X2, mRNA	Penaeus japoni...	4549	8520	97%	0.0	79.65%	16970	XM_043036606.1
PREDICTED: Penaeus japonicus ryanodine receptor-like (LOC122266745), transcript variant X1, mRNA	Penaeus japoni...	4549	8603	97%	0.0	79.65%	16970	XM_043036605.1
PREDICTED: Penaeus japonicus ryanodine receptor-like (LOC122266745), transcript variant X4, mRNA	Penaeus japoni...	4543	8598	97%	0.0	79.64%	16964	XM_043036608.1
PREDICTED: Hyalella azteca ryanodine receptor-like (LOC108667979), partial mRNA	Hyalella azteca	2252	3332	50%	0.0	77.29%	7129	XM_018155091.1
PREDICTED: Penaeus monodon ryanodine receptor-like (LOC119591821), mRNA	Penaeus mono...	1967	1967	22%	0.0	79.99%	2790	XM_037940565.1
PREDICTED: Homarus americanus ryanodine receptor-like (LOC121873476), partial mRNA	Homarus ameri...	1953	1953	16%	0.0	84.82%	2007	XM_042377061.1
PREDICTED: Pollicipes pollicipes ryanodine receptor-like (LOC119093627), transcript variant X3, mRNA	Pollicipes pollici...	1454	2251	50%	0.0	73.78%	11143	XM_037216609.1
PREDICTED: Pollicipes pollicipes ryanodine receptor-like (LOC119093627), transcript variant X2, mRNA	Pollicipes pollici...	1454	2251	50%	0.0	73.78%	11170	XM_037216608.1
PREDICTED: Amphibalanus amphitrite ryanodine receptor-like (LOC122384982), mRNA	Amphibalanus a...	1410	1410	32%	0.0	73.68%	4434	XM_043372865.1
PREDICTED: Pollicipes pollicipes ryanodine receptor-like (LOC119093627), transcript variant X1, mRNA	Pollicipes pollici...	1369	2166	44%	0.0	74.83%	11203	XM_037216607.1
Homarus americanus ryanodine receptor (RyR) mRNA, partial cds	Homarus ameri...	1280	1280	11%	0.0	84.25%	4916	AF051936.1
PREDICTED: Pollicipes pollicipes ryanodine receptor-like (LOC119094482), mRNA	Pollicipes pollici...	1179	1660	30%	0.0	74.53%	6963	XM_037217542.1
PREDICTED: Hyalella azteca ryanodine receptor-like (LOC108675337), partial mRNA	Hyalella azteca	1166	1326	15%	0.0	78.53%	2112	XM_018163339.1
Adoxophyes orana ryanodine receptor 2 (RyR2) mRNA, complete cds	Adoxophyes or...	1064	1509	33%	0.0	73.04%	16071	MG013971.1
Adoxophyes orana ryanodine receptor 1 (RyR1) mRNA, complete cds	Adoxophyes or...	1064	1509	33%	0.0	73.04%	16071	MG013970.1
PREDICTED: Trichoplusia ni ryanodine receptor (LOC113505095), mRNA	Trichoplusia ni	1040	1040	26%	0.0	73.10%	16256	XM_026887609.1
Spodoptera frugiperda isolate c9822_g2_i2 ryanodine receptor (RyR) mRNA, complete cds	Spodoptera frug...	948	948	26%	0.0	72.46%	15330	MK805909.1

Figure 4.17. BLASTn results for the scaffold sequence obtained from RYR 1,2,3 and 4 amplicons.



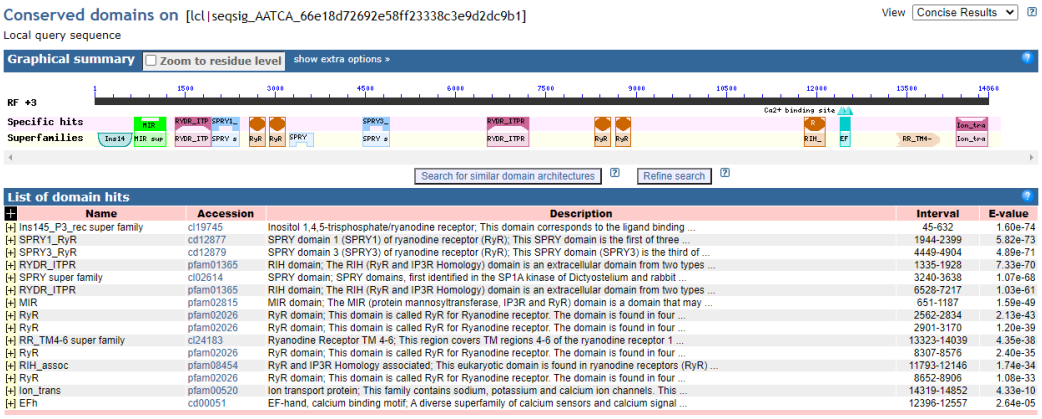
Name	Accession	Description	Interval	E-value
Ins145_P3_rec super family	cl19745	Inositol 1,4,5-trisphosphate/ryanodine receptor; This domain corresponds to the ligand binding ...	45-632	1.28e-74
SPRY1_RyR	cd12877	SPRY domain 1 (SPRY1) of ryanodine receptor (RyR); This SPRY domain is the first of three ...	1944-2399	4.65e-73
SPRY3_RyR	cd12879	SPRY domain 3 (SPRY3) of ryanodine receptor (RyR); This SPRY domain (SPRY3) is the third of ...	4449-4904	3.91e-71
RYDR_ITPR	pfam01365	RIH domain; The RIH (RyR and IP3R Homology) domain is an extracellular domain from two types ...	1335-1928	5.85e-70
SPRY super family	cl02514	SPRY domain; SPRY domains, first identified in the SP1A kinase of Dictyostelium and rabbit ...	3240-3638	8.55e-69
RYDR_ITPR	pfam01365	RIH domain; The RIH (RyR and IP3R Homology) domain is an extracellular domain from two types ...	6528-7217	8.23e-62
MIR	pfam02815	MIR domain; The MIR (protein mannosyltransferase, IP3R and RyR) domain is a domain that may ...	651-1187	1.27e-49
RyR	pfam02026	RyR domain; This domain is called RyR for Ryanodine receptor. The domain is found in four ...	2562-2834	1.71e-43
RyR	pfam02026	RyR domain; This domain is called RyR for Ryanodine receptor. The domain is found in four ...	2901-3170	9.64e-40
RyR	pfam02026	RyR domain; This domain is called RyR for Ryanodine receptor. The domain is found in four ...	8307-8576	1.93e-35
RyR	pfam02026	RyR domain; This domain is called RyR for Ryanodine receptor. The domain is found in four ...	8652-8906	8.67e-34
RIH_assoc	pfam08454	RyR and IP3R Homology associated; This eukaryotic domain is found in ryanodine receptors (RyR) ...	11793-11924	1.17e-14

Figure 4.18. Graphic of Conserved Domains Analysis of the scaffold sequence obtained from RYR 1,2,3 and 4 amplicons.

All of the fragment sequences were assembled and a single continuous sequence, 14859 bp long, was obtained. As a result of this assembly, a sequence, containing RYR 1,2,3,4,5 amplicons and the part with ion transfer domain, has successfully been revealed.

PREDICTED: Penaeus japonicus ryanodine receptor-like (LOC122266745), transcript variant X5, mRNA	Penaeus japonicus	10587	10587	97%	0.0	79.98%	16898	XM_043036609.1
PREDICTED: Penaeus japonicus ryanodine receptor-like (LOC122266745), transcript variant X8, mRNA	Penaeus japonicus	10504	10504	97%	0.0	79.88%	16898	XM_043036612.1
PREDICTED: Penaeus japonicus ryanodine receptor-like (LOC122266745), transcript variant X7, mRNA	Penaeus japonicus	10499	10499	97%	0.0	79.88%	16892	XM_043036611.1
PREDICTED: Penaeus vannamei ryanodine receptor-like (LOC113815056), mRNA	Penaeus vannamei	6942	9963	90%	0.0	80.61%	17394	XM_027367149.1
PREDICTED: Penaeus japonicus ryanodine receptor-like (LOC122266745), transcript variant X3, mRNA	Penaeus japonicus	6613	10668	97%	0.0	79.60%	16970	XM_043036607.1
PREDICTED: Penaeus japonicus ryanodine receptor-like (LOC122266745), transcript variant X2, mRNA	Penaeus japonicus	6541	10513	97%	0.0	79.47%	16970	XM_043036606.1
PREDICTED: Penaeus japonicus ryanodine receptor-like (LOC122266745), transcript variant X1, mRNA	Penaeus japonicus	6541	10596	97%	0.0	79.47%	16970	XM_043036605.1
PREDICTED: Penaeus japonicus ryanodine receptor-like (LOC122266745), transcript variant X4, mRNA	Penaeus japonicus	6536	10590	97%	0.0	79.46%	16964	XM_043036608.1
PREDICTED: Penaeus japonicus ryanodine receptor-like (LOC122266745), transcript variant X6, mRNA	Penaeus japonicus	5594	10625	95%	0.0	79.77%	16889	XM_043036610.1
PREDICTED: Homarus americanus ryanodine receptor-like (LOC121879297), mRNA	Homarus americanus	5515	14082	99%	0.0	83.53%	17771	XM_042385894.1
PREDICTED: Penaeus monodon ryanodine receptor-like (LOC119591819), mRNA	Penaeus monodon	4575	4575	38%	0.0	81.04%	6067	XM_037940564.1
Homarus americanus ryanodine receptor (RyR) mRNA, partial cds	Homarus americanus	4004	4004	28%	0.0	83.69%	4916	AF051936.1
PREDICTED: Hyalella azteca ryanodine receptor-like (LOC108667979), partial mRNA	Hyalella azteca	2252	3332	40%	0.0	77.29%	7129	XM_018155091.1
PREDICTED: Penaeus monodon ryanodine receptor-like (LOC119591821), mRNA	Penaeus monodon	1967	1967	18%	0.0	79.99%	2790	XM_037940565.1
PREDICTED: Homarus americanus ryanodine receptor-like (LOC121873476), partial mRNA	Homarus americanus	1953	1953	13%	0.0	84.82%	2007	XM_042377061.1
PREDICTED: Pollicipes pollicipes ryanodine receptor-like (LOC119093627), transcript variant X3, mRNA	Pollicipes pollicipes	1454	2251	40%	0.0	73.78%	11143	XM_037216609.1
PREDICTED: Pollicipes pollicipes ryanodine receptor-like (LOC119093627), transcript variant X2, mRNA	Pollicipes pollicipes	1454	2251	40%	0.0	73.78%	11170	XM_037216608.1
PREDICTED: Amphibalanus amphitrite ryanodine receptor-like (LOC122384982), mRNA	Amphibalanus amp...	1410	1410	26%	0.0	73.68%	4434	XM_043372865.1
PREDICTED: Pollicipes pollicipes ryanodine receptor-like (LOC119093627), transcript variant X1, mRNA	Pollicipes pollicipes	1369	2166	35%	0.0	74.83%	11203	XM_037216607.1
PREDICTED: Penaeus monodon ryanodine receptor-like (LOC119592110), mRNA	Penaeus monodon	1216	2087	18%	0.0	81.35%	3329	XM_037940930.1

Figure 4.19. BLASTn results for the assembled sequence for crayfish ryanodine mRNA.



Name	Accession	Description	Interval	E-value
[+] Ins145_P3_rec super family	cl19745	Inositol 1,4,5-trisphosphate/ryanodine receptor; This domain corresponds to the ligand binding ...	45-632	1.60e-74
[+] SPRY1_RyR	cd12877	SPRY domain 1 (SPRY1) of ryanodine receptor (RyR); This SPRY domain is the first of three ...	1944-2399	5.82e-73
[+] SPRY3_RyR	cd12879	SPRY domain 3 (SPRY3) of ryanodine receptor (RyR); This SPRY domain (SPRY3) is the third of ...	4449-4904	4.89e-71
[+] RYDR_ITPR	pfam01365	RIH domain; The RIH (RyR and IP3R Homology) domain is an extracellular domain from two types ...	1335-1928	7.33e-70
[+] SPRY super family	cl02614	SPRY domain; SPRY domains, first identified in the SP1A kinase of Dictyostelium and rabbit ...	3240-3638	1.07e-68
[+] RYDR_ITPR	pfam01365	RIH domain; The RIH (RyR and IP3R Homology) domain is an extracellular domain from two types ...	6528-7217	1.03e-61
[+] MIR	pfam02815	MIR domain; The MIR (protein mannosyltransferase, IP3R and RyR) domain is a domain that may ...	651-1187	1.59e-49
[+] RyR	pfam02026	RyR domain; This domain is called RyR for Ryanodine receptor. The domain is found in four ...	2562-2834	2.13e-43
[+] RyR	pfam02026	RyR domain; This domain is called RyR for Ryanodine receptor. The domain is found in four ...	2901-3170	1.20e-39
[+] RR_TM4-6 super family	cl24183	Ryanodine Receptor TM 4-6; This region covers TM regions 4-6 of the ryanodine receptor 1 ...	13323-14039	4.35e-38
[+] RyR	pfam02026	RyR domain; This domain is called RyR for Ryanodine receptor. The domain is found in four ...	8307-8576	2.40e-35
[+] RIH_assoc	pfam08454	RyR and IP3R Homology associated; This eukaryotic domain is found in ryanodine receptors (RyR) ...	11793-12146	1.74e-34
[+] RyR	pfam02026	RyR domain; This domain is called RyR for Ryanodine receptor. The domain is found in four ...	8652-8906	1.08e-33
[+] Ion_trans	pfam00520	Ion transport protein; This family contains sodium, potassium and calcium ion channels. This ...	14319-14852	4.33e-10
[+] EFh	cd00051	EF-hand, calcium binding motif; A diverse superfamily of calcium sensors and calcium signal ...	12396-12557	2.64e-05

Figure 4.20. Graphic of Conserved Domains Analysis of the assembled sequence for crayfish ryanodine mRNA.

By using the conventional cloning method, a huge part of the crayfish RyR mRNA has been revealed. However, the attempts failed to expose 3' end of the target mRNA. Abundant number of short reads, originally developed from muscle cDNA library, has been aligned to a 1 kb fragment of the 3' end cloned sequence. Iterative calculations efficiently extended the end of the sequence to cover the stop codon with an extensive level of coverage. The calculated sequence extension was quality controlled. The extension sequence gave a full translation with a distinct stop codon and almost an absolute similarity to the other sequences from neighboring species. A similar approach has been fallowed for extending the UTR region in the 5' end. The final sequence (Figure 4.21), been used as a reference, and the abundant number of short reads, originally developed from muscle cDNA library, have been aligned in

reference-based mode. Observation of a high coverage in the alignment confirmed the fidelity of the extension calculations.

TATCACCGTGGTATGGACTTCTTATCAGTGTAAGAGTGTTTTTTCATATTTGATCAGTGTG
AATCAAGCGTCATTATGGCGGACAGTGAAGGGTCCTCGGAGCAAGATGATGTCTCCTTC
CTCCGGACGGAGGACATGGTGTGCCTCTCCAGCACCGCCGTCGGCGAGAGGGTCTGCC
TGGCAGCTGAAGGCTTCGGGAACCGTACCTGCTTCCTGGAGAACATCGCTGATAAGAAC
AACCCTCCGGACCTATGCCAGTGCGTGTTCGTAATAGAACAGGCGCTGTCGGTGCGAGC
CCTGCAGGAGCTGGTCACCGCCGCCGCTAGCGAAGAGGGTAAAGGTACGGGTTCAGGC
CACCGCACTCTCCTCTACGGCAACGCTGTCCTCCTCCGACACATGAACTCAATGATGTGC
CTAGCTTGCCTTTCAACAAGTTCCTCCAGAGACAAACTGGCCTTCGATGTCGGCCTTCAG
GAGCACACCAAAGGAGAGTCGTGCTGGTGGACCATCCACCCCGCCAACAAACAGAGAT
CTGAAGGCGAGAAGGTTCGTGTCGGTGACGACCTCATCCTGGTCTCCGTGGCTACAGAA
CGCTACTTGCAAGCGACCCGCGAGGACGAGCAGAGCATCGTCAACGCTTCCTTCCACGT
CACCCACTGGTCCGTCAGCCCCTTCGGTACCGGTCTCTCTAGGCTCAAGTTTGTGGCTTG
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CCTCCACCTGGTCGGAGCAGCAAGGCCAGAACATCGTGGTGTATGAGGGCGGGTCGGT
GACGTCACAGGCAAGGTCGCTGTGGCGTCTTGAGTTAGCACGGACGAAGTGGGCGGGC
GGCTACATCAACTGGTTCCACCCCATGCGCATCCGACACATCACCACCGGCAGATACCTC
GGAATCAACGAACAAAACGAACTCGTTCTCTTGCACAGAGACGAGGCAACGATGGCGG
CGACCGCGTTTTACCTGAGGGAGGAAAAGGACGACAACAAAGTGCTGCTCGAGGACAA
GGACCTGGAGGTGATCGGTACTCCGCTCATCAAATACGGCGACTCCACCGTCATCGTCC
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CGGTCTTGTCTTCTACAGGAGTCAAGAGGAAGAGTCCCGCACTGGTCGGGTCATCCGCA
AGTGCTCACACCTCTTCAACAGCTTCATCAAAGGACTGGACCACATTCAGACCTCCCGAA
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CTCATCAATTATTTCGCCTACCCCGCTGATGACTTAGAACACAACGAAAGGCAGTTCTCG
CTACGTGCTCTGAGGAACCGTCAAGACCTCTTCCAAGAGGAAGGGATTCTCAACCTGAT
CCTGGACGCCATCGACAAGATCACAGTCATCACCCAGCAGGGGTACCTGGTGGCTCTCG
CTGGAGAGGAGGCCGGACTCGACTGGGATATCATCTCAGGATACCTTTACCAGCTGCTG
GCCGCCGTCATCAAGGGCAACCACACCAACTGCTCGCAGTTCGCCAACAGCCACCGCCT
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AAGGTGATTATCGCGCTGCTGGAGAAGTACGGCCGTGACCCCAAGGTCCTGGACGTAC
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AGCGCACGACCCAACATCTTCGTGGGCTTCGTGGAAGGCTCAGCCATCTACCAGAAGTG
GTACTACGAGGTGACTCTCGACCACATCGAGCAGATATCACACCTGTCTCCACACTTGCG

TCTGGGATGGGCCAATACCAAGGGCTACGTTAGCTACCCTGGAGGTGGTGAGAAGTGG
GGTGGCAATGGCGTCGGTGACGACCTCTACTCTTACGGATTCGACGGTTCCTACTTGTG
GACGGGCGGGAGATATTCCCTGGTCAACCCTATTGATTCGGAACCGCTGATCAAGAAAG
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GTGTCCGCATCTGCCAAACTCAGCGCGAGGTTCTTGCTGGGAGGCGAACACGGACGTCT
CAAGTACCCACCGCCCGACGCCCACTCCCCCGTCTCTGAATGTCTTCTACCCTTCCAGACC
CTGACCATCGACCCGTGCTTCTACTTCGGGGAGCAGCACAAGGGGACGCTGGCGGGGC
CGCTCCTCATCCAGGACGACCTGGTGTTCGTTCCCAAGCCTGTCGACACCTCAGCCATCC
AGCTGCCGGGGTACATCGAGCAGGTTCGAGACAAGCTGGCCGAGAACATCCACGAGAT
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ATGGACAAGCCTCCCAGCCGTATCCGCACCGTCAGGCTACCCAACGATCCCTTCCTGCAG
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GAAGAGCTGGTGGACCAGCTGGCGGAGAACACCCACAATATCTGGGCCCGGGAGCGT
ATCCTACAAGGCTGGACATACGGCCTCAACGAGGACCCGGACACGCATCGCTCACCTCA
CCTCGTCAGCTATGGAAACGTTGACGAAGCCATCAAAAAGGCCAACCGAGACACAGCA
TCCGAGACCATTCGGACTCTTCTTGTTTATGGCTACATCCTGGAGGCTCCCACGGGTGAC
CAGGCTGAAGCGGCGGCCGCTGCCATAGAGAGCACCAAGCGAGGCACCGTCCACCGCA
CCTACCGCCTGGAGAACACCAACGCCGTCACTTCTGGCAAGTGGTACTTTGAGATGGAA
GTGCTGACCTCTGGACCGATGCGTGTAGGCTGGATAGAGACGAGCAGTCCGCCCGGGA
CGGAGCTCGGCTGCGACGACAAGTCTTGGGCTTTCGACGGCTTCCGTCATATCAAGCAC
CATATGGGAGGGTCGGAGCCTTACGGACGACGCGCCCAGCCGGGCGACATTGTGGGG
GTAATGATGGACCTGCATGACAAGACCATCAGCTTCTCGCTCAACGGGGAGTTGATGAT
GGATGCCAGTGGGTCAGAGACGGCGTTCAGCGACGTGCAGGGCGACGCCTTCGTCCCC
GCCTGCACTCTTGGCGTCGGCCAGAAGGCGCACCTTGTCTTTGGCCAAGACATCAATCA
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Figure 4.21. Complete sequence of crayfish RyR mRNA. Start and stop codons are highlighted in green.

MADSEGSSEQDDVSFLRTEDMVCLSSTAVGERVCLAAEGFGNRTCFLENIADKNNPPDLCQ
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INFLLMCCDCNHDGKIDYVEFTERFHNPAKEIGFNLAVLLTNLSEHMPNDPRLARFLETAGS
VLNYFEPLLGRIEIMGSSKRIERVYFEIKEENIDQWEKPQIKESKRGFFYAIVTEGDKEKLEAFV
NFCEDAIFEMQHAAALMEEEDDALAKKCDADALKYLTEDEEEKTGMDLIKAKIGGVKDQM
LETFSILAPSNLKKKIKEIKQMTPAELAVGFCRLLFLMMYHSVFGVFYLSRKVWRATMRLMQ
GPPVEQAEQKEEKSGPFVRLAIPALPDVAHADLPQPHAQPKLEGEQLSLEDKPKDIIDDEKM
KPVLDALAELKDDITPEQAIAAVKAAEKKSVEAAQQEAMQKTEEQPSAAASEPSPVSQVDLS
SYNKRAVSFLARNFYNLKYAALVLAFCINFILLFFKASALGGVEEEEEDVAVHNPFAFGSGDLL
GSGDAAVLGDDEGDELGSGNFTLGDDTDDEEDEEEVEEWIHMDDRYFYLEHVIRLFSVTHS
IVALCMLLAYYNLKIPLVIFKREKDVARRLEFDGIYVAEQPEDDDIKAHWDKLVISAKSFPNNY
WDKFVKKKVRQKYSETYDFDAISNLLGMETTMSFKQEEASTGIIGYMTSVDWRYQVWKAG
VTITDNQFLYNLWYLTFSMLGNINYFFFAAHLLDVAVSIPSLKTILQSVTHNGKQLILTCMLLTI
IVYCYTVIAFNFFRKFYISEEDDVVDQKCHDMLTCFVFHLYKGVRAGGGIGDEIESPDGDDYE
LYRIIFDITFFFFIIVILLAIIQGLIIDAFGELRDQLESVKENLESNCFICGIGSDYFDAVPHGFDMH
VLKEHNLANYMFFLMHLINKDETEYTGQETYVWNMYQQRCWDFFPVGDCFRKQYEEELS
GGGSAS*

Figure 4.22. Amino acid sequence of crayfish ryanodine channel.

The cloned sequence, used as a reference, and Miseq short reads of RYR1,2,3,4 and 5 amplicons were aligned by using DNAStar platform (Figure 4.23).

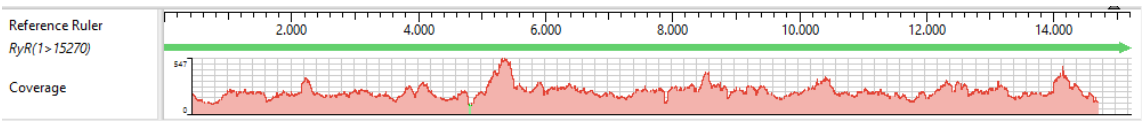

Figure 4.23. The *SeqMan* Assembly display of the short reads of RYR 1,2,3,4&5 amplicons to the cloned crayfish RyR mRNA sequence.

The theoretical Mw is calculated as 569.8 kDa. The transmembrane segments of the RyR were predicted via TMHMM (Figure 4.24), which indicates that the putative crayfish RyR contains six transmembrane helixes.

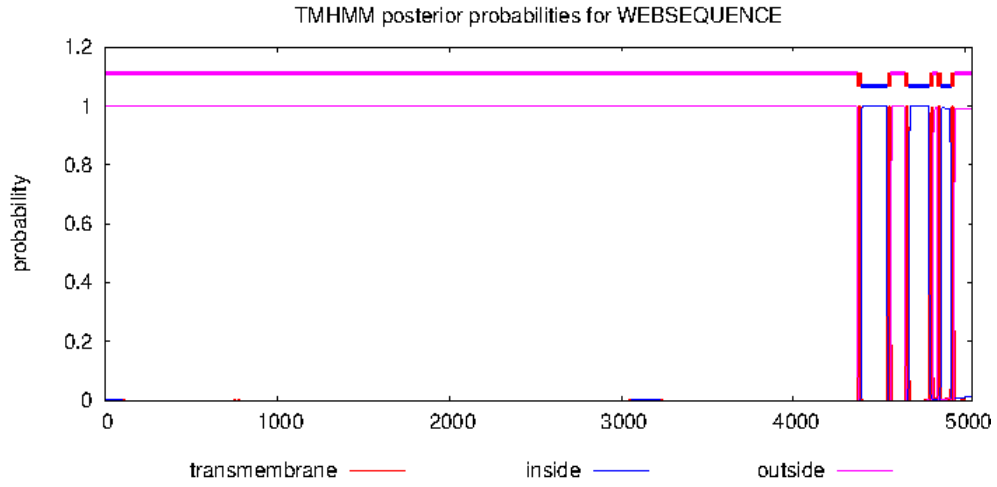

Figure 4.24. Graphic of TMHMM analysis of the crayfish RyR protein.

The phylogenetic tree was also constructed to examine the evolutionary relationship between the putative crayfish RyR and the other RyRs (Figure 4.25). Firstly, it branched into two nodes, one of which belong to the vertebrates and the other for the invertebrates. In addition, grouped RyR isoforms can be distinguished.

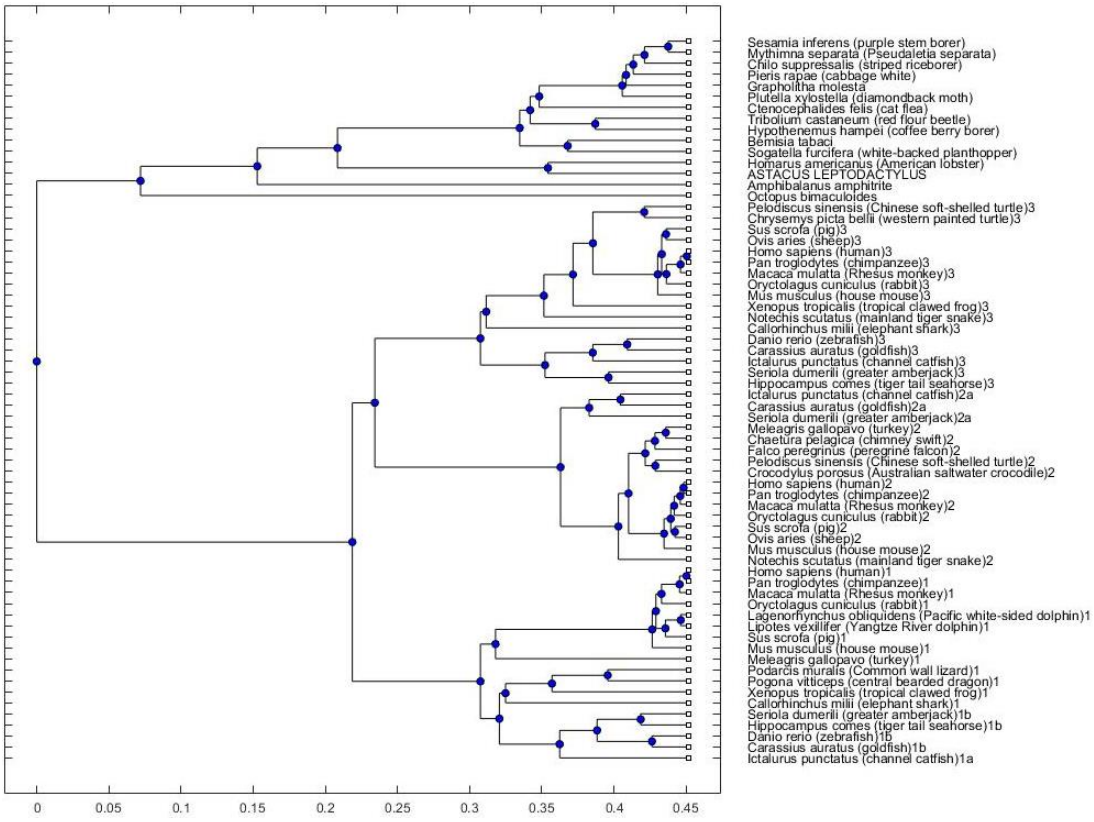

Figure 4.25. The phylogenetic tree of RyR channels in the animal kingdom.

5. DISCUSSION

In the present study a complete mRNA sequence, 15236 bp, has originally been cloned in the cDNA templates from *Astacus leptodactylus* muscle samples. This is the longest mRNA sequence presently available in our model animal and among those cloned our laboratory. Quality and the fidelity of the cloned sequence has been analyzed by aligning 10 M short reads of cDNA library. A homogenous and continuous distribution of the coverage map along the length of cloned sequence indicated that the sequence is relevant. Further, the largest repeat motif is 15 bp in size which indicates that the alignment is free of redundant and repetitive articulations. An ORF region, coding a continuous protein sequence, between 76-15201 bp has been identified. Some non-coding segments flanking the ORF in both 3' and 5' directions have been identified. Thus, it is conceivable to propose that the cloned sequence has basic properties of a mRNA and has appropriate quality. The BLAST analysis of the nucleotide sequence indicated a strong similarity to other RyR channel mRNAs reported for other species ranging from invertebrates to human.

Conversion of the coded ORF sequence to amino acids revealed a protein sequence of 5042 amino acids. BLASTp analysis showed that the putative crayfish RyR shares high similarity with other RyRs, especially with the RyRs from H. americanus (91.19%) and *P. japonicus* (88.02%). The amino acid sequence of the putative crayfish RyR shows similarity with the three RyR isoforms of *H. sapiens*, 45.53%, 45.64% and 44.27%, respectively. It has been reported that a typical RyR protein possesses a set of conserved structural domains and unique sequence motifs, closely related to function of RyR channels. Those, typical fragments are also conserved in the cloned protein sequence (Figure 4.20). Further, some functional domains observed in other RyR channel sequences are present in the cloned sequence.

The N-terminal region of the cloned RyR sequence contains IP3R (Inositol 1,4,5-trisphosphate receptor) /RyR superfamily domain (cl19745) between residues 11-206; MIR (protein mannosyltransferase, IP3R and RyR) domain (pfam02815) between

residues 213-391, and two RIH (RyR and IP3R homology) domains (pfam01365) at 441-638 and 2172-2401, respectively. In addition, there are three SP1A kinase/RyR (SPRY) domains at positions 644-7059 (SPRY1, cd12877), 1076-1208 (SPRY2, cl02614) and 1479-1630 (SPRY3, cd12879), and four copies of ryanodine receptor (RyR) domain (pfam02026) at positions 850-940, 963-1052, 2765-2854 and 2880-2964. At the C-terminal region of the cloned sequence are present an RIH-associated domain (pfam08454) between residues 3927–4044, EF-hand (calcium binding motif, cd00051) 4128-4181, RR_TM4-6 (ryanodine receptor TM4-6) region (cl24183) 4437-4675 and ion transport protein (pfam00520) 4769-4946 present.

In reference to topology analysis, the transmembrane segments of putative RyR channel have been predicted (Figure 4.24). The sequence may contain six transmembrane segments (TMS1: 4372-4394, TMS2: 4544-4566, TMS3: 4647-4669, TMS4: 4788-4810, TMS5: 4836-4858, TMS6: 4916-4935) which are located at RR_TM4-6 and ion transfer protein domains of the peptide. The pore helix of the channel, responsible for ion selectivity, is located between the putative TMS5 and TMS6, at ER/SR lumen. Another well conserved binding motif, GXRXGGGXGD, residing in this loop region (23, 57), is present as GVRAGGGIGD between residues at 4888-4897 in the cloned sequence. It has been reported to be important for both ryanodine binding and the channel conduction and, building the pore-forming segment of the RyRs. Those findings strongly indicate that the cloned mRNA conceivably translates to a functional Ca^{2+}-selective ion channel. The residues corresponding to I^{4897}, R^{4913}, and D^{4917} in rabbit RyR1 (58) and those (I^{4982}, R^{4998}, and D^{5002}) in diamondback moth of a typical RyR channel, playing an important role in the activity and conductance, have also conserved in the cloned sequence at I^{4895}, R^{4911} and D^{4915} positions. In addition, a glutamate residue, likely involved in the Ca^{2+} sensitivity, at position 4032 in rabbit RyR1 (58) and at position 4174 in diamondback moth RyR (59), is present in the cloned sequence (E^{4083}). The amino acid sequence of the transmembrane segment 5-6 was also analyzed in BLASTp as it has the pore forming segment of the channel. It has high similarity to both H. sapiens and M. musculus, in the range of 72.28-75.25%.

The phylogenetic tree displays that although similarity of the cloned sequence was largest in neighboring species, a substantial similarity was present to all mammalian and human RyRs (Figure 4.25). Similarity of the protein sequence was larger than that observed when nucleic acid sequences were compared. In addition, the figure shows that three RyR isoforms have diverged from each other. These results also fit with the evolution of animals by classical systematics. According to Figure 4.25, A. leptodactylus RyR clustered with other crustaceans RyRs, and has a common node with H. americanus RyR as they are genetically closest than other RyRs. As a result, branching out differently from the RyR isoforms suggest that there is a single isoform of the crayfish RyR, as in the case with other invertebrates (60, 61).

6. CONCLUSION

Analysis of the cloned sequence indicates that a mRNA has been cloned. Identification multiple membrane spanning segments in the coded protein sequence relevant to that of a pore forming transmembrane peptide. Presence of a calcium selectivity filter favors the idea that the cloned sequence should be a type of calcium channel. Further, presence of conserved domains solely confined to RyR channels and apparent similarity to nucleic acid and protein sequences of RyR channels from a wide range of species indicates that the cloned mRNA should code a protein for a RyR channel. Thus, it is conceivable to propose that a putative RyR mRNA in *Astacus leptodactylus* has originally been cloned in the present study.

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8. APPENDIX

APPENDIX 1: Supplementary Material

Table 8.1. List of RyR protein sequence sources used for phylogenetic tree.

	Species	RyR	GenBank Accession Number	aa size
1	*Homo sapiens* human	RYR 1	NP_000531.2	5038
2		RYR 2	NP_001026.2	4967
3		RYR 3	NP_001027.3	4870
4	*Mus musculus* house mouse	RYR 1	NP_033135.2	5035
5		RYR 2	NP_076357.2	4966
6		RYR 3	NP_001306085.1	4868
7	*Oryctolagus cuniculus* rabbit	RYR 1	NP_001095188.1	5037
8		RYR 2	NP_001076226.1	4968
9		RYR 3	XP_008267020.1	4873
10	*Sus scrofa* pig	RYR 1	NP_001001534.1	5035
11		RYR 2	XP_020928342.1	4987
12		RYR 3	XP_020955566.1	4871
13	*Danio rerio* zebrafish	ryr1b	XP_017207446.1	5109
14		ryr3	XP_009293048.1	4863
15	*Ovis aries* sheep	RYR 2	XP_027818181.1	4975
16		RYR 3	XP_014952400.2	4866
17	*Meleagris gallopavo* turkey	RYR 1	NP_001290128.1	5050
18		RYR 2	XP_031408049.1	4933
19	*Macaca mulatta* Rhesus monkey	RYR 1	XP_028695840.1	5040
20		RYR 2	XP_014982093.1	5028
21		RYR 3	XP_014997278.2	4870
22	*Xenopus tropicalis* tropical clawed frog	ryr1	XP_004917160.1	5044

23		ryr3	XP_031747158.1	4884
24	*Pan troglodytes* chimpanzee	RYR 1	XP_009433809.2	5037
25		RYR 2	XP_016797014.2	4996
26		RYR 3	XP_016783375.1	4870
27	*Ictalurus punctatus* channel catfish	ryr1a	XP_017344262.1	5078
28		ryr2a	XP_017339435.1	4971
29		ryr3	XP_017311641.1	4859
30	*Seriola dumerili* greater amberjack	ryr1b	XP_022594118.1	5072
31		ryr2a	XP_022621736.1	4980
32		ryr3	XP_022621746.1	4874
33	*Podarcis muralis* Common wall lizard	RYR 1	XP_028597881.1	5039
34	*Lagenorhynchus obliquidens* Pacific white-sided dolphin	RYR 1	XP_026935263.1	5019
35	*Pogona vitticeps* central bearded dragon	RYR 1	XP_020642744.1	4936
36	*Lipotes vexillifer* Yangtze River dolphin	RYR 1	XP_007471136.1	5032
37	*Pelodiscus sinensis* Chinese soft-shelled turtle	RYR 2	XP_025043878.1	4981
38		RYR 3	XP_025045224.1	4911
39	*Hippocampus comes* tiger tail seahorse	ryr1b	XP_019716998.1	5100
40		ryr3	XP_019738677.1	4837
41	*Carassius auratus* goldfish	ryr1b	XP_026143398.1	5123
42		ryr2a	XP_026078039.1	4961
43		ryr3	XP_026089089.1	4780
44	*Chrysemys picta* Painted turtle	RYR 3	XP_023956876.1	4799
45	*Crocodylus porosus* Australian saltwater crocodile	RYR 2	XP_019405355.1	4965
46	*Notechis scutatus* mainland tiger snake	RYR 2	XP_026525091.1	4955
47		RYR 3	XP_026520093.1	5067

48	*Chaetura pelagica* chimney swift	RYR 2	XP_009995897.1	4955
49	*Falco peregrinus* peregrine falcon	RYR 2	XP_027644916.1	5076
50	*Callorhinchus milii* elephant shark	ryr1	XP_007909255.1	5008
51		ryr3	XP_007886252.1	4886
52	*Octopus bimaculoides*	ryr2-like	XP_014787737.1	5242
53	*Ctenocephalides felis* cat flea	ryr-like	XP_026469278.1	5087
54	Homarus Americanus	ryr-like	XP_042241828.1	5619
55	Amphibalanus amphitrite		KAF0307467.1	4233
56	Plutella xylostella		NP_001296002.1	5123
57	Tribolium castaneum		NP_001308588.1	5094
58	Chilo suppressalis		AKC03558.2	5133
59	Hypothenemus hampei		QEE14187.1	5107
60	Sesamia inferens		AXA98483.1	5139
61	Mythimna separata		AWV67093.1	5123
62	Pieris rapae		XP_022127229.1	5105
63	Bemisia tabaci		AQR59331.1	5122
64	Grapholitha molesta		ALM96708.1	5141
65	Sogatella furcifera		AHW99829.1	5128

APPENDIX 2: Thesis Originality Report

Cloning of Astacus leptodactylus ryanodine receptor gene

ORİJİNALLİK RAPORU

%18	%13	%12	%8
BENZERLİK ENDEKSİ	İNTERNET KAYNAKLARI	YAYINLAR	ÖĞRENCİ ÖDEVLERİ

BİRİNCİL KAYNAKLAR

1	www.openaccess.hacettepe.edu.tr:8080 İnternet Kaynağı	%2
2	kclpure.kcl.ac.uk İnternet Kaynağı	%2
3	Ke-Yi Wang, Xuan-Zhao Jiang, Guo-Rui Yuan, Feng Shang, Jin-Jun Wang. "Molecular Characterization, mRNA Expression and Alternative Splicing of Ryanodine Receptor Gene in the Brown Citrus Aphid, Toxoptera citricida (Kirkaldy)", International Journal of Molecular Sciences, 2015 Yayın	%1
4	Submitted to Universiti Malaysia Kelantan Öğrenci Ödevi	%1
5	Submitted to Queen's University of Belfast Öğrenci Ödevi	%1
6	etheses.whiterose.ac.uk İnternet Kaynağı	%1
7	Cagil Coskun, Nuhan Purali. "Cloning and molecular characterization of a putative	<%1

APPENDIX 3: Digital Receipt

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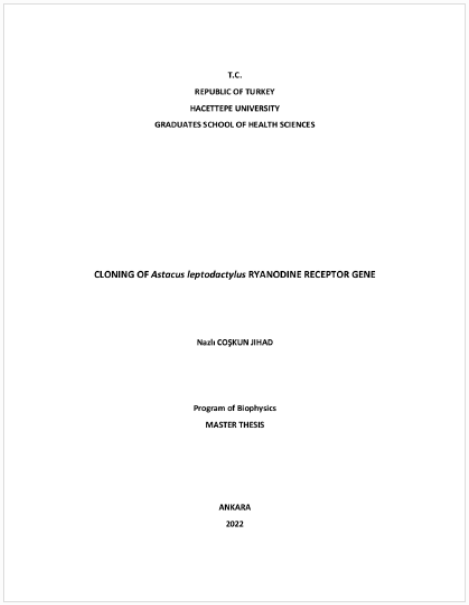
Gönderen:	Nazli Coskun Jihad
Ödev başlığı:	TEZ
Gönderi Başlığı:	Cloning of Astacus leptodactylus ryanodine receptor gene
Dosya adı:	Nazl_COS_KUN_JIHAD-_Msc_Thesis.docx
Dosya boyutu:	2.38M
Sayfa sayısı:	45
Kelime sayısı:	6,533
Karakter sayısı:	55,443
Gönderim Tarihi:	06-Oca-2022 10:28ÖÖ (UTC+0300)
Gönderim Numarası:	1738040693



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REPUBLIC OF TURKEY
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Nazlı COŞKUN JIHAD

Program of Biophysics
MASTER THESIS

ANKARA
2022

9. CURRICULUM VITAE