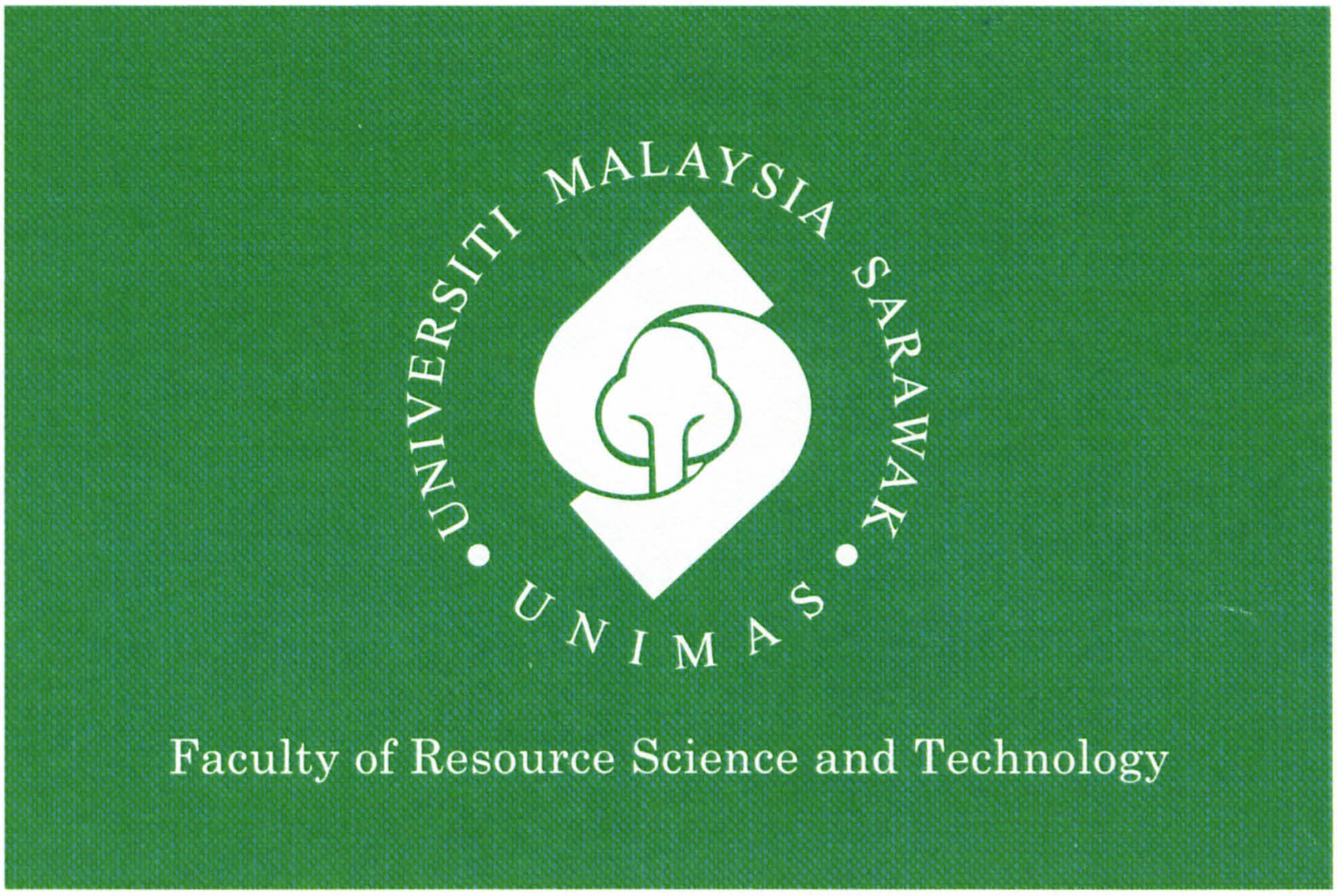


# Isolation and Cloning of *ABCF1* Gene from *Rasbora Sarawakensis*

**Clarissa Patrick Balinu**
**(40955)**

**Bachelor of Science with Honours**
**(Resource Biotechnology)**
**2016**

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# Isolation and Cloning of *ABCF1* Gene from *Rasbora Sarawakensis*

**Clarissa Patrick Balinu**
**(40955)**

A thesis submitted in partial fulfilment of the Final Year Project 2 (STF3015) Resource Biotechnology

**Supervisor: Dr. Chung Hung Hui**

Resource Biotechnology
Department of Molecular Biology

Faculty of Resource Science and Technology
Universiti Sarawak Malaysia
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This declaration is made on the 1 day of August 2016.

**Student's Declaration:**

I, CLARISSA PATRICK BALINU, 40955, FACULTY OF RESOURCE SCIENCE AND TECHNOLOGY hereby declare that the work entitled Isolation and Cloning of *ABCF*1 Gene from *Rasbora Sarawakensis* is my original work. I have not copied from any other students' work or from any other sources except where due reference or acknowledgement is made explicitly in the text, nor has any part been written for me by another person.

1/8/2016
Date submitted

Clarissa Patrick Balinu (40955)

**Supervisor's Declaration:**

I Dr CHUNG HUNG HUI hereby certifies that the work entitled Isolation and Cloning of *ABCF*1 Gene from *Rasbora Sarawakensis* was prepared by the above named student, and was submitted to the "FACULTY" as a partial fulfillment for the conferment of Bachelor of Science with Honours (Resource Biotechnology), and the aforementioned work, to the best of my knowledge, is the said student's work.

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## List of Abbreviations

| | |
|---|---|
| ABC | ATP-Binding Cassette |
| AGE | Agarose Gel Electrophoresis |
| ATP | Adenosine Triphosphate |
| DNA | Deoxyribonucleic Acid |
| dNTP | Deoxyribonucleotide Triphosphate |
| MDR | Multi-Drug Resistance |
| mRNA | Messenger Ribonucleic Acid |
| NBD | Nucleotide-Binding Domain |
| NBF | Nucleotide Binding Folds |
| NCBI | National Center for Biotechnology Information |
| PBS | *Phosphate Buffered Saline* |
| PBT | Polybutylene Terephthalate |
| PCR | Polymerase Chain Reaction |
| RNA | Ribonucleic Acid |
| TBST | Tris Buffered Saline with Tween |
| TMD | Transmembrane Domain |
| TNF | Tumour Necrosis Factor |
| °C | Celsius (temperature) |
| rpm | Revolution per minute |

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# Isolation and Cloning of *ABCF*1 Gene from *Rasbora Sarawakensis*

**Clarissa Patrick Balinu (40955)**
Resource Biotechnology
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

## Abstract

Ribosome assembly and protein translation process are regulated by *ABCF1* gene. Since the function of *ABCF1* gene is ribosome assembly regulation, the loss of this function may cause the failure to make proteins in an organism. The purpose of this study is to clone the *ABCF1* gene of *Rasbora sarawakensis* into pGEM-T easy vector and analyse the extracted gene sequence from *R. sarawakensis*. Total RNA was extracted from a whole fish homogenate via Tri reagent and phenol chloroform precipitation. The cDNA generated from reverse transcription was amplified with PCR using degenerate primers targeting the conserved region of the gene. The PCR amplified amplicons of size approximately 803 bp and was inserted into pGEM-T easy vector. Transformation was performed using in-house prepared *E. coli* JM109 competent cell which has the efficiency of $1.57 \times 10^4$ transformants/μg. The white colonies were run in colony PCR and confirmed to have an insert. Further confirmation was conducted by performing *NotI* restriction digestion and the result shows two discreet bands. Afterwards, the plasmid that was obtained from plasmid miniprep was sent for sequencing and the result was corroborated by using BLAST and it shows highest similarity to gene of *Sinocyclocheilus anshuiensis*. Based on this study, further study of expression analysis can be conducted which can lead to the understanding of the temporal and spatial patterns of gene expression that can help assign function to genes, thus establishing *R. sarawakensis* as a model of study to detect eco-toxicity of Sarawakian rivers.

Keywords: *ABC* transporter, *ABCF*1, Cloning, *Rasbora sarawakensis*, PCR.

## Abstrak

*Perhimpunan ribosome dan proses terjemahan protein dikawal selia oleh gen ABCF1. Dengan membawa fungsi sebagai pengawal selia ini, kehilangan gen ini boleh membawa kepada kegagalan untuk memproses protein dalam organisma tersebut. Tujuan kajian ini adalah untuk mengklon gen ABCF1 dari R. sarawakensis ke dalam vektor pGEM-T easy dan menganalisis sekuen gen yang diekstrak daripada R. sarawakensis. RNA telah diekstrak daripada homogenate ikan menggunakan reagen Tri dan kaedah mendapan fenol kloroform. Kemudian, cDNA yang dihasilkan melalui transkripsi terbalik diamplifikasi melalui PCR bersama dengan primer degenerasi dengan target pada gene tersebut. PCR menghasilkan amplicon 803 bp yg kemudiannya diklonkan ke vector pGEM-T easy. Transformasi dilakukan dengan menggunakan kompeten sel E.coli JM109 yang menghasilkan kecekapan* $1.57 \times 10^4$ *transformants/μg. Koloni putih digandakan melalui kolony PCR untuk mengesan kehadiran gen. Pencernaan restriksi juga turut dilakukan dengan enzim NotI dan menghasilkan dua band menunjukkan kehadiran gen yang diingini. Setelah itu, plasmid yang diekstrak melalui penjujukan dan keputusannya dianalisa dengan menggunakan BLAST menunjukkan keputusan persamaan yang tinggi dengan gen ABCF1 Sinocyclocheilus anshuiensis. Berdasarkan kajian ini, analisis ekspresi lanjutan boleh dilangsungkan agar dapat membawa kepada pemahaman yang ebih mendalam tentang expresi spatial dan temporal dan fungsi gen boleh terus dikaji, dan menjadikan Rasbora sarawakensis sebagai organisma model untuk mengkaji eko-ketoksikan sungai di Sarawak.*

*Kata kunci: ABC pengangkut, ABCF1, klon, Rasbora sarawakensis, PCR*

## 1.0 Introduction

Ribosomes are the important element in translation process which allows the conversion of mRNA with coded information into proteins. The synthesis of ribosomes in eukaryotes is one of the most demanding processes in cellular activities and it appears to be very complex (Warner, 1999). The process of ribosome synthesis includes ribosome assembly. However, even though the structures of ribosomes are known in much detail, the molecular mechanisms driving the ribosome assembly still remains elusive. This is due to the challenges to coordinate the assembly of ribosomal RNA (rRNA) and the ribosomal proteins (RP), as well as the cellular environment that has to be considered to regulate the assembly (Warner, 1999).

- One of the genes that are involved in ribosome assembly is the *ABCF* gene. The *ABCF* gene subfamily contains three members, *ABCF1*, *ABCF2*, and *ABCF3* and they are found in all chordates and at least one subfamily gene in all eukaryotes (Annilo *et al.*, 2006). All the members of *ABCF* gene is the gene in ABC superfamily that does not possess transport activities through plasma membrane. In this study, ATP-binding cassette sub-family F member 1 (*ABCF1*) gene of *Rasbora sarawakensis* will be the research subject on understanding more on the gene itself in more depth. The physiological function of *ABCF*1 gene is known to be involved in the assembly of ribosomes and also involved in protein translation (Annilo *et al.*, 2006). Moreover, the *ABCF*1 gene is regulated by tumour necrosis factor-alpha (TNF-alpha) (Semov *et al.*, 2002). Thus this implicating that the gene is involved in the activity of immune system. *ABCF1* mRNA expression can be increased when the Tumour Necrosis Factor alpha (TNF-α), a pro-inflammatory cytokine is stimulated (Wilcox, 2010). Therefore from this information, we can deduce that *ABCF1* expression level may be able to be taken as an indication of inflammatory levels found in *Rasbora sarawakensis* to detect eco-toxicological substances present in the Sarawak rivers.

*ABCF1* gene is highly conserved in all eukaryotic organisms (Dean, 2001). Because less mutation of *ABCF1* gene is found, thus the discoveries of diseases due to the mutation are very limited. However, since the function of *ABCF1* gene is ribosome assembly, the loss of this function may cause the failure to make proteins. Proteins are important in any living organisms as it is needed for numerous functions in cells such as cell growth, repair damaged tissue or directing chemical processes (Strunk & Karbstein, 2009).

The function of this gene specifically in this species is not yet documented but can only be predicted. By finding the sequences of *ABCF1* of *Rasbora Sarawakensis*, this may explain the pattern of this gene throughout different species of teleost species as well as further ancestor species such as insects and humans. By comparing the sequences of *ABCF*1 gene throughout the hierarchy of the species phylogenetic tree, we may conclude some general properties of *ABCF*1 gene in a broader view.

Besides, the findings through this study can bring us to more understanding of the species' environments, which is the Sarawak waters in which this species have as their habitat, if the further study is continued. Also through this research, another sequence of different species of *ABCF1* gene is going to be contributed and eventually lead to understanding of the protein structure that is analysed through this research. This may, in further studies, have many benefits to the native society, as well as to the world until as far as understanding how the proteins interact and their mechanism of doing its role or function.

The hypothesis of this study is to aim that the sequence obtained show almost similar sequences of other teleost species' *ABCF1* gene of a partial sequencing. This hypothesis is based on the data gathered on *ABCF*1 gene sequences between species of teleost.

Moreover, in further study, the gene expression in early stage of development in *R. sarawakensis* can be analysed by determining the temporal and spatial gene expression. The temporal and spatial patterns of gene expression can help assign function to genes involved in physiological changes, tumorigenesis, cellular responses to stimuli and wide variety of other cellular events. Other than that, more information on the taxonomy and physiology of *Rasbora sarawakensis* for novel therapeutic targets will be found through this study.

The aims of this project are:

i. To isolate *ABCF1* gene of *Rasbora sarawakensis.*

ii. To clone the *ABCF1* gene of *Rasbora sarawakensis.*

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# 2.0 Literature Review

## 2.1 ATP-Binding Cassette (ABC) Protein Family

ATP-binding cassette (ABC) protein superfamily is categorised as one of the largest protein family existed (Higgins, 2001). According to Linton and Higgins (1998), 5% of the *Escherichia coli* genome encodes for the components of ABC proteins. This protein superfamily can be found in all prokaryotes, as well as eukaryotes, and are membrane bound proteins (Vasiliou *et al.*, 2009). The ABC protein superfamily is known to be transport proteins. The ABC transport proteins contain nucleotide-binding domains (NBD) and transmembrane domain (TMD). Each comprises with α-helices. The core unit of ABC transporters consists of four domains, which is two NBDs and two TMDs. Even though ABC transporter family are recognized as genes that encodes for proteins for transporting substrate across the membrane, some are referred as "half-transporters" (two subunits bind together as a homodimer or a heterodimer), whereas the others are full-transporters.

The ABC gene consists of seven subfamilies, namely *ABCA*, *ABCB*, *ABCC*, *ABCD*, *ABCE*, *ABCF*, *ABCG*, and *ABCH* gene subfamily. Each has different functions and expressions. For example, some of the proteins confer multidrug resistance (MDR) in cancers (Gros *et al.*, 1986). The MDR conferring proteins are found in the cell membrane. The proteins in this family mainly involved in the control of transportation of substances, specifically drugs and certain metals through cell membranes (Gottesman *et al.*, 2002). The proteins translocate substances including sugars, amino acids, metal ions, peptides, proteins, and hydrophobic compounds across the extracellular membranes as well as the intracellular membranes (Dean *et. al.*, 2001). For tissues involved in the excretory system, ABC transporter family seems to present abundantly, for example in kidneys and liver.

They are also expressed in gut epithelium, and capillary endothelia which form a barrier between blood and brain (Taipalensuu *et. al.*, 2001; Löscher & Potschka, 2005).

The ABC transporters are always moving to the apical part of epithelial cells that are polarised (Leslie *et al.*, 2005). This suggests that they are limiting the chemical uptake and increases elimination of substances through the membrane contributing to defence against toxicants (Leslie *et al.*, 2005). Organisms without ABC drug transporters are usually healthy but may experience hypersensitivity to certain toxicants or small pathophysiological changes (Kaminski, 2006).

Table 2.1 below shows human ABC gene and their basic features. This will give an insight of what are some of the importance of ABC gene in an organism.

**Table 2.1:** Human ABC transporters and their basic features (Glavinas *et al.*, 2004)

| Family | Member | Alias | Expression | Function |
|---|---|---|---|---|
| ABCA | ABCA1 | ABC1 | Ubiquitous | Removal of cholesterol and PLs onto HDL particles |
| | ABCA2 | ABC2 | Brain | |
| | ABCA3 | ABC3,ABCC | Lung | |
| | ABCA4 | ABCR | Rod photoreceptors | |
| | ABCA5 | | Muscle, heart, testes | |
| | ABCA6 | | Liver | Drug resistance |
| | ABCA7 | | Spleen, thymus | Surfactant protection |
| | ABCA8 | | Ovary | N-retinylidiester-PE efflux |
| | ABCA9 | | Heart | |
| | ABCA10 | | Muscle, heart | |
| | ABCA11 | | Stomach | |
| | ABCA12 | | Low in all tissues | |
| ABCB | ABCB1 | MDR1,PGP | Adrenal,kidney,brain | Multidrug resistance |
| | ABCB2 | TAP1 | Ubiquitous,ER | Peptide transport into the ER |
| | ABCB3 | TAP2 | Ubiquitous,ER | Peptide transport into the ER |
| | ABCB4 | PGP3,MDR3 | Liver | Phosphatidylcholine transport |
| | ABCB5 | | Ubiquitous | |
| | ABCB6 | MTABC3 | Mitochondria | Iron transport |
| | ABCB7 | ABC7 | Mitochondria | Heme transport |
| | ABCB8 | MABC1 | Mitochondria | Heme transport |
| | ABCB9 | | Heart,brain | |
| | ABCB10 | MTABC2 | Mitochondria | |
| | ABCB11 | SPGP,BSEP | Liver | Bile salt transport |
| ABCC | ABCC1 | MRP1 | Ubiquitous | Drug resistance |
| | ABCC2 | MRP2, cMOAT | Liver | Organic anion transport |
| | ABCC3 | MRP3, cMOAT-2 | Lung, intestine, liver | Drug resistance |
| | ABCC4 | MRP4, MOAT-B | Prostate | Nucleoside transport |
| | ABCC5 | MRP5, MOAT-C | Ubiquitous | Nucleoside transport |
| | ABCC6 | MRP6 | Kidney, liver | |
| | ABCC7 | CFTR | Exocrine tissues | Chloride ion transport |
| | ABCC8 | SUR | Pancreas | Sulfonylurea receptor |
| | ABCC9 | SUR2 | Heart, muscle | |
| | ABCC10 | MRP7 | Low in all tissues | |
| | ABCC11 | MRP8 | Low in all tissues | |
| | ABCC12 | MRP9 | Low in all tissues | |
| ABCD | ABCD1 | ALD | Peroxisomes | VLCFA transport regulation |
| | ABCD2 | ALD1,ALDR | Peroxisomes | |
| | ABCD3 | PMP70, PXMP1 | Peroxisomes | |
| | ABCD4 | PMP69, P70R | Peroxisomes | |
| ABCE | ABCE1 | OABP | Ovary, testes, spleen | Oligoadenylate-binding protein |
| ABCF | ABCF1 | ABC50 | Ubiquitous | |
| | ABCF2 | | Ubiquitous | |
| | ABCF3 | | Ubiquitous | |
| ABCG | ABCG1 | ABC8, Human white | Ubiquitous | Cholesterol transport |
| | ABCG2 | ABCP, MXR, BCRP | Placenta, intestine | Drug resistance |
| | ABCG4 | White2 | Liver | |
| | ABCG5 | Steroline 1 | Liver, intestine | Sterol transport |
| | ABCG8 | Steroline 2 | Liver, intestine | Sterol transport |

HDL: high density lipoprotein
VLCFA: very long chain fatty acid

## 2.2 ATP-binding Cassette, Sub-family F, member 1 (*ABCF1*) gene

Sub-family F of ABC transport protein is a type of protein that lacks TMDs and consists of μNBDs. This shows that sub-family *ABCF* does not involve in the transport of substances across cellular membranes. *ABCF* is generally known to carry the function of regulation of gene expression (Garcia *et al.*, 2004). *ABCF* subfamily consists of 3 members, which are *ABCF*1, *ABCF*2, and *ABCF*3. The expression of *ABCF*1 has shown to increase upon tumour necrosis factor alpha (TNFα) stimulation (Paytubi *et al.*, 2008). This is significant as TNFα is a pro-inflammatory cytokine produced by macrophages and T cells and has a number of functions in the immune response (Paytubi *et al.*, 2009). *ABCF*1 are thought to function in translational initiation as it binds to translation elongation initiation factor 2 (eIF2) (Duttaroy & Spener, 2003).

## 2.3 *Rasbora sarawakensis*

*Rasbora sarawakensis* is the native species of fish found in Borneo. Although the name suggest it is from Sarawak state, but it also ranges into neighbouring country such as West Kalimantan of Indonesia. Some of the water sources which *R. sarawakensis* can be found are Sungai Sarawak and Batang Kayan in Sarawak, and the Mempawah and Melawi in Kalimantan Barat. This species is a freshwater fish which inhabits in a slow-moving water stream in the forest with thick marginal vegetation. This species usually found in rivers with shaded dense rainforest above the water body. Their habitat most often are covered with thick layer fallen leaves and branches of trees thus causing the water to be quite brown due to the tannins released from decomposing organic matter.

A matured *R. sarawakensis* fish usually can grow up to 4.5cm at maximum. This makes an advantage of taking it as the research model as the housing area required to contain the fish is smaller, making the husbandry costs lower. Besides, the costs to buy

experimental chemicals, drugs are lower due to the lower dosage required. The volume of water is also reduced for smaller fish and causes smaller volume of water to be disposed. In terms of labware and reagents, their usages are minimized (Hill *et al.*, 2002). Moreover, smaller embryo size is suitable to be tested in single culture plates or series of petri dishes in order to provide relicates of embryos at time (Hill *et at.*, 2005). This allows a high throughput screens for smaller molecule screening, and discovery of drugs in which the fish grow in small microformat screening plates (Hill *et al.*, 2005).

The most suitable maintenances or accommodation of their habitat for keeping it in a tank are dark tank, well-planted setup. Some floating plants and driftwood roots can be added into the substrate for more natural feel. The tank would not need to be fast and filtration is not necessarily to be strong. The water of the tank should have the temperature of 22-26°C, pH value of 6.0-7.5 and hardness of 2-12°H. This species mostly feeds on Daphnia, bloodworm and suchlike. This will encourage the fish into breeding condition. The sex of the fish can be identified by comparing the body of the fish, whereby the females have noticeable rounder belly and probably a little larger than the males. Spawning can be initiated by adding cool water in the tank for every few hours and feeding the fish with live and frozen foods. Eggs will be eaten by the adults after a few days to avoid fry to be sucked in the mechanism. The eggs of *R. sarawakensis* can be incubated in 22-26°C and hatches in between 28 to 48 hours later.

*R. sarawakensis* is chosen because of its nativity to Borneo water. Moreover, *R. sarawakensis* is not widely studied. In a further extend of time; the continual study of this species could contribute to the findings of the features such as toxicity, water content and composition, and quality of Bornean Rivers. Therefore, to reach to that point, much information is needed from the fish's biology to achieve novel therapeutical target.

**Figure 2.1**: Rasbora sarawakensis (Adapted from Rasbora sarawakensis, 2012)

# 3.0 Materials and Methods

## Materials

TRI reagent

Chloroform

Isopropanol

Reverse transcriptase

Ultrapure water

Ethanol

Hydrogen peroxide

PBT

PBS

Paraformaldehyde

Methanol

pGEM®-T Easy Vector System

## 3.1 Maintenance of Rasbora Sarawakensis

*Rasbora Sarawakensis* was provided by the Department of Molecular Biology, UNIMAS. The fish tank was cleaned every week to maintain a clean condition of the aquatic habitat. The condition of water in terms of pH value was maintained in between 6.0-7.5 pH value and hardness of water ranges between 2°H to 12°H. The temperature was maintained in the range of 22°C-28°C. The feeding schedule for the fish was twice a day, every day at 9 am and 5 pm. The aquarium contains plants including floating plants to allow less light penetrating the aquarium providing environment similar to its natural habitat.

## 3.2 Primer design

Firstly, Clustal Omega software (http://www.ebi.ac.uk/Tools/msa/clustalo/) was used for sequence alignment to find the conserved region of *ABCF1* gene between different species of teleost where the sequences was taken from National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) to form degenerate primer. Then, Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/) was used to analyse all the suitable primer pairs designed for hairpin, palindromes, dimmers and melting temperature (Tm). Table 3.1 shows the degenerate code that were used to replace the non-conserved regions.

**Table 3.1:** Degenerate code for non-conserve region

| Non-conserved region | Degenerate code |
|---|---|
| **A,G** | R |
| **A,C** | M |
| **G,C** | S |
| **A,T** | W |
| **A,T,C** | H |
| **G,T,C** | B |
| **A,T,G,C** | N |
| **C,T** | Y |
| **G,T** | K |
| **G,A,T** | D |
| **G,A,C** | V |

## 3.3 Total RNA Isolation

Tissue samples of *R. Sarawakensis* were first cut into smaller pieces and transferred into a microcentrifuge tube to be homogenized. Under a fume cupboard, the tissue samples were added with 500 µl of TRI Reagent and homogenized in the microcentrifuge tube by crushing it repeatedly using pipette tips until the tissues were disrupted enough. TRI Reagent was added again for a volume of 500 µl to the homogenate. The homogenate was then centrifuged for 12,000 rpm at 4°C for 10 minutes. The supernatant was then