

博士論文 (要約)

**Targeted mutagenesis of the *CYP1A* gene in Javanese  
medaka, *Oryzias javanicus*, to understand the  
metabolism of organic pollutants**

(有機汚染物質代謝機構解明のためのジャワメダカ *Oryzias  
javanicus* *CYP1A* 遺伝子への選択的変異導入)

スハイラ ビンティ ルスニ

SUHAILA BINTI RUSNI

博士論文 (要約)

**Targeted mutagenesis of the *CYP1A* gene in Javanese  
medaka, *Oryzias javanicus*, to understand the  
metabolism of organic pollutants**

(有機汚染物質代謝機構解明のためのジャワメダカ *Oryzias  
javanicus* *CYP1A* 遺伝子への選択的変異導入)

スハイラ ビンティ ルスニ

SUHAILA BINTI RUSNI

Graduate School of Frontier Sciences  
The University of Tokyo

# Contents

<b>General Introduction</b>	1
<b>Chapter 1</b>	12
Strain selection and tissue-specific expression	
<b>Chapter 2</b>	31
The response of <i>O. javanicus</i> to PAHs	
<b>Chapter 3</b>	45
Genome editing and knockout strain establishment	
<b>Chapter 4</b>	72
Toxicity test on the knockout strain	
<b>General Discussion</b>	88
<b>Acknowledgments</b>	99
<b>Summary in Japanese</b>	10
<b>References</b>	105

## List of Abbreviations

CYP1A	Cytochrome P450 Family 1A
CRISPR/Cas 9	Clustered regularly interspaced short palindromic repeats-associated Cas 9 protein
COD	Chemical oxygen demand
DMSO	Dimethyl sulfoxide
DO	dissolved oxygen
DSB	Double strand break
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
HMA	Heteroduplex mobility assay
HPLC	High performance liquid chromatography
LC <sub>50</sub>	Lethal concentration of 50% death
NHEJ	Non-homologous end joining
PAHs	Polycyclic aromatic hydrocarbons
PCBs	polychlorinated biphenyls
PCDD/PCDF	polychlorinated dibenzo-p-dioxins and dibenzofurans
qPCR	Real-time quantitative PCR
RT-PCR	Reverse transcription
SFS	Fluorescence spectroscopy and synchronous fluorescence scanning
sgRNA	Single guide RNA
SNP	Single nucleotide polymorphism

## **General Introduction**

## **1) Marine pollution**

Marine pollution has become one of the most severe problems in many countries for decades. The coastal zone is facing the highest risk of multiple anthropogenic impacts, although the open ocean also has a high possibility of being polluted. Especially in developing countries, the situation of marine pollution is still serious. For example, oil spills from petroleum tankers and waste discharge from industrial factories along the coastal areas worsen the organic pollution in the marine environment and disturb the ecological system there (Bakke et al., 2013).

Malaysia is an example of a developing country that has been impacted by marine pollution related to industrialization and urbanization processes. This country, which consisting of the Peninsular Malaysia, Borneo, and many islands, has long coastlines, and the coastal area is often affected by polluted water. Coastal marine pollution has been a severe problem, especially in the Straits of Malacca along with the Peninsular Malaysia and the coast facing the South China Sea along the east coast of Sabah and Sarawak, both of which include big cities and are with heavy marine traffic.

## **2) Organic marine pollutants**

Kasmin (2010) stated that marine pollutants are land, atmospheric, and marine origin, mainly the waste product of factories, agriculture farms, and oil spills. Among such pollutants, organic pollutants are major components, many of which are very harmful to living organisms and natural ecosystems. Major organic pollutants found in the marine environment are polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/PCDF), and also other halogenated compounds such as pesticides and herbicides (Goks, 2016;

Hwang and Wade, 2008; Karami, 2008, Lin et al., 2002). These compounds, especially PAHs, cause adverse effects when in contact with or bioaccumulated by aquatic organisms such as fish, and finally affect humans who consume the fish. They may interrupt the endocrine and reproductive system and may also cause cancer.

To metabolize lipid-soluble chemical compounds, including organic pollutants, taken into the body, specific processes are required to transform them into polar, ionizable and easily excretable compounds. This transformation processes involve phase I and phase II mechanisms. In phase I, the target chemical is modified by the addition of reactive or polar groups and become a substance that fits into phase II. In phase II, enzymatic reactions will cause coupling of the compounds produced by phase I with another substance such as glutathione, amino acids, or glucuronic acid. The phase II products are generally polar and larger in size than the initial substrate and become ready to be removed from the body. This excretion and biotransformation are usually referred to as the detoxification process, which is important to reduce the pollutants' effect that might harm the organism. However, when the level of pollutants in the environment exceeded the organism's metabolizing ability, the organism will not be able to complete the biotransformation process and finally die (Di Giulio et al., 1995). Therefore, pollution levels in the marine environments should be continuously monitored and controlled to preserve marine organisms and the ecosystem.

### **3) Bioindicator system to detect pollutants**

A monitoring system is essential to regulate environmental pollution and to maintain good water quality. Various systems to survey the pollution status have been established by many sectors using physical, chemical, and biological methods. Physical

and chemical methods are used to analyze the water characteristics and detect the pollutants, for example, measurement of dissolved oxygen (DO), chemical oxygen demand (COD), and pH of the water, and detection and quantification of specific chemicals that are likely to exist in the area. More detailed detection of small aromatic hydrocarbons was performed using gas chromatography (GC) since the late 1950s, as reported by Lee et al. (1981) and Means (1998), then followed by a gas chromatography-mass spectrometry (GC-MS) to analyze the unsubstituted and alkylated derivatives of PAHs, and heterocyclic PAHs (Alzaga et al., 2004; Means, 1998).

Later, a more advanced method by high-performance liquid chromatography (HPLC) combined with either mass spectrometry (Kerns et al., 1997) or fluorescence detection (Jonsson et al., 2003) to analyze the -OH derivatives of PAH. Other than that, using fluorescence spectroscopy and synchronous fluorescence scanning (SFS) is another convenient way that can detect the concentration of a single species or complex mixtures of PAHs (Wang et al., 2005; William et al., 1994). These methods enable the detection of the level of particular pollutants that exist in water, sediment, or biological samples. However, even using such fine analytical technologies, the effects of the pollutants on the living organisms and concentrations of the compounds that affect living organisms are not indicated.

Biological methods, in which the effects of the pollutants included in environmental samples on live organisms are monitored, are promising methods in representing the influence of the pollutants on living organisms. Fish and invertebrates (Moore, 1979; Moore and Farrar, 1985) are often used as models to reveal the impact of chemicals in the aquatic system because they are major members of aquatic ecosystems and accumulate pollutants by taking up from the environments or through the food chain



(Segner, 1998). It is also known that bioconcentration of pollutants occurs in these organisms, and it makes the detection process more sensitive.

#### **4) Javanese medaka, *Oryzias javanicus*, the ideal bioindicator**

Bioindicator systems using fish have been proposed to be an effective way to study chemical pollutants' effects on living organisms. Especially, fish is a group of vertebrates, in which human is included. Thus, the results obtained in fish models are useful to predict the phenomena that will occur in human. Among bony fishes, Japanese medaka (*Oryzias latipes*) is one of the major model fish species and has been used frequently as a test fish. For Japanese medaka, inhabiting mainly freshwater areas in Japan, Korea, and China (Naruse, 1993), enormous biological information has been accumulated because it has been utilized in various fields of research, such as toxicology, genetics, and genomics studies (Kinoshita et al. 2009; Naruse et al., 2016). Many studies have proved this fish's effectiveness as the bioindicator for various chemicals and heavy metals in the freshwater area.

However, as my study purpose is to determine the effect of organic pollutant on the marine organism and to understand the impact of the pollutants on the coastal and seawater area, Japanese medaka is not an ideal representative due to its preference for freshwater than seawater, although it has some adaptability to seawater (Inoue and Takei, 2002; 2003). Therefore, I decided to use a related species in the same genus, Javanese medaka, *Oryzias javanicus*, which can survive in seawater and freshwater environments and prefers the former (Inoue and Takei, 2002; 2003), to study organic pollutant effects in seawater and marine areas. This species is widely distributed along the coastal regions of Malaysia, Indonesia, Thailand, and Singapore (Iwamatsu et al., 1982; Magtoon and

Termvidchakorn, 2009 and Roberts, 1998) and can be found at all periods of the year. It is advantageous as a bioindicator because of the easiness to being maintained in the laboratory for experimental purposes similar to Japanese medaka. The clear and fast response to chemical pollutant exposure which already been reported (Yusof et al., 2014; 2018), makes it as an ideal candidate for use as a bioindicator (Yusof et al., 2012; Yusof et al., 2013). This is also supported by a study done by Woo et al. (2009) that recognized the changes in gene expression of *O. javanicus* towards various kinds of heavy metals. Availability of the whole genome sequence information, recently reported by Takehana et al. (2020) and Lee et al. (2020), is also an advantage of this species, because genome information will help in understanding the metabolic processes responding to pollutants.

## **5) Cytochrome P450 Family 1A (CYP1A)**

An important system discovered to metabolize lipophilic xenobiotic compounds in a wide variety of organisms from bacteria to vertebrates is the cytochrome P450 (CYP) superfamily (Diotel et al., 2010), consisting of heme-containing proteins engaged in the oxidative metabolism of numerous endogenous and exogenous lipophilic substrates. Some of the CYP superfamily members have been proposed as a pollution indicator since the mid-1970s, and its molecular basis, gene regulation processes, and reaction to organic xenobiotics have been reported (Goksøyr, 1995). A member of the superfamily, CYP1A has been identified as one of the essential enzymes catabolizing xenobiotic compounds. While CYP1A occurs ubiquitously in vertebrates, elucidation of its function in fish is vital because fish reside, throughout the lifecycle, in water, where pollutants are contained (Kim et al., 2013; Roy et al., 2018; Imbery et al., 2019). CYP1A is known to participate in the phase I metabolism of exogenous substrates, including aromatic hydrocarbons,

drugs, and chemical carcinogens (Rewitz et al., 2006), and is owned by most fish (Goldstone et al., 2007). Generation of *CYP1A* gene expression by pollutants has been reported in various fish (Yang et al., 2017; Boulanger et al., 2019; Franco and Lavado, 2019; Gaaied et al., 2019; Leggieri et al., 2019). Therefore, this thesis focuses specifically on the gene of *CYP1A* in the Javanese medaka, *O. javanicus*.

## **6) Overview of genome editing and comparison of the existing technique**

Genome editing technologies have attracted the interests of the scientific community in many fields and are widely used for various purposes, including biological, medical, and agricultural sciences. Its primary goal is to modify or manipulate specific parts of DNA sequences in order to discover, understand, and utilize their functions. This technology allows researchers to edit a specific part of genome sequences by precisely adding, removing, or substituting with other DNA sequences, basically utilizing targeted engineered endonuclease (EENs), which is reviewed by Chandrasegaran and Carroll (2015). Such nucleases have enabled researchers to modify the target area in any living organism's gene of interest (Provasi et al., 2012; Takasu et al., 2010).

Several gene-editing techniques have been developed using this concept, such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and currently developed clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) system. Through these methods, DNA double-strand breaks (DSBs) are induced at the target genomic locus (Wright et al., 2014), which simultaneously activates the DNA repair mechanism in the cells. Generally, there are two major pathways for repairing the DNA, either by non-homologous end joining (NHEJ) or homologous directed repair (HDR). Eukaryotes often use NHEJ, which prone

to cause errors during the repairing process of the double-strand breaks, and such errors are utilized to induce gene knockout. Namely, due to the absence of templates for DNA repair, NHEJ tends to cause fragment deletion or frameshift mutations, which can be utilized to generate knockout genotypes (Segal and Meckler, 2003).

Although several typical techniques generate genome-edited organisms using targeted nucleases, not all the approaches are efficient and successfully induce targeted mutagenesis. A minimum of two fundamental roles is needed; one is the specific binding capability on the target DNA, and the other is the adequate cleavage ability at the target point. Referring to these criteria, ZFNs are made up of two linked protein domains, namely, DNA binding and DNA cleavage domains. The DNA binding domain contains zinc finger repeat that can detect three base pairs of genomic DNAs. Meanwhile, the cleavage domain is the nuclease domain of the *FokI* restriction enzyme, which required two independent zinc finger repeats. However, lacking any binding events may lead to failure of the induction of DNA double-strand breaks.

TALENs, which appeared after ZFNs, are almost similar, which utilize the *FokI* nuclease domain for editing the target gene (Mussolino and Cathomen, 2012). The DNA binding domain is originated from transcription activator-like effectors (TALE) from *Xanthomonas* proteobacteria (Sugio et al., 2007). This method can overcome limitations in ZFNs by recognizing the DNA by the tandem of individual TALE repeats. Each of them has the capability to one single base in the genomic sequence. Besides, it has a broader sequence spectrum that can be targeted compared to the previous technique. Although these techniques have gained the possibility to modify specific sequences on the genome of living organisms, they are still with a major disadvantage, which is their high potential to cause off-target effects.

CRISPR/Cas 9 system, a relatively recent genome editing technology, rapidly became popular because it reduced the disadvantage of the previous methods. The founder of this technique, Emmanuelle Charpentier and Jennifer Doudna just won The Nobel Prize in Chemistry 2020, for development of this gene technology that have high precision in editing genomes. This system was developed utilizing the adaptive immune defense system of bacteria (Sapranaukas et al., 2011), which uses the endonuclease to cut a specific non-host DNA sequence (Segal & Meckler, 2003). Only about 20 nucleotides are needed for the single guide RNA (sgRNA) design to guide endonuclease to the target site. Double strand breaks formed on the targeted region will be repaired but usually incomplete and cause deletion, insertion, or a substitution on the DNA sequence. Pennisi (2013) stated that this new approach in genome editing is simpler, faster, and efficient besides cheaper in cost and the ability to target multiple sites at once.

#### **7) The objective of the study:**

My study aims to understand more about the *CYP1A* gene function and metabolism of organic pollutants in which CYP1A is involved in fish using *O. javanicus*, as the candidate for the bioindicator to show the response and effect when exposed to the organic pollutants. The organic pollutant metabolism controlling gene, Cytochrome P450 family 1 (*CYP1A*) gene of this fish, is knocked out using CRISPR/Cas 9 system. The process will enable us to detect the harmful level of particular pollutants and provide information on the fish metabolism against those substances. It may finally contribute to predicting the potential effects on humans.

In Chapter 1, I report the cloning and sequencing of *CYP1A* cDNA of different Javanese medaka strains that originated from separate locations, Penang, Malaysia and

Jenepono, Indonesia. By comparing them with a sequence reported by Tuan et al. (2014), single nucleotide polymorphism (SNPs) sites were identified. Subsequently, I examined the primary expression site of the *CYP1A* gene by reverse transcription (RT-PCR) on the strain originating from Penang. This organ was used as the target organ for the following studies.

Chapter 2 of this study describes the toxicity tests using adult *O. javanicus* against representative PAHs, phenanthrene and pyrene. In the first experiment, LC<sub>50</sub>s of the *O. javanicus* for the test pollutants were determined. Subsequently, the second exposure test was performed for the concentration below the LC<sub>50</sub>s. Their responses in terms of the survival rate, physiological changes of every 24 hours of the test period are recorded until 96 hours. Changes in expression levels of *CYP1A* mRNA are also examined by real-time quantitative PCR (qPCR) on the test fish of the second exposure test. As I found the correlation between the doses of the test pollutants and mRNA levels, I discuss the potential of the *CYP1A* expression level to be used as the marker on the PAHs contamination.

In Chapter 3, I established a *CYP1A* gene-knockout strain of *O. javanicus* using the CRISPR/Cas 9 system to understand the function of *CYP1A* in fish. The first process of the genome-editing by CRISPR/Cas9 is the microinjection of specifically designed single-guide RNA (sgRNA) along with the Cas 9 enzyme. I designed sgRNA for the *CYP1A* gene using the *CYP1A* cDNA sequences determined in Chapter 1, avoiding the SNPs location. The injected individuals were analyzed using heteroduplex mobility assay (HMA) analysis to detect the mutation's existence. The mutation patterns were further analyzed by PCR amplification and sequencing of the corresponding region. I chose individuals with a four-base deletion on their *CYP1A* sequence for the knockout strain

establishment. Since the process of the establishment of the knockout strain consists of the crossing of mutant individuals and genotype analyses for several generations, I established an environmental-DNA-based genotyping method that enables to distinguish homozygous and heterozygous knockout and wild-type genotypes using sequence-specific primers, avoiding anesthesia and fin-clipping. I completed this part with the establishment of the homozygous knockout-*CYP1A* generation.

Finally, in Chapter 4, the application of the successfully generated *CYP1A*-knock out *O. javanicus* on the toxicity test is carried out by exposing the fish to the model PAHs, phenanthrene and pyrene. This is the most crucial part of this study, in which the effectiveness of the knockout attempts is being tested. In this part, I compare the responses of the three types of *O. javanicus* fish — homozygous knockout, heterozygous knockout, and wild-type *CYP1A*. The sensitivity of the test fish on PAHs compound is determined by comparing their survivability, changes in physiological responses, and also the effects on the internal organs.

After completing all the steps and integrating all these chapters, I propose the use of *O. javanicus* as an ideal test organism to the PAHs compound, and developed *CYP1A*-knocked out *O. javanicus* to understand more on *CYP1A* function and metabolism of the chemical substances by using this fish. I discussed the outcome of the study and relating the four chapters and their significant results. I elaborate more on the importance of the *CYP1A* gene and the effects on the fish after it is being knocked out. After all, the mode of toxicity of the test fish on different PAHs compounds and the detailed function of the *CYP1A* gene are explored.

## **Chapter 1**

### **Strain selection and tissue-specific expression**



## 1.1 Introduction

Japanese medaka, *Oryzias latipes* has been studied as a model fish species in Japan for decades, and most of its major genes have been identified, including the target gene in this study, the cytochrome P450 family 1A (*CYP1A*) gene (AY297923 in 2003). For Javanese medaka, *O. javanicus*, the *CYP1A* cDNA sequence was reported later by Tuan et al. (2014). However, considering the wide and dispersed distribution areas of Javanese medaka (Kinoshita et al. 2009), sequence variations are likely to exist, as sequence polymorphisms observed in xenobiotic-metabolizing enzymes of Japanese medaka strains from different localities (Katsumura et al. 2014). In this chapter, I determined the sequences of the *CYP1A* cDNA of the two Javanese medaka strains available from the National BioResource Project (NBRP) Medaka because precise sequence information is crucial for gene editing by using the CRISPR/Cas 9 system. In this genome-editing system, the single guide RNA and target sequence should match completely, as any difference between them may lead to failure of sequence detection or inaccurate recognition, which may cause off-target mutation (Ansai and Kinoshita, 2014). Besides identifying the major organ in which the *CYP1A* gene functions, I conducted reverse-transcription (RT)-PCR on major organs of the Javanese medaka strain originated from Penang, Malaysia.

## 1.2 Materials and methods

### 1.2.1 Experimental animals

Javanese medaka, *Oryzias javanicus* strains originating from Penang, Malaysia and Jeneponto, Sulawesi, Indonesia (Strain IDs, RS831, and RS858, respectively), were obtained from the National BioResource Project (NBRP) Medaka at the Natural Institute

for Basic Biology (NIBB), Okazaki, Japan, and cultured in the medaka breeding room in Atmosphere and Ocean Research Institute (AORI), The University of Tokyo. All experiments were conducted according to the Guidelines for the Care and Use of Animals of the University of Tokyo and approved by the animal experiment committee at the AORI, The University of Tokyo. The fish were cultured in natural seawater under the photoperiod of 14 hours light and 10 hours dark, and the temperature was set to be 26°C. The seawater salinity was maintained around 31 ppt, and fishes were fed twice a day with the brine shrimp's larvae, *Artemia salina* (Kinoshita et al., 2000).

#### 1.2.2 cDNA sequences determination of Penang and Jenepono strains

Total RNA was extracted from the liver of *O. javanicus* from Penang and Jenepono using TRIsure (Bioline, London, UK). The cDNA template was created using the SMART cDNA Library Construction Kit (Clontech, Palo Alto, CA, USA). Japanese medaka, *O. latipes* genome sequence (NCBI reference sequence: NC\_019861.1), and mRNA sequence (Accession No: AY297923) were aligned together with known Japanese medaka *CYP1A* mRNA (Tuan et al. 2014; Accession No: KJ689303) using MAFFT software version 7 to determine the location of exons and introns. Then, six forward and six reverse primers were designed on the location of the exons, as listed in Table 1.1. Polymerase Chain Reaction (PCR) was carried out with the reaction mixture contained 1 µL of genomic DNA as a template, with 0.75 µL of primer, 12.5 µL of 2× KOD FX Neo Buffer, 5 µL dNTP, and 0.5 µL KOD FX enzyme and 4.5 µL of Milli-Q water in the total volume of 25 µL. The thermal cycler conditions were set as follows: one cycle at 94 °C for 2 min for preheating, 27 cycles with 98 °C for 10 sec, annealing temperature shown in Table 1.1 for 30 sec, and extension at 68 °C for 1 min, and a final extension at 68 °C

for 7 min. The reaction was stopped at 4 °C. Electrophoresis was conducted on 1.5 % agarose gels containing gel green (Wako, Osaka, Japan) to confirm amplicon lengths. To 5 µL of PCR product, 0.5 µL Exosap-IT and 1.5 ml of MilliQ water were added and then incubated at 37 °C for 50 min and 15 min at 80 °C.

### 1.2.3 DNA sequencing

Cycle sequencing was performed using BigDye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystem). Reaction mixtures containing 1 µL Exosap-treated DNA product, 0.5 µL of 5X BigDye buffer, 0.8 µL primer, 0.5 µL BigDye Ready Reaction Mix, was summed up with Milli-Q to a total of 5 µL. The samples were then incubated at 96 °C for 1 min, followed by 25 cycles at 96 °C for 10 sec, 55 °C for 5 sec, and 60 °C for 4 min. The reaction was stopped at 4 °C, and ethanol precipitation was performed. DNA sequencing was carried out by using ABI PRISM 3130X1 Genetic Analyzer (Applied Biosystems). Results obtained were analyzed by Genetyx-Mac Ver. 16.0.9 software (GENETYX Corporation, Tokyo, Japan).

### 1.2.4 Single Nucleotide Polymorphisms (SNPs) detection

The *CYP1A* cDNA sequences of the two strains and the known sequence (KJ689303) were aligned using the multiple sequence alignment program, MAFFT version 7, to determine if any difference in nucleotide exists. The changes in amino acids caused by differences in nucleotides also examined using the same program.

### 1.2.5 Genomic DNA extraction

The caudal fin piece, about 2 mm from the posterior end, was excised from adult fish of the two Javanese medaka strains with a total body length of about 2.0 cm after anesthesia using tricaine methanesulfonate. The fish was directly returned to their water tank after the fin cutting process. DNA was extracted from the fin tissue using DNeasy Blood & Tissue Kit (Qiagen, Limburg, Nederland). The DNA extraction process was carried out following the Spin-Column Protocol for purification of total DNA from animal tissues (Qiagen, Maryland, USA). PCR amplification of exon sequences was done as described in the previous section, except that the fin DNA was used instead of the liver cDNA. The *CYP1A*-coding sequences of the two strains were compared using the multiple sequence alignment program, MAFFT version 7 (Kato and Standley, 2013).

### 1.2.6 Cloning of deletion-containing sequence

Sequences of the gene segment containing the site of a nucleotide deletion in the known sequence (KJ689303) were confirmed by the following steps: The PCR process was carried out by using 5F (Table 2.1) and CDS III primers included in the SMART cDNA Library Construction Kit (Clontech, Palo Alto, CA, USA). The PCR condition was as follows: pre-heating at 94 °C for 2 min, 35 thermal cycles of 98 °C for 10 sec, 57 °C for 30 sec, 68 °C for 1 min, and a final extension at 68 °C for 7 min. The reaction stopped at 4 °C. The sample was treated with Exosap-IT Kit (USB Corporation, Cleveland, Ohio, USA), and nested PCR was conducted using the PCR product of CYP1A\_5F and CDS III primers as a template, 4R-F (Table 2.1) and CDS III as primers, under the same PCR conditions as above. The amplified product was electrophoresed on 1.5% agarose gel, and the DNA band of target length was cut out and placed in a 1.5 ml

microcentrifuge tube and subjected to the gel extraction process using QIAquick Spin (QIAGEN, Limburg, Nederland). Extracted DNA was used in the sub-cloning process into pGEM-T Easy vector (Promega, Madison, WI, USA) for TA-cloning. The ligated vector was transformed into a competent cell from *E. coli* XL-1 Blue strain and cultured overnight on LB agar at 37 °C. For direct colony PCR, each colony was picked and added into the reaction mix containing SP6 and T7 primers and Amplitaq Gold (Applied Biosystems, Foster City, CA, USA) according to the protocol supplied with the enzyme. The colony DNA sample in mixture solution was incubated in the reaction condition of pre-heating at 95 °C for 5 min, 25 cycles of 95 °C for 15 sec, 55 °C for 15 sec, and 72 °C for 90 sec. Final extension at 72 °C for 7 min was added, and the reaction stopped at 4 °C. The electrophoresis process was carried out, and the size of the bands was determined. The product obtained was treated with Exosap-IT, and the sequence was determined by ABI PRISM 3130X1 Genetic Analyzer Machine (Applied Biosystems) as described above.

#### 1.2.7 Organ dissection

Adult Javanese medaka of Penang strain, whose details were described in 2.2.1, were dissected under a stereomicroscope. Their internal organs, i.e., liver, muscle, gill, fin, intestine, brain, and skin (Figure 1.4), were dissected and collected from five individuals and placed in a 1.5 ml microcentrifuge tube per each stated organ. The tubes were frozen in liquid nitrogen and kept in -80 °C freezers until the RNA extraction process.

#### 1.2.8 RNA extraction

Total RNA was extracted using TRIsure (Bioline, London, UK). Complementary DNA was synthesized from 320 ng total RNA using SuperScript III First-strand Synthesis System for RT-PCR (Life Technologies) following the protocol supplied with the kit. The cDNA templates were used for PCR reactions.

#### 1.2.9 Reverse-transcription (RT-) PCR

The expression level of the gene of interest, *CYP1A*, is compared with that of a housekeeping gene, beta-actin (internal control). For this purpose, beta-actin forward and reverse primers (Table 1.1) that amplifying a 715 bp product were used. For the *CYP1A* gene target region, primer CYP1A\_F2 and CYP1A\_R2 (Table 1.1) amplify a part of *CYP1A* cDNA with the length of 552 bp nucleotides were used. KOD FX Neo was used as the polymerase enzyme, and the reaction mixture was prepared following the protocol supplied with the enzyme. Thermal conditions were one cycle at 94 °C for 2 min, 27 cycles at 98 °C for 10 sec, 59 °C and 56 °C annealing temperature for beta-actin and target gene primers, respectively, for 30 sec, and at 68 °C for 30 sec, and a final sequence extension for 7 min at 68 °C. The reaction was stopped at 4 °C, and the amplified products were electrophoresed on 1.5% agarose gel containing gel green (Wako, Japan).

## 1.3 Results

### 1.3.1 *CYP1A* sequences comparison

The full-length *CYP1A* cDNAs were sequenced, and 1,566 bp (Appendix) open reading frame encoding 521 amino acid residues was identified from the two Javanese medaka strains of Penang, Malaysia and Jenepono, Indonesia. The sequences were deposited into DDBJ under accession nos. LC505990 and LC505991, respectively. The alignment process was performed to compare the obtained sequences with the reference sequence reported by Tuan et al. (2014) (Figure 1.2; Figure 1.3). The result of cDNA sequence alignment (Figure 1.2) showed that the Penang fish that I studied contains the most similar cDNA sequence to the reference sequence; only 1 substitution was detected. However, the Indonesian strain included a higher number of nucleotide substitutions, i.e., 9 SNPs against the reference sequence (Table 1.2). Two SNPs that differ between Penang and Jenepono strains resulted in amino acid substitutions (Figure 1.3). The sequences of both strains analyzed in this study included one nucleotide (adenine) deletion, against the reference sequence, near the end of ORF (Figure 1.3). To confirm the deletion's existence, I amplified the genomic segment containing the deletion site from the two strains and sequenced. The sequence of the genomic segment was completely matched to the cDNA sequence in both strains. Thus, the deletion is not an artifact because it is observed in both cDNA and genomic sequences of both strains.

### 1.3.2 Tissue-specific gene expression

The result of the RT-PCR was indicated in Figure 1.5 shows the liver to exhibit the highest expression. Expression in the gill and intestine was also high. In the muscle, fin, brain, and skin, amplified products were detected, but the level was low.

## 1.4 Discussion

### 1.4.1 Variation in *CYP1A* cDNA sequences

The first step in this thesis is to determine the cDNA sequences of the *CYP1A* of the two strains of Javanese medaka to study the effect of xenobiotics on the *CYP1A* gene. Although a sequence of *CYP1A* cDNA was already reported previously (Tuan et al., 2014), we need to know the precise sequence of the fish that we use for the knockout experiment as mentioned above. From the results of sequence determination by direct PCR and cloning of the two strains of *O. javanicus*, several SNPs caused by substitutions and a base deletion were detected compared to the sequence reported in the previous study (Tuan et al., 2014) (Figure 1.2). Although most of the substitutions were silent, except two accompanied amino acid substitutions, a nucleotide deletion had resulted in a frameshift mutation near the carboxyl terminus on both strains (Figure 1.3). As the sequences determined in this study is rather similar to other fish *CYP1A* sequences, including that of Indian medaka (JQ905051, with a C-terminal DGH sequence) and Japanese medaka (AY297923, with a C-terminal DGC sequence), the sequences identified in this study are likely to be the major *CYP1A* sequences of Javanese medaka. Ingelman-Sundberg et al. (2007) proposed that functional CYP polymorphisms might include gene duplications, deletions and may end up with an inactive gene product. Besides, amino acid changes determined in this study due to variation in the nucleotide sequence may also alter activity and substrate specificity. Therefore, I decided to use only the Penang strain for subsequent experiments to avoid genetic variation.



#### 1.4.2 The main organ for *CYP1A* expression

RT-PCR results in seven organs (Figure 1.5) indicated that all the tested organs contain *CYP1A* mRNA, but their expression levels are different. The liver showed the highest concentration of the *CYP1A* mRNA, followed by gills and intestine. Other organs, such as muscles, fins, brain, and skin, also contained *CYP1A* mRNA, but at low levels. These results are consistent with a report for goldfish, in which the highest levels of *CYP1A* were detected in the liver, intestine, and gill (Oh et al., 2009). In the European flounder, *CYP1A* mRNA was found in the entire body, although it is located primarily in the liver cells (Williams et al., 2000). The expression of *CYP1A* in the liver has also been documented in a variety of fish and mammals. Considering the liver's primary function as the central organ for metabolism and excretion of xenobiotics and toxic substances (Hampton et al., 1989; Hinton, 1994), high levels of *CYP1A* expression in the liver is a reasonable result.

Other extrahepatic tissue that also facilitates toxic metabolism in the body should not be overlooked. Those organs, including the alimentary canal, gills, endocrine systems, brain, and olfactory, have also been reported to exhibit CYP1A enzyme activities (Sarasquete and Segner, 2000). The accumulation of xenobiotic compounds in aquatic organisms typically caused by contaminated food, water source, and indigested sediments. For example, xenobiotics ingested in the diet are sent to the digestive tract, such as the intestine, and the capability of the fish gut to metabolize xenobiotics has been reported (Van Veld et al., 1987). The gill is also the primary site of contact to environmental water, and a certain level of *CYP1A* mRNA and CYP1A enzymatic activity has been detected in rainbow trout gills (Leggieri et al., 2019). Besides, xenobiotics accumulation in the brain might develop neurotoxic effects on the fish (Sarasquete and Segner, 2000). Moreover,

fish skin, which also contacts environmental water, has been documented to possess low levels of mRNA of *CYP1A* (Sadek and Allen-Hoffmann, 1994). The results that I acquired in this study are consistent with such previous reports.

However, the study found to contradicts the present result is the study by Tuan et al. (2014), using the same species, Javanese medaka, in which a low level of *CYP1A* expression in the liver was reported. The reason for the inconsistency is unknown at present. Nonetheless, since we found that the liver is the primary site of *CYP1A* expression, we used the liver as the primary organ for gene expression analyses in this study.

**Table 1.1 Primers used in this chapter.**

Name	Sequence (5' to 3')	Annealing Temperature (°C)	Purpose
CYP1A_1F	GAGACATCAAGAGTGGTAATTC	51.8	1
CYP1A_1R	CCTTGTTTAATGAGAGCCTGTC	51.8	1
CYP1A_2F	ACTGAAATGAGCAAGCGGTTT	55.5	1, 2
CYP1A_2R	TGTTTTGTTGGGTAGATACTGC	55.5	1, 2
CYP1A_3F	GCATGCTGGAGGAACACATCTG	50.3	1
CYP1A_3R	GTCTCTTTTCTATGTCAGGG	50.3	1
CYP1A_4F	GCATCGTGAATGATCTCTTTG	51.0	1
CYP1A_4R	GTAACCATTCAGAGATGTGTC	51.0	1
CYP1A_5F	CCGAAATCCTACCATGTCTGATAG	56.9	1
CYP1A_5R	CCTGTAATGCCATTGAATCTG	56.9	1
CYP1A_6F	GCCACGTTAGAGCATCACTG	55.3	1
CYP1A_6R	GCCCTTTAAACATACAACCTGTC	55.3	1
CYP1A_4R-F	GACACATCTCTGAATGGTTAC	57.0	1
SP6	ATTTAGGTGACACTATAGAA	55.0	3
T7	TAATACGACTCACTATAGGG	55.0	3
Beta-actin F	GTGGATCAGCAAGCAGGAG	59.0	4
Beta-actin R	CAAGTCGGAACACATGTGCAC	59.0	4

**Table 1.2 Nucleotide substitutions and deletion in *CYP1A* cDNA sequences of *Oryzias javanicus* strains from two locations.**

	Substitution	Deletion
Penang	1	1*
Jeneponto	9	1*

Numbers represent differences in comparison with the reference sequence by Tuan et al. (2014). \*Causes a frameshift mutation.



**Figure 1.1** Original habitat locations of *Oryzias Javanicus* in this study. Location (a) is Penang, (b) is Jeneponto.

Image by Google Maps (<https://www.google.co.jp/maps>).

Reference	1	ATGGCATTAAATGATACTGCCATTTCATCGGTCTCTGTCAAGTGTGGAGGGTTTGATTGCC
Penang	1	ATGGCATTAAATGATACTGCCATTTCATCGGTCTCTGTCAAGTGTGGAGGGTTTGATTGCC
Jeneponto	1	ATGGCATTAAATGATACTGCCATTTCATCGGTCTCTGTCAAGTGTGGAGGGTTTGATTGCC
Reference	61	TTGGCTACAGTGTGTTTGGTTTATCTGCTCCTCAAGCATTTTAAACAAAGAGATCCCCGGG
Penang	61	TTGGCTACAGTGTGTTTGGTTTATCTGCTCCTCAAGCATTTTAAACAAAGAGATCCCCGGG
Jeneponto	61	TTGGCTACAGTGTGTTTGGTTTATCTGCTCCTCAAGCATTTTAAACAAAGAGATCCCCGGG
Reference	121	GGCCTTCGTGCGGAGCCGGGCCCCACACCACTGCCCATCATTGGGAATCTGCTGGAGCTG
Penang	121	GGCCTTCGTGCGGTGCCGGGCCCCACACCACTGCCCATCATTGGGAATCTGCTGGAGCTG
Jeneponto	121	GGCCTTCGTGCGGTGCCGGGCCCCACACCACTGCCCATCATTGGGAATCTGCTGGAGCTG
Reference	181	GGCAGCAGACCCTACCTGAGCCTCACTGAAATGAGCAAGCGGTTTGGAGACGTCTTCCAA
Penang	181	GGCAGCAGACCCTACCTGAGCCTCACTGAAATGAGCAAGCGGTTTGGAGACGTCTTCCAA
Jeneponto	181	GGCAGCAGACCCTACCTGAGCCTCACTGAAATGAGCAAGCGGTTTGGAGACATCTTCCAA
Reference	241	ATCCAGATCGGCATGCGTCCCGTTGTCGTTCTGAGTGGCAACGAAACCGTTTCGACAGGCT
Penang	241	ATCCAGATCGGCATGCGTCCCGTTGTCGTTCTGAGTGGCAACGAAACCGTTTCGACAGGCT
Jeneponto	241	ATCCAGATCGGCATGCGTCCCGTCTGTCGTTCTGAGTGGCAACGAAACCGTTTCGACAGGCT
Reference	301	CTCATTAACAAGGAGACGACTTTTCCGGCAGGCCTGATTTGTATAGCTTCCAGTTCATC
Penang	301	CTCATTAACAAGGAGACGACTTTTCCGGCAGGCCTGATTTGTATAGCTTCCAGTTCATC
Jeneponto	301	CTCATTAACAAGGAGACGACTTTTCCGGCAGGCCTGATTTGTATAGCTTCCAGTTCATC
Reference	361	AATGACGGCAAGAGCCTGGCTTTTCCAGCACAGATCAAGCAGGAGTTTGGCGGGCCCGCAGA
Penang	361	AATGACGGCAAGAGCCTGGCTTTTCCAGCACAGATCAAGCAGGAGTTTGGCGGGCCCGCAGA
Jeneponto	361	AATGACGGCAAGAGCCTGGCTTTTCCAGCACAGATCAAGCAGGAGTTTGGCGGGCCCGCAGA
Reference	421	AAGTTGGCCTACAGTGCTTTGCGCTCTTTCTCAAGCCTAGAGGGCAGCAATGCAGAATAC
Penang	421	AAGTTGGCCTACAGTGCTTTGCGCTCTTTCTCAAGCCTAGAGGGCAGCAATGCAGAATAC
Jeneponto	421	AAGTTGGCCTACAGTGCTTTGCGCTCTTTCTCAAGCCTCAGAGGGCAGCAATGCAGAATAC
Reference	481	TCATGCATGCTGGAGGAACACATCTGCAAAGAGACAGAGTACCTGATCAGAGAGATTAAG
Penang	481	TCATGCATGCTGGAGGAACACATCTGCAAAGAGACAGAGTACCTGATCAGAGAGATTAAG
Jeneponto	481	TCATGCATGCTGGAGGAACACATCTGCAAAGAGACAGAGTACCTGATCAGAGAGATTAAG
Reference	541	AAAGTAATGCAGACAGAAGGCAAATTCGACCCCTATCGATACATTGTTGTGTCTGTGGCC
Penang	541	AAAGTAATGCAGACAGAAGGCAAATTCGACCCCTATCGATACATTGTTGTGTCTGTGGCC
Jeneponto	541	AAAGTAATGCAGACAGAAGGCAAATTCGACCCCTATCGATACATTGTTGTGTCTGTGGCC
Reference	601	AACGTTATCTGTGGCATGTGCTTCGGACGGCGCTATGACCACCATGACCAGGAGCTGGTT
Penang	601	AACGTTATCTGTGGCATGTGCTTCGGACGGCGCTATGACCACCATGACCAGGAGCTGGTT
Jeneponto	601	AATGTTATCTGTGGCATGTGCTTCGGACGGCGCTATGACCACCATGACCAGGAGCTGGTT
Reference	661	GGCCTGGTAAACCTCAGTGAAGATTTTGTCCAAGCAACAGGCAACGGCAACCCAGCCGAC
Penang	661	GGCCTGGTAAACCTCAGTGAAGATTTTGTCCAAGCAACAGGCAACGGCAACCCAGCCGAC
Jeneponto	661	GGCCTGGTAAACCTCAGTGAAGATTTTGTCCAAGTAAACAGGCAACGGCAACCCAGCCGAC
Reference	721	TTCATCCCCGCCCTGCAGTATCTACCCAACAAAACAATGAAAAAGTTTGTGACATCAAC
Penang	721	TTCATCCCCGCCCTGCAGTATCTACCCAACAAAACAATGAAAAAGTTTGTGACATCAAC
Jeneponto	721	TTCATCCCCGCCCTGCAGTATCTACCCAACAAAACAATGAAAAAGTTTGTGACATCAAC
Reference	781	AACCGCTTCAACAACCTTTGTTTCAAGATCGTCAGCGAGCACTATGCCACTTATAATAAG
Penang	781	AACCGCTTCAACAACCTTTGTTTCAAGATCGTCAGCGAGCACTATGCCACTTATAATAAG
Jeneponto	781	AACCGCTTCAACAACCTTTGTTTCAAGATCGTCAGCGAGCACTATGCCACTTATAATAAG
Reference	841	GACAACATCCGTGACATTACAGACTCTCTTATTGATCACTGTGAGGACAGAAAACCTGGAT
Penang	841	GACAACATCCGTGACATTACAGACTCTCTTATTGATCACTGTGAGGACAGAAAACCTGGAT
Jeneponto	841	GACAACATCCGTGACATTACAGACTCTCTTATTGATCACTGTGAGGACAGAAAACCTGGAT

Reference	901	GAAAATTCCAACATCCAGATGTCAGACGAAAAGGTCGTTGGCATCGTGAATGATCTCTTT
Penang	901	GAAAATTCCAACATCCAGATGTCAGACGAAAAGGTCGTTGGCATCGTGAATGATCTCTTT
Jeneponto	901	GAAAATTCCAACATCCAGATGTCAGACGAAAAGGTCGTTGGCATCGTGAATGATCTCTTT
Reference	961	GGAGCAGGTTTCGACACAATCTCTACTGCTCTGTCTTGGTCAGTGGGGTATTTGGTGGCC
Penang	961	GGAGCAGGTTTCGACACAATCTCTACTGCTCTGTCTTGGTCAGTGGGGTATTTGGTGGCC
Jeneponto	961	GGAGCAGGTTTCGACACAGTCTCTACTGCTCTGTCTTGGTCAGTGGGGTATTTGGTGGCC
Reference	1021	CACCCTGACATAGAAAAGAGACTTTTTGAAGAACTTAAGGAAAACATCGGCCTGGACCGA
Penang	1021	CACCCTGACATAGAAAAGAGACTTTTTGAAGAACTTAAGGAAAACATCGGCCTGGACCGA
Jeneponto	1021	CACCCTGACATAGAAAAGAGACTTTTTGAAGAACTTAAGGAAAACATCGGCCTGGACCGA
Reference	1081	AATCCTACCATGTCTGATAGAAACAACCTACCTCTCCTGGAGGCTTTTATTTTGGAGATC
Penang	1081	AATCCTACCATGTCTGATAGAAACAACCTACCTCTCCTGGAGGCTTTTATTTTGGAGATC
Jeneponto	1081	AATCCTACCATGTCTGATAGGAACAACCTACCTCTCCTGGAGGCTTTTATTTTGGAGATC
Reference	1141	TTTCGCCATTCTCATTTCTCCCATTCAACAATCCACACTGCTCAACAAAGGACACATCT
Penang	1141	TTTCGCCATTCTCATTTCTCCCATTCAACAATCCACACTGCTCAACAAAGGACACATCT
Jeneponto	1141	TTTCGCCATTCTCATTTCTCCCATTCAACAATCCACACTGCTCAACAAAGGACACATCT
Reference	1201	CTGAATGGTTACTATATCCCTAAAGACACATGTGTCTTCATCAACCAGTGGCAGATAAAC
Penang	1201	CTGAATGGTTACTATATCCCTAAAGACACATGTGTCTTCATCAACCAGTGGCAGATAAAC
Jeneponto	1201	CTGAATGGTTACTATATCCCTAAAGACACATGTGTCTTCATCAACCAGTGGCAGATAAAC
Reference	1261	CATGACCCGAAACTGTGGCAGGATCCATCATCCTTTAACCAGATCGTTTCCTGAATGAA
Penang	1261	CATGACCCGAAACTGTGGCAGGATCCATCATCCTTTAACCAGATCGTTTCCTGAATGAA
Jeneponto	1261	CATGACCCGAAACTGTGGCAGGATCCATCATCCTTTAATCCAGATCGTTTCCTGAATGAA
Reference	1321	GATGGAACTGAGGTCAATCGGCTAGAAGGAGAGAAAAGTGTGGCCTTTGGTCTGGGAAAAG
Penang	1321	GATGGAACTGAGGTCAATCGGCTAGAAGGAGAGAAAAGTGTGGCCTTTGGTCTGGGAAAAG
Jeneponto	1321	GATGGAACTGAGGTCAATCGGCTAGAAGGAGAGAAAAGTGTGGCCTTTGGTCTGGGAAAAG
Reference	1381	CGACGTTGCATTGGGGAGGTCATCGCACGAAATGAAGTTTTCTCTTTTGGCAATCATG
Penang	1381	CGACGTTGCATTGGGGAGGTCATCGCACGAAATGAAGTTTTCTCTTTTGGCAATCATG
Jeneponto	1381	CGGCGTTGCATTGGGGAGGTCATCGCACGAAATGAAGTTTTCTCTTTTGGCAATCATG
Reference	1441	ATTCAGAAATTGAGATTTGAGGAAGTGCCAGGGGAGCCTATGGACTTGACCCAGAGTAC
Penang	1441	ATTCAGAAATTGAGATTTGAGGAAGTGCCAGGGGAGCCTATGGACTTGACCCAGAGTAC
Jeneponto	1441	ATTCAGAAATTGAGATTTGAGGAAGTGCCAGGGGAGCCTATGGACTTGACCCAGAGTAC
Reference	1501	GGGCTTACCATGAAGCAAAGCGCTGCCACGTTAGAGCATCACTGCGGTCAAAGGATGG
Penang	1501	GGGCTTACCATGAAGCAAAGCGCTGCCACGTTAGAGCATCACTGCGGTCAAAGGATGG
Jeneponto	1501	GGGCTTACCATGAAGCAAAGCGCTGCCACGTTAGAGCATCACTGCGGTCAAAGGATGG
Reference	1561	ACACTGAAGCTGTTTCATAATGCACCATTATGA
Penang	1560	ACACTGA
Jeneponto	1560	ACACTGA

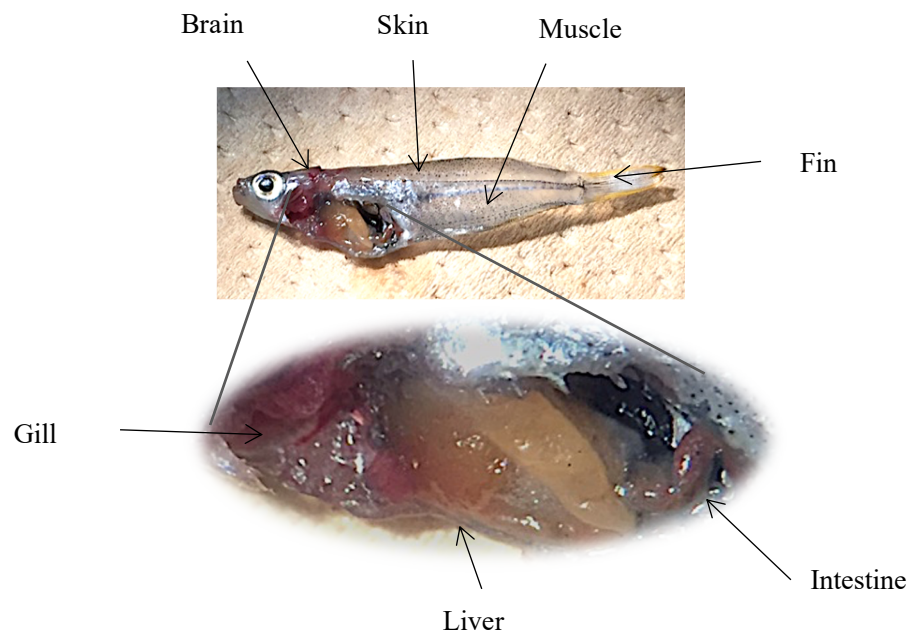
**Fig 1.2 Full-length cDNA sequences of *CYP1A* genes of different fish strains and single nucleotide polymorphisms (SNPs) represented by coloured regions. The yellow shaded area indicates the difference between the studied sequences and the reference sequence (Tuan et al., 2014). Hyphens indicate a gap.**



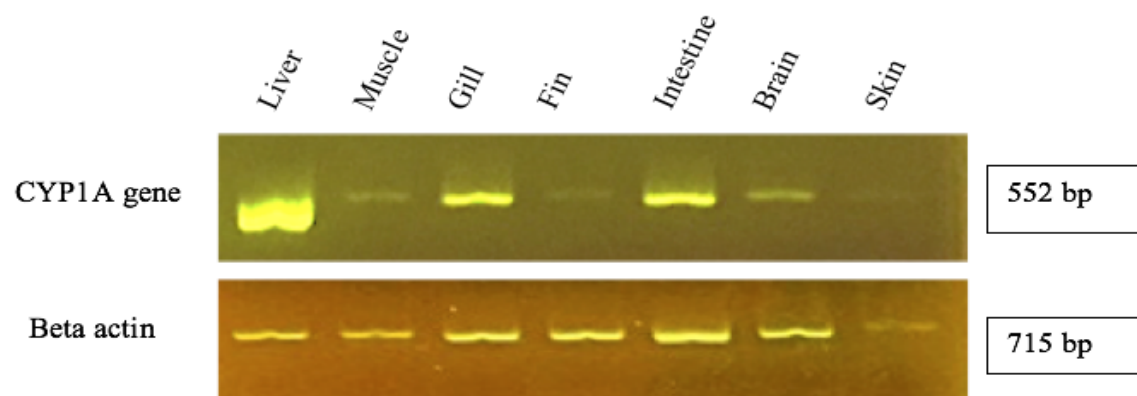
Reference	1	MALMILPFIGPLSVLEGLIALATVCLVYLLLKHFNKEIPGGLRRQPGTPLPIIGNLLEL
Penang	1	MALMILPFIGPLSVLEGLIALATVCLVYLLLKHFNKEIPGGLRRLPGTPLPIIGNLLEL
Jeneponto	1	MALMILPFIGPLSVLEGLIALATVCLVYLLLKHFNKEIPGGLRRLPGTPLPIIGNLLEL
Reference	61	GSRPYLSLTEMKSRFGDVFIQIGMRPVVVLSGNETVRQALIKQGDDFSGRPDLYSFQFI
Penang	61	GSRPYLSLTEMKSRFGDVFIQIGMRPVVVLSGNETVRQALIKQGDDFSGRPDLYSFQFI
Jeneponto	61	GSRPYLSLTEMKSRFGDIFQIGMRPVVVLSGNETVRQALIKQGDDFSGRPDLYSFQFI
Reference	121	NDGKSLAFSTDQAGVWRARRKLAYSALRSFSSLEGSNAEYSCMLEEHICKETEYLIREIK
Penang	121	NDGKSLAFSTDQAGVWRARRKLAYSALRSFSSLEGSNAEYSCMLEEHICKETEYLIREIK
Jeneponto	121	NDGKSLAFSTDQAGVWRARRKLAYSALRSFSSLEGSNAEYSCMLEEHICKETEYLIREIK
Reference	181	KVMQTEGKFDPYRYIVVSVANVICGMCFGRRYDHHQELVGLVNLSEDFVQATGNGNPAD
Penang	181	KVMQTEGKFDPYRYIVVSVANVICGMCFGRRYDHHQELVGLVNLSEDFVQATGNGNPAD
Jeneponto	181	KVMQTEGKFDPYRYIVVSVANVICGMCFGRRYDHHQELVGLVNLSEDFVQATGNGNPAD
Reference	241	FIPALQYLPNKTMKKFVDINNRFNNFVQKIVSEHYATYNKDNIRDITDSLIDHCEDRKLD
Penang	241	FIPALQYLPNKTMKKFVDINNRFNNFVQKIVSEHYATYNKDNIRDITDSLIDHCEDRKLD
Jeneponto	241	FIPALQYLPNKTMKKFVDINNRFNNFVQKIVSEHYATYNKDNIRDITDSLIDHCEDRKLD
Reference	301	ENSNIQMSDEKVVGIVNDLFGAGFDTISTALSWSVGYLVAHPDIEKRLFEELKENIGLDR
Penang	301	ENSNIQMSDEKVVGIVNDLFGAGFDTISTALSWSVGYLVAHPDIEKRLFEELKENIGLDR
Jeneponto	301	ENSNIQMSDEKVVGIVNDLFGAGFDTVSTALSWSVGYLVAHPDIEKRLFEELKENIGLDR
Reference	361	NPTMSDRNNLPLLEAFIEIFRHSSFLPFTIPHCSTKDTSLNGYYIPKDTCVFINQWQIN
Penang	361	NPTMSDRNNLPLLEAFIEIFRHSSFLPFTIPHCSTKDTSLNGYYIPKDTCVFINQWQIN
Jeneponto	361	NPTMSDRNNLPLLEAFIEIFRHSSFLPFTIPHCSTKDTSLNGYYIPKDTCVFINQWQIN
Reference	421	HDPKLWQDPSSFNPDRFLNEDGTEVNRLEGEKVLAFGLGKRRCIGEVIARNEVFLFLAIM
Penang	421	HDPKLWQDPSSFNPDRFLNEDGTEVNRLEGEKVLAFGLGKRRCIGEVIARNEVFLFLAIM
Jeneponto	421	HDPKLWQDPSSFNPDRFLNEDGTEVNRLEGEKVMFAFGLGKRRCIGEVIARNEVFLFLAIM
Reference	481	IQKLRFEFVPGEPMDLTPEYGLTMQKQKCHVRASLRSGWTLKLFIMHHL
Penang	481	IQKLRFEFVPGEPMDLTPEYGLTMQKQKCHVRASLRSDGH
Jeneponto	481	IQKLRFEFVPGEPMDLTPEYGLTMQKQKCHVRASLRSDGH

**Figure 1.3 Alignment of the peptide sequences of CYP1A, deduced from cDNA sequences, among *Oryzias javanicus* strains from different localities.** Residues that differ from the reference sequence reported by Tuan et al. (2014) are black in color. Arrow indicates the deletion location.





**Figure 1.4 Internal organs of Javanese medaka dissected for tissue specific expression study.**



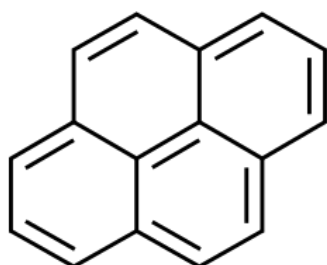
**Figure 1.5 Reverse-transcriptase PCR (RT-PCR) results of *CYP1A* expression in different organs of Japanese medaka.**

## **Chapter 2**

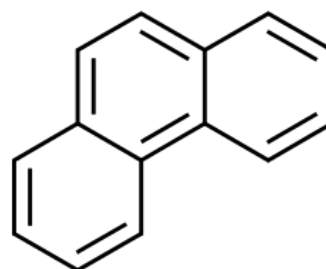
### **The response of *O. javanicus* to PAHs**

## 2.1 Introduction

As the confirmation of the *CYP1A* sequence and detection of the main organ expressing the gene had been carried out in the previous chapter, the response of the *CYP1A* gene of *O. javanicus* to polycyclic aromatic hydrocarbons (PAHs) is to be studied in this section. Polyaromatic hydrocarbons (PAHs), a group of persistent organic pollutants (POPs), have attracted global attention as major marine pollutants imposing harmful effects on living organisms and the environment. Most of them are derived from human activities, such as the burning of fossil fuels, incomplete combustion of coal, tobacco, and garbage; besides from the natural sources, which are the volcanic activities, forest fires, and oil seeps (Rand et al., 1995), and ubiquitously distributed in the environment (Latimer and Zheng, 2003; Menzie et al., 1992; Chapman, 1996). The direct contamination of aquatic organisms is commonly occurring through industrial waste releases, oil spills, and domestic wastewater discharges. Such PAHs cause adverse effects on aquatic organisms and other consumers who consume marine products. Besides the significant fish kills, various effects, including carcinogenesis, mutagenesis, and immune-suppression, are also recognized in the risk assessment related to this compound's effects (Abdel-Shafy and Mansour, 2016; Wolinska et al., 2011).



Pyrene



Phenanthrene

**Figure 2.1 Chemical structures of pyrene and phenanthrene**

Phenanthrene (three fused benzene rings) and pyrene (four fused benzene rings) were chosen as the model pollutants for the toxicity test (Figure 2.1). These two compounds are ubiquitous in the environment, which is released as a byproduct of the combustion of organic compounds. Phenanthrene is often detected as the top abundant PAH compound in sediments, seawater, and estuarine waters, and it was also detected in the air (Latimer and Zheng, 2003). Pyrene is another well-known PAH with the potency to cause dioxin-like toxicity in fish (Hendon et al., 2008; Incardona et al., 2006; Shi et al., 2012; Zhang et al., 2012) and affect cardiac function (Zhang et al., 2012). Pyrene and phenanthrene concentrations in the industrialized area's surface water can reach around  $\mu\text{gL}^{-1}$  up to several hundred  $\mu\text{gL}^{-1}$  (Rigaud et al., 2020).

In the first section of this chapter, I exposed the normal wild-type fish to various concentrations of the two representative PAHs, recorded mortality and behavioral changes, and determined  $\text{LC}_{50}$  for the two compounds. Then, I exposed the fish to lower concentrations than  $\text{LC}_{50}$  and measured changes in the expression level of *CYP1A* mRNA by real-time quantitative PCR (qPCR), to understand the effect of the pollutants on the *CYP1A* gene expression. The response of the wild-type fish conducted in this chapter will become the reference for the knockout fish experiment discussed in chapter 4.

## **2.2 Methodology**

### **2.2.1 Test organism**

The strain of Javanese medaka, *O. javanicus*, originated from Penang, Malaysia, was selected to be the test organism against the PAHs compounds in this section. Adult fish with a total body length of 2 cm and aged around six months were chosen. The fish were maintained in natural seawater under the photoperiod of 14 hours light and 10 hours

dark, and the room temperature was set to be 26°C. The seawater's salinity was set to 31, and fishes were fed twice a day with the brine shrimp, *Artemia salina*.

#### 2.2.2 Range-finding test and LC<sub>50</sub> determination

In this study, the toxicity test followed guidelines for Testing of Chemicals on Fish, Acute Toxicity Test, the Organization for Economic Cooperation and Development 203 (OECD, 1992). Phenanthrene and pyrene (Sigma, Saint Louis, Missouri, U.S.A) were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO was adjusted to 0.375% (v/v) for all tests. As the LC<sub>50</sub> of Javanese medaka on the selected chemicals was still unknown, the range-finding test (2.8, 11.2, and 19.6 µM) including two controls: seawater and seawater containing dimethyl sulfoxide (DMSO) was carried out. For the test, six fish were put into a 1L glass beaker containing a 1L test solution. The water temperature was kept at 26°C, and the photoperiod was maintained to be 14 hours light and 10 hours dark. The animals were not fed during the test, and the mortality rate and swimming behavior were monitored daily. The median lethal concentration (LC<sub>50</sub>) value is calculated by Probit analysis with probit conversion from mortality in 96 hours of the concentrations tested.

#### 2.2.3 Acute toxicity test

An acute toxicity test was conducted using six fish and different phenanthrene and pyrene concentrations (0.8, 1.2, 1.6, and 2.0 µM) for 96 hours, including two controls; one with seawater and the other with seawater containing dimethyl sulfoxide (DMSO). Test conditions other than phenanthrene and pyrene concentrations were the same as those described in the former section. The chemical solutions were replaced every 24

hours, and no feeding was provided to the fish during the test. The fish survival and changes in the movement were examined every 24 hours, and the dead fish was removed from the test vessel. After the exposure period, the fish were rendered unconscious with ice, and their livers were excised. Total RNA was extracted from the liver using TRIsure (Bioline, London, UK).

#### 2.2.4 mRNA quantification by real-time quantitative PCR (qRT-PCR)

The *CYP1A* mRNA in the liver total RNA of all the fish exposed to phenanthrene or pyrene (except one fish exposed to 1.2  $\mu$ M pyrene whose RNA was degraded) and the control fish exposed to DMSO-containing seawater was quantified using real-time quantitative PCR analysis. The reaction was performed in 384-well plated using SYBR® Premix Ex Taq™ II (Tli RNase H Plus) (Takara Bio Inc) and analyzed by Applied Biosystems Prism 7900 Sequence Detection System (Applied Biosystems, USA). Twenty nanograms of total RNA were reverse-transcribed using Multiscribe Reverse Transcriptase (Applied Biosystems, USA) in 18- $\mu$ L reaction mixtures. The amount of cDNA template used was 1  $\mu$ L, and primers (10  $\mu$ M) are 0.4  $\mu$ L (real-time RT-F/R and Oja $\beta$ -actin-F/R; Table 2.1) in a total reaction mixture of 10  $\mu$ L. Reaction conditions were set up as follows: 95°C/30 s, 45 cycles at 95°C/5 s, and 60°C/30 s. Every real-time quantitative PCR reaction utilized serially diluted cDNA to generate the relative standard curve using 200 ng/ $\mu$ L up to 7-fold dilution. The amount of gene expression calculated using the standard curve and the relative expression of the target gene normalized by the levels of  *$\beta$ -actin* mRNA (Oja $\beta$ -actin). Relative differences between experimental and control groups were calculated and shown as the relative changes.

### 2.2.5 Statistical analysis

All data are presented as means  $\pm$  S.E, and the significance of differences among experimental groups was analyzed using one-way ANOVA with Tukey's post-test.  $P < 0.05$  was considered significant. The R application was used for statistical analyses.

## 2.3 Results

### 2.3.1 LC<sub>50</sub> determination and exposure test to model organic pollutants

Range finding tests using concentrations of 2.8, 11.2, and 19.6  $\mu\text{M}$  on the selected pollutants revealed that LC<sub>50</sub> of the fish against pyrene and phenanthrene were 2.5 and 3.9  $\mu\text{M}$ , respectively. Higher concentrations than that caused abnormal swimming behavior, tissue degradation, changes in the color of internal organs, and lead to death within 48 hours. As the LC<sub>50</sub>s were determined, a range of sublethal concentrations from 0.8 to 2.0  $\mu\text{M}$  was designed for the next exposure experiment to the two model PAHs.

In the acute toxicity test against pyrene, 33.3% of fish exposed to 1.6 and 2.0  $\mu\text{M}$  solutions (Figure 2.2) died after 72 hours of exposure, with the remaining fish displayed abnormal swimming behaviors. On the last day of the test (96 hours), dead fish were found at all four solutions containing pyrene, with the number of deaths was in a dose-dependent manner, but all the fish were found alive and healthy in the two control solutions. In the test on phenanthrene (Figure 2.3), all the fish were survived until 72 hours of exposure test, but 17% found dead after 96 hours in the concentration of 1.2 and 2.0  $\mu\text{M}$ .



### 2.3.2 qRT-PCR for evaluation of mRNA expression

The *CYP1A* mRNA levels in the liver excised from the fish exposed to pyrene and phenanthrene are shown in Figures 2.4 and 2.5, respectively. In the case of pyrene exposure (Figure 2.4), mRNA expression also increased in a dose-dependent manner from 0 to 1.2  $\mu\text{M}$  but decreased at the highest concentration, 2.0  $\mu\text{M}$ , which is likely to be over the range that the fish can adapt. For the case of phenanthrene exposure, although the mRNA level in the fish exposed to 0.8  $\mu\text{M}$  was not different from that of the control, a linear increase was observed from 0.8 to 2.0  $\mu\text{M}$  (Figure 2.5).

The statistical test using one-way ANOVA and Tukey's post-test indicated no significant difference among the mean of each group. Nevertheless, a positive coefficient correlation was observed between the gene expression level and pyrene concentration between 0 to 1.6  $\mu\text{M}$  ( $r=0.983$ ,  $p<0.05$ ). The *CYP1A* mRNA expression also exhibited an increasing tendency with phenanthrene concentrations between 0.8 to 2.0  $\mu\text{M}$  ( $r=0.943$ ), although statistical support was not significant ( $p=0.057$ ) (Figs. 2.4 and 2.5).

## 2.4 Discussion

### 2.4.1 Acute toxicity test on the adult wild-type *O. javanicus*

I chose pyrene and phenanthrene, two of the simplest PAHs and commonly found in the aquatic environment, as model organic pollutants to assess the response of *O. javanicus*. A pyrene test on common gobies, *Pomatoschistus microps*, yielded the  $\text{LC}_{50}$  of 0.871 mg/L (Oliveira et al., 2012). The  $\text{LC}_{50}$  of phenanthrene has been determined in larval of rainbow trout, *Oncorhynchus mykiss* and largemouth bass, *Micropterus salmoides* (0.04 mg/L and 0.18 mg/L, respectively) (Black et al., 1983). However, as the  $\text{LC}_{50}$ s of these compounds on adult *O. javanicus* were unknown, I tested the survival of

*O. javanicus* up to 10 mg/L pyrene and phenanthrene in the “range-finding test.” I found that the concentration of 5 mg/L or higher is lethal for this species after performing the preliminary experiment using the pyrene and phenanthrene in the concentration of 1, 5, and 10 mg/L, which equivalent to 2.8, 11.2, and 19.6  $\mu$ M. As the results of this test, LC<sub>50</sub>s of pyrene and phenanthrene for *O. javanicus* were shown to be 2.5  $\mu$ M (0.7 mg/L) and 3.9  $\mu$ M (0.5 mg/L), respectively. Other studies of the effects of polycyclic aromatic hydrocarbons (PAHs) on aquatic organisms also reported higher toxicity for pyrene, apparently due to its extra aromatic ring, compared to phenanthrene (Millemann et al., 1984; Zhao et al., 2017).

#### 2.4.2 Dose-dependent *CYP1A* expression

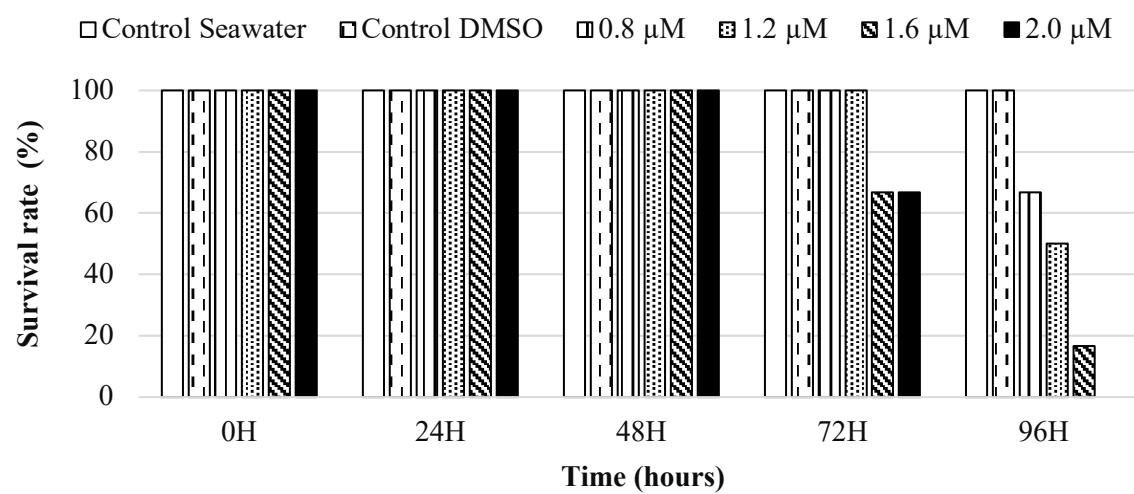
The dose-dependent response of *O. javanicus* *CYP1A* mRNA expression to pyrene and phenanthrene were demonstrated by real-time quantitative PCR (qPCR) assessment. For this purpose, those two pollutants’ concentrations were set up at lower than LC<sub>50</sub> because higher concentrations of those substances destroy the liver tissue, which it became difficult to obtain the undegraded RNA. The qPCR result showed a positive correlation between the mRNA level of the *CYP1A* gene and the concentrations of pyrene and phenanthrene. A previous study showed that phenanthrene causes limited gene expression changes but other remarkable side effects such as influencing the cardiac rhythm and inhibiting ovary development and fecundity in female medaka (Sun et al., 2015). Thus, in terms of mRNA induction, the present result is clearer than the previous study. Concerning Pyrene, induction of the *CYP1A* gene expression was reported for up to 5 days in tilapia (Zapata-Pérez et al., 2002). Also, a study done by Zhang et al. (2012) showed that low-level pyrene failed to alter gene expression, although it affected the

development of the cardiovascular system. Considering the positive correlation between mRNA expression and the dose of both PAHs, *CYP1A* mRNA levels can be a direct indicator of PAH pollution. The potential of using *CYP1A* gene expression as an indicator of organic pollutants has been suggested in *Tilapia* (Zapata-Pérez et al., 2002) and Indian medaka (Kim et al., 2013). Besides, other studies also have reported a correlation of *CYP1A* mRNA expression and the level of pollutants (Woo et al., 2009 and Sun et al., 2015). Therefore, *CYP1A* mRNA expression levels, examined in this study, may be utilized as a potential marker of PAHs pollution.

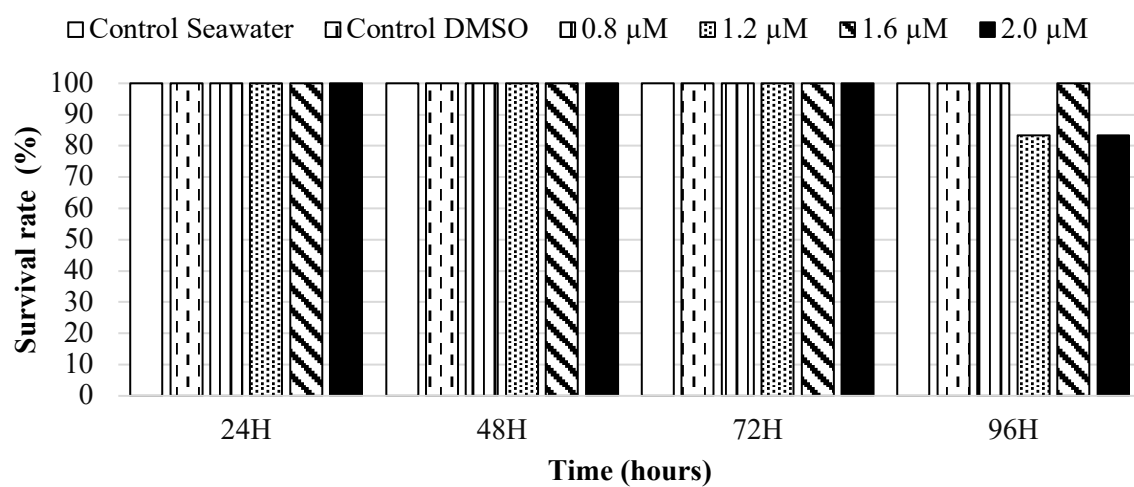
Although stimulation of *CYP1A* gene expression was demonstrated in this chapter, it is also crucial to understand the full scope of *CYP1A* function as it is playing a vital role in the catabolism of various xenobiotics and polycyclic environmental contaminants, such as dioxins (Sakaki et al., 2002; Kubota et al., 2005) and PAHs, although PCBs inhibit it (Besselink et al., 1998; Richardson et al., 2010). One possible approach to understand the function of *CYP1A* would be to perform loss-of-function experiments by disrupting the gene. *O. javanicus* is as useful for such genetic analyses as congeneric taxa (Inoue and Takei, 2002, 2003; Kinoshita et al., 2009), and application of the knockout technique, CRISPR/Cas9 system, has also been demonstrated in closely related *O. latipes* (Ansai and Kinoshita, 2014). I also applied this knockout technique to the *CYP1A* gene in *O. javanicus*. The results will be described in chapter 3.

**Table 2.1 Primer sets for real-time PCR**

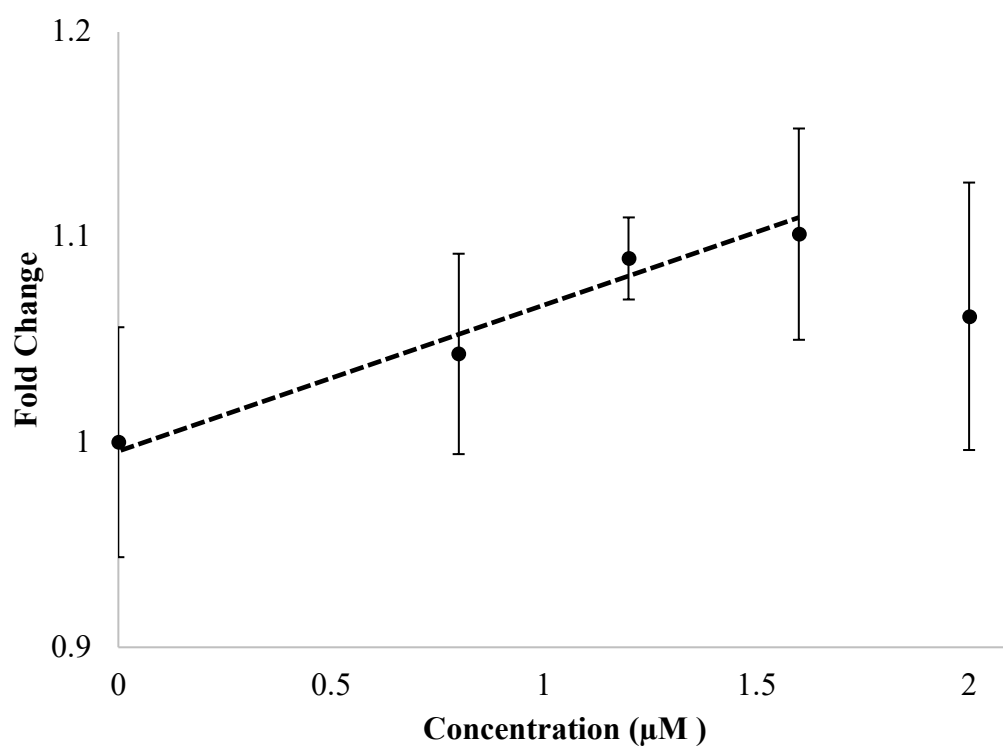
Name	Sequence (5' to 3')	Annealing Temperature (°C)
RT-F	CGACTTTTCCGGCAGGC	50.0
RT-R	GCCAACTTTCTGCGGGC	50.0
Oja- $\beta$ -actin F	GATCTGGCATCACACCTTCTACAA	50.0
Oja- $\beta$ -actin R	TACATGGCAGGGGTGTTGAAGGTC	50.0



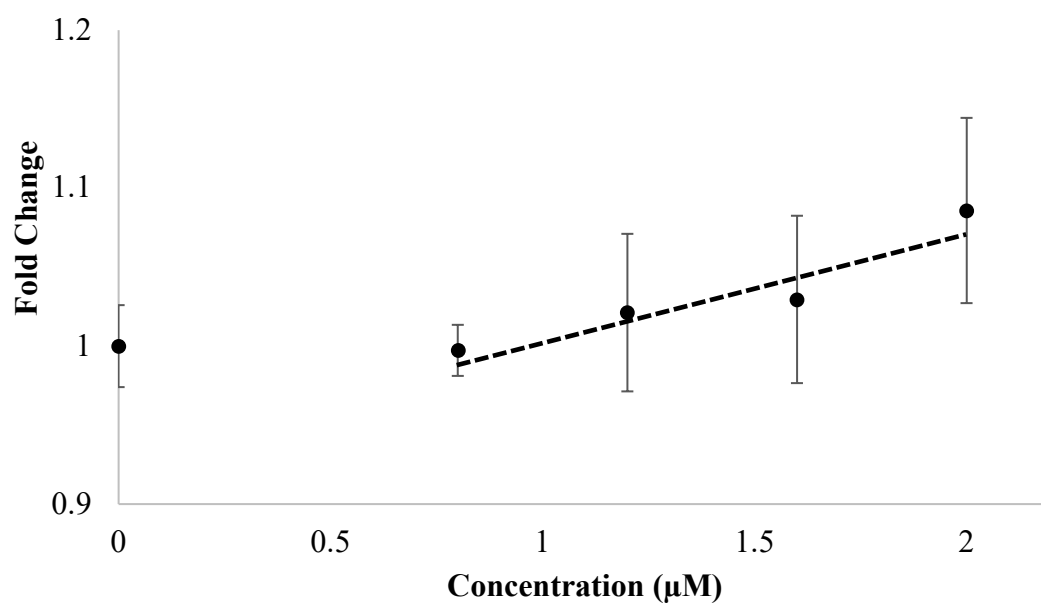
**Figure 2.2** Survival rate of Javanese medaka exposed to different concentrations of pyrene.



**Figure 2.3 Survival rates of Japanese medaka exposed to different concentrations of phenanthrene.**



**Figure 2.4** *CYP1A* expression level after exposure to pyrene for 96 hours.



**Figure 2.5** *CYP1A* expression level after exposure to phenanthrene for 96 hours.



**In this abridged version thesis,  
Chapter 3, Chapter 4, and General Discussion (Page 45-98) are excluded.**

**Reason:** Those parts are scheduled to be published in a journal article.

## ACKNOWLEDGEMENTS

In completing this thesis, I received numerous help and support from many kind-hearted and excellent people I met throughout this Ph.D. journey. I express my gratitude to God, The Most Gracious and The Most Merciful, for allowing me to carry out this challenging but exciting moment in my life to increase knowledge and prepare myself to be a better human being.

I want to express my deepest appreciation to Professor Koji Inoue, my supervisor for master and Ph.D. studies. I had a great experience to be his student and had learned a lot of skills and knowledge that I had never learned before. I am so grateful to get his guidance and wise advice on my study and research work. I also appreciate his unyielding support and warm encouragement in all aspects. I hope to maintain the right attitude and practice all the skills learned during this study period in my career and future life.

I am also grateful for having support from Dr. Masato Kinoshita from Kyoto University and got his help with the establishment of the Javanese medaka fish that is very important in this study. I am lucky to have the chance to gain knowledge on the basic technique for genome editing directly from him, one of the experts in this field. Besides, I wish to extend my special thanks to Professor Kiyoshi Naruse from National Institute for Basic Biology, for providing the Javanese medaka fish strains and advise for genotyping methods; as well as to Dr. Yusuke Takehana from Nagahama Institute of Bioscience and Technology for his guidance in the experimental procedure using Javanese medaka fish, and for providing the fish whole genome sequence. I also feel indebted to Dr. Marty Wong and members of Laboratory of Physiology for teaching me the basic technique in medaka culturing and helped in the fish maintenance process.

Besides, I would like to express my gratitude to all members of the Laboratory of Molecular Marine Biology for being very cooperative in sharing knowledge and assistance in various aspects. I received many useful comments and suggestions regarding my research work from Associate Professor Dr. Chuya Shinzato and Assistant Professor Dr. Toshiyuki Takagi. I also received much kind support from Dr. Azusa Kinjo, Ms. Sassa Mieko, Ms. Misako Seimiya, and the previous and current students in the laboratory. Apart from this, I acknowledge with gratitude all staff and lecturers in Natural Environmental Studies Department, especially Professor Shigeaki Kojima and Associate Professor Susumu Yoshizawa, for their useful advice in my study and for reviewing my thesis.

My appreciation is extended to my beloved family members, especially my husband, Abdul Rauf, and my son, Afif Izzuddin, for their unconditional support and understanding regarding my passion for study and research work. We had faced this journey together; hence I consider this achievement as our shared success. I gratefully thank my parents, Rusni Bin Hassan and Zainab Binti Md. Ali, my parents-in-law, Abdul Rahman bin Mohamad Taib and Natrah Binti Awang Yazid, and all other family members. Without their generous support and encouragement, I may not be able to complete this study smoothly.

Last but not least, I would like to express my appreciation to The University of Tokyo for giving me the chance to continue my study here and also provided the scholarship of Todai Fellowship until I finish my study. I truly hope with the knowledge I gained; I may share it with others and use it to benefit humankind, as well as contributing to making this world a better place for every creature.

## 論文の内容の要旨

論文題目 Targeted mutagenesis of the *CYP1A* gene in Javanese medaka, *Oryzias javanicus*, to understand the metabolism of organic pollutants  
(有機汚染物質代謝機構解明のためのジャワメダカ *Oryzias javanicus* *CYP1A* 遺伝子への選択的変異導入)

氏 名 スハイラ ビンティ ルスニ

本研究では、海洋生物における有機汚染物質の代謝機能を解明するために、海水魚のモデルとして有用なジャワメダカ (*Oryzias javanicus*) において、有機汚染物質代謝に重要な役割を果たすと考えられているCytochrome P450 1A (*CYP1A*) 遺伝子のcDNA配列の解析、同遺伝子の汚染物質に対する発現応答の解析、同遺伝子のノックアウト系統の作出、そして、作出したノックアウト系統の汚染物質への反応を調べ、有機汚染物質の物質ごとの有害性発現機序の違いを野生型系統とノックアウト系統の比較により明らかにできることを見出した。

人為的化学物质による海洋汚染の有害性は数十年前に顕在化したが、今日においても依然大きな問題であり、とくに発展途上国においては深刻な状況が続いている。様々な汚染物質がこれまで報告されているが、タンカー事故による石油流出や、工業廃水や農業廃水から環境に放出される汚染物質の主要なものは、有機化合物である。なかでも、多環芳香族炭化水素 (PAHs) は、石油起源の成分として、あるいは有機物の燃焼によっても生成し、環境中に広く検出される汚染物質である。

環境中の汚染物質の検出は、主として各種クロマトグラフィーなどの化学分析によって行われる。しかし、化学分析の結果から生物や生態系に対する影響を直接評価することはできない。その点を解決するには、生物を用いる環境評価、すなわち、環境水や化学成分に生物を曝露することで、環境水中の有害成分の存在や、その影響を明らかにするという

手法が有効である。生物を用いる環境評価には、メダカやゼブラフィッシュなどの小型淡水魚が従来用いられてきたが、海を対象とする研究において近年注目されている種が、ジャワメダカである。ジャワメダカは、東南アジアに広く分布するメダカの近縁種であるが、メダカ同様に維持管理が容易であり、かつ淡水より海水を好むため、海水中での汚染物質の影響や代謝機構の研究に最適である。

本研究では、海洋生物の有機汚染物質の代謝機構をより深く理解するために、ジャワメダカの*CYP1A*遺伝子に注目した。有機汚染物質の代謝は、取り込んだ汚染物質を修飾して毒性の低い物質、あるいは反応や排出が容易な物質に変換するphase Iと、変換された物質を抱合体化して排出するphase IIのふたつの段階から構成されるが、CYP1Aは、phase Iを担う主要な酵素であることが知られている。

本学位論文は、General Introduction、Chapter 1～4、およびGeneral Discussionから構成される。Chapter 1では、まずマレーシアのペナン産およびインドネシアのスラヴェシ産のジャワメダカを基礎生物学研究所のNational BioResource Project (NBRP) Medakaから入手し、それぞれから*CYP1A* cDNAを単離し、配列比較を行った。その結果、遠く離れた産地の系統間には、配列の一部に変異があることがわかった。また、主要な発現組織は肝臓であることがわかった。従って、以降の実験には、ペナン産の系統のみを用い、発現の解析は肝臓を主な対象とすることにした。

Chapter 2では、ペナン産のジャワメダカを用いて、最も構造が単純なPAHsであるPhenanthreneとPyreneをモデル化合物として曝露実験を行い、それぞれの毒性を調べた。その結果、PhenanthreneとPyreneの半数致死濃度 (LC50) はそれぞれ3.9  $\mu\text{M}$  (0.7 mg/L) および2.5  $\mu\text{M}$  (0.5 mg/L) であることがわかった。次に、LC50よりも低い濃度 (0.8～2.0  $\mu\text{M}$ ) での曝露実験を行い、肝臓中の*CYP1A* mRNA量を定量リアルタイムPCRにより測定したところ、それぞれの物質の濃度とmRNA量の間に正の相関または相関傾向が認められた。従って、*CYP1A*遺伝子は、環境中の汚染物質に応答して発現が上昇することがわかった。

Chapter 3では、Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated protein 9 (CRISPR/Cas9) システムを用いて、ペナン系統のジャワメダカの*CYP1A*遺伝子のノックアウト系統の確立を試みた。標的配列に結合するsingle guide RNA (sgRNA) を、配列のミスマッチを避けるためにChapter 1で検出された配列変異部位を避けて設計し、Cas9 RNAとともに受精卵に顕微注入を行った。注入を行った卵について、当該配列をPCRで増幅し、Heteroduplex Mobility Assay (HMA) を行って、変異導入の成功を確認した。注入し

た卵を成魚まで飼育し、相互に交配してF1世代を得た。F1世代では挿入、欠失、置換など様々なタイプの変異が検出されたが、そのなかからタンパク質のフレームシフトが起こる4塩基欠失をもつ個体を選抜し、野生型個体と交配してF2を得た。F2のなかから配列変異を持つ個体を選抜し、相互に交配することで、F3世代を得た。その際、その遺伝子型の分析を簡便に行うために、野生型および変異型の配列に特異的なPCRプライマーを設計し、ひれから抽出したDNAに対してPCRとアガロース電気泳動を行い、増幅配列の有無を確認することで、遺伝子型がわかるように工夫した。その結果、F3世代において、初めて変異を両方の染色体に持つホモ型変異体（ノックアウト個体）が得られたが、ホモ型変異体を得られる確率は低く、とくにオスのホモ型変異体は全く得られなかった。

ホモ型変異体を育てるためには、ホモ型変異体をふ化後なるべく早く選抜し、ヘテロ型変異体や、変異のない野生型とは別に飼育することが必要と考えられた。しかし、従来の尾びれを切り取ってDNAを抽出する方法では、尾びれの採取が可能な大きさに育つまで遺伝子型が判定できなかった。その問題を解決するために、本研究では「環境DNA」手法を用いて、各個体を個別の容器に入れ、その飼育水からDNAを抽出し、PCRを行うことで遺伝子型を分析する方法を考案した。それにより、各個体にひれ切除に伴うダメージを与えることなく、発生後期の胚や、稚魚の時期に遺伝子型を判定できるようになった。この方法でホモ型変異体を稚魚期に選別し、注意深く飼育することで、雌雄両方のホモ型変異体をF4、F5世代で得ることができた。

Chapter 4では、前章で確立したホモ型、ヘテロ型のCYP1A遺伝子変異個体、および野生型個体を用い、モデル化合物であるPhenanthreneとPyreneへの曝露実験を行った。有機汚染物質代謝の重要な因子であるCYP1Aを欠く個体では、汚染物質の代謝能力が低下するため、汚染物質の影響が鋭敏に検出できると期待された。実際、Pyreneに曝露した場合、死亡個体や遊泳行動の異常はホモ型変異体に最も早く現れ、野生型個体が最も影響が少ない結果となった。一方、Phenanthreneへの曝露実験では、結果は全く逆になった。すなわち、曝露の影響は野生型個体に最も早くかつ強く現れ、ホモ型変異個体には曝露の影響は殆ど認められなかった。また、曝露実験後、解剖を行って内臓の観察を行うと、肝臓の組織の崩壊が野生型個体やヘテロ型変異体において見られたのに対し、ホモ型変異体では顕著な影響は認められなかった。加えて、phase IIで生じた抱合体が集まる胆のうについても、野生型個体やヘテロ型変異体では変色と肥大が見られたのに対し、ホモ型変異体では顕著な変化は認められなかった。これらの結果は、Pyreneは、その物質そのものの有害性が高く、

CYP1Aが作用することで生じる代謝物質は元の物質より有毒性が低いのに対し、Phenanthreneは、その物質そのものの有害性は極めて低く、CYP1Aが作用して生じる代謝物のほうが有害性が高いことを示唆する。すなわち、有害性が認められる様々な物質において、それぞれの作用機序は一律ではなく、CYP1Aにより有害性の高い物質に変換されることで有毒性が発揮される物質も存在することがわかった。

以上のように、本研究で確立したCYP1A遺伝子ノックアウト系統は、有機汚染物質全般に対して鋭敏なアッセイ系統にはならなかったが、有害物質への曝露の効果を野生型系統と比較することにより、毒性物質の作用が元の化合物そのものの作用なのか、代謝によって変換された化合物の作用によるのかを区別することができることがわかった。本研究で確立したCYP1A遺伝子ノックアウト系統は、汚染物質の有毒性の機序や、魚類の汚染物質代謝のメカニズムをより深い理解く理解するために有用であると考えられる

## References



- Abdel-Shafy, H. I., & Mansour, M. S. M. (2016). A review on polycyclic aromatic hydrocarbons: Source, environmental impact, effect on human health and remediation. *Egyptian Journal of Petroleum*, 25(1), 107–123.
- Alzaga, R., Montuori, P., Ortiz, L., Bayona, J. M., & Albaigés, J. (2004). Fast solid-phase extraction–gas chromatography–mass spectrometry procedure for oil fingerprinting: application to the Prestige oil spill. *Journal of Chromatography A*, 1025, 133–138.
- Angel, J.R.J., Vinay, T. N., Raghavan, R., Thomas, D., Avunje, S., Aravind, R., Shekhar, M. S., & Vijayan, K. K. (2019). First record of the Javanese ricefish, *Oryzias javanicus* (Bleeker, 1854) (Beloniformes: Adrianichthyidae) in the natural waters of India. *Journal of Applied Ichthyology*. 35, 1034–1038.
- Ansai, S., Ochiai, H., Kanie, Y., Kamei, Y., Gou, Y., Kitano, T., Yamamoto, T., & Kinoshita, M. (2012). Targeted disruption of exogenous EGFP gene in medaka using zinc-finger nucleases. *Developmental Growth Differentiation*, 54, 546–556.
- Ansai, S., & Kinoshita, M. (2014). Targeted mutagenesis using CRISPR/Cas system in medaka. *Biology Open*, 3(5), 362–371.
- Arkoosh, M. E. Clemons, P. Huffman & A. Kagley. (2001). Increased susceptibility of juvenile Chinook salmon to vibriosis after exposure to chlorinated and aromatic compounds found in contaminated urban estuaries. *Journal of Aquatic Animal Health*, 13, 257–268.
- Bakke, T., Klungsøyr, J., & Sanni, S. (2013). Environmental impacts of produced water and drilling waste discharges from the Norwegian offshore petroleum industry. *Marine Environmental Research*, 92, 154–169.
- Besselink, H. T., Denison, M. S., Hahn, M. E., Karchner, S. I., Vethaak, A. D., & Koeman, J. H., Brouwer, A. (1998). Low inducibility of cypla activity by polychlorinated biphenyls (PCBs) in flounder (*Platichthys flesus*): Characterization of the Ah receptor and the role of CYP1A inhibition. *Toxicological Sciences*, 43(2), 161–171.
- Black, J. A., Birge, W. J., Westerman, A. G., & Francis, P. C. (1983). Comparative aquatic toxicology of aromatic hydrocarbons. *Toxicological Sciences*. 3(5), 353–358.
- Boelsterli, U. (2002). Mechanistic Toxicology: The Molecular Basis of How Chemicals Disrupt Biological Targets; Taylor Francis (UK).
- Boulanger, E., Barst, B. D., Alloy, M. M., Blais, S., Houde, M., & Head, J. A. (2019). Science of the Total Environment Assessment of environmentally contaminated sediment using a contact assay with early life stage zebra fish (*Danio rerio*). *Science of the Total Environment*, 659, 950–962.
- Boverhof, D. R., Chamberlain, M. P., Elcombe, C. R., Gonzalez, F. J., Heflich, R. H., Hernández, L. G., & Gollapudi, B. B. (2011). Transgenic animal models in

- toxicology: Historical perspectives and future outlook. *Toxicological Sciences*, 121(2), 207–233.
- Buhler DR, & Williams DE. (1989). Enzymes involved in metabolism of PAH by fishes and other aquatic animals: Oxidative enzymes (or phase I enzymes). In Varanasi U, ed, *Metabolism of PAH in the Aquatic Environment*. CRC, Boca Raton, FL, USA, pp 1–39.
- Carroll, D. (2011). Genome engineering with zinc-finger nucleases. *Genetics*, 188, 773–782.
- Chandrasegaran, S., & Carroll, D. (2015). “Origins of Programmable Nucleases for Genome Engineering,” *Journal of Molecular Biology*, 428(5), 963–989.
- Chapman, D. (1996). *Water Quality Assessments: A Guide to Use of Biota, Sediments and Water in Environmental Monitoring*. Second Edition. Cambridge: University Press.
- Cho, S. W., Kim, S., Kim, J. M., & Kim, J. S. (2013). Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. *Nature Biotechnology*, 31(3), 230–232.
- Cho, S.W., Kim, S., Kim, Y., Kweon, J., Kim, H.S., Bae, S. & Kim, J.S. (2014). Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases. *Genome Research*, 24, 132–141.
- Cong L., Ran F. A., Cox D., Lin S., Barretto R., & Habib N. (2013). Multiplex genome engineering using CRISPR/Cas systems. *Science*, 339, 819–823.
- Diotel N, Le Page Y, Mouriec K, Tong SK, Pellegrini E, Vaillant C, Anglade I, Brion F, Pakdel F, Chung BC, & Kah O (2010). Aromatase in the brain of teleost fish: expression, regulation and putative functions. *Front Neuroendocrinol* 31, 172–192.
- Di Giulio, R. T., Benson, W. H., Sanders, B. M., & Veld, P. A. V. (1995). In *Fundamentals of Aquatic Toxicology: Effects, Environmental Fate, and Risk Assessment*; 2nd ed.; Rand, G., Ed.; Taylor & Francis, Washington, pp 523–561.
- Ekpe, O. D., Kim, K. Y., Jung, J. H., Yim, U. H., & Oh, J. E. (2020). Formation and distribution of phenanthrene and its metabolites (monohydroxy-phenanthrenes) in Korean rockfish (*Sebastes schlegelii*). *Environmental Pollution*, 263, 114–588.
- Franco, M. E., & Lavado, R. (2019). Science of the Total Environment Applicability of in vitro methods in evaluating the biotransformation of polycyclic aromatic hydrocarbons (PAHs) in fish: Advances and challenges. *Science of the Total Environment*. 671, 685–695.

- Gaaied, S., Oliveira, M., Bihanic, F.L., Cachot, J., & Banni, M. (2019). Gene expression patterns and related enzymatic activities of detoxification and oxidative stress systems in zebrafish larvae exposed to the 2,4-dichlorophenoxyacetic acid herbicide. *Chemosphere*, 224, 289-297.
- Goldstone JV, Goldstone HM, Morrison AM, Tarrant A, Kern SE, Woodin BR, & Stegeman JJ (2007). Cytochrome P450 1 genes in early deuterostomes (tunicates and sea urchins) and vertebrates (chicken and frog): origin and diversification of the CYP1 gene family. *Molecular Biology and Evolution*, 24, 2619-2631
- Goksoyr, A. (1995). Use of cytochrome P450 1A (CYP1A) in fish as a biomarker of aquatic pollution. *Archives of Toxicology*, 17, 80–95.
- Goks, A. (2016). Use of cytochrome P450 1A (CYP1A) in fish as a biomarker of aquatic pollution. *Archives of Toxicology*, 4274.
- Guengerich FP, & Shimada T. (2008). Oxidation of toxic and carcinogenic chemicals by human cytochrome P450 enzymes. *Chemical Research in Toxicology*, 4, 391.407.
- Hampton, J.A., Lantz, C.R. & Hinton DE (1989). Functional units in rainbow trout (*Salmo gairdneri*) liver III. Morphometric analysis of parenchyma, stroma and component cell types. *American Journal of Anatomy*, 185, 58-73.
- Hankinson O. (1995). The aryl hydrocarbon receptor complex. *Annual Review of Pharmacology and Toxicology*, 35, 307.340.
- Hawkins, S. A., Billiard, S. M., Tabash, S. P., Brown, R. S., & Hodson, P. V. (2002). Altering cytochrome P4501A activity affects polycyclic aromatic hydrocarbon metabolism and toxicity in rainbow trout (*Oncorhynchus mykiss*). *Environmental Toxicology and Chemistry*, 21(9), 1845–1853.
- Hecht, S.S., Carmella, S.G., Villalta, P.W. & Hochalter, J.B. (2010). Analysis of phenanthrene and benzo[a]pyrene tetraol enantiomers in human urine: relevance to the bay region diol epoxide hypothesis of benzo[a]pyrene carcinogenesis and to biomarker studies. *Chemkumarical Research in Toxicology*, 23, 900-908.
- Hendon, L.A., Carlson, E.A., Manning, S., & Brouwer, M., (2008). Molecular and developmental effects of exposure to pyrene in the early life-stages of *Cyprinodon variegatus*. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 147, 205–215.
- Hinton, D.E. (1994). Cells, cellular responses and their markers in chronic toxicity of fishes. In: Malins DC, Ostrander GK, eds. *Aquatic Toxicology*. Boca Raton, USA: Lewis Publishers, 207-239.
- Hwang, H. M. & Wade, T.L. (2008). Aerial distribution, temperature dependent seasonal variation, and sources of polycyclic aromatic hydrocarbons in pine needles from the Houston metropolitan are, Texas, USA. *Journal of Environmental Science and*

*Health Part A: Toxic/Hazardous Substances and Environmental Engineering*, 43, 1243-1251.

- Imbery, J. J., Buday, C., Miliano, R. C., Shang, D., Round, J. M., Kwok, H., & Aggelen, G.V., Helbing, C. C. (2019). Evaluation of Gene Bioindicators in the Liver and Caudal Fin of Juvenile Pacific Coho Salmon in Response to Low Sulfur Marine Diesel Seawater-Accommodated Fraction Exposure. *Environmental Science and Technology*, 53(3), 1627–1638.
- Incardona, J.P., Carls, M.G., Holland, L., Linbo, T.L., Baldwin, D.H., Myers, M.S., Peck, K.A., Tagal, M., Rice, S.D., & Scholz, N.L., (2015). Very low embryonic crude oil exposures cause lasting cardiac defects in salmon and herring. *Scientific Reports*, 5, 13499.
- Ingelman-Sundberg, M., Sim, S. C., Gomez, A. & Rodriguez-Antona, C. (2007). Influence of cytochrome P450 polymorphisms on drug therapies: Pharmacogenetic, pharmacoepigenetic and clinical aspects. *Pharmacology and Therapeutics*, 116(3), 496-526.
- Inoue, K. & Takei, Y. (2002). Diverse adaptability in *Oryzias* species to high environmental salinity. *Zoological Science*, 19, 727-734.
- Inoue, K. and Takei, Y. (2003). Asian medaka fishes offer new models for studying seawater adaptation. *Comp. Biochem. Physiol. B* 136, 635-645.
- Iwamatsu, T., Imaki, A., Kawamoto, A. & Inden, A. (1982). On *Oryzias javanicus* collected at Jakarta. *Zoologicae Japonenses*, 55, 190-198.
- Jonsson, G., Beyer, J., Wells, D., & Ariese, F. (2003). The application of HPLC-F and GC-MS to the analysis of selected hydroxy polycyclic hydrocarbons in two certified fish bile reference materials. *Journal of Environmental Monitoring*, 5, 513-520.
- Jothy, A. A. (1976). Report on marine pollution problems in Malaysia. In *International Workshop on Marine Pollution in East Asian Waters*, 25.
- Joung, J.K. & Sander, J.D. (2013). TALENs: a widely applicable technology for targeted genome editing. *Nature Reviews Molecular Cell Biology*, 14, 49-55.
- Karami, A., Eghtesadi, P., Negarestan, H., Ranaei siadat, O. & Maghsoudlou, A. (2008). The role of three dimensional geometric descriptors of selected PAHs on inducing mortality in juvenile angelfish (*Pterophyllum scalare*). *Journal of Biological Sciences*, 8, 314-320.
- Kasmin, S. (2010). Enforcing ship-based marine pollution for cleaner sea in the Strait of Malacca. *Environment Asia*, 3 (Special issue), 61-65.

- Katsumura, T., Oda, S., Nakagome, S., Hanihara, T., Kataoka, H., Mitani, H., Kawamura, S., Oota, H. (2014) Natural allelic variations of xenobiotic-metabolizing enzymes affect sexual dimorphism in *Oryzias latipes*. *Proceedings of the Royal Society B-Biological Sciences*, 281, 20142259.
- Katoh, K., & Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Molecular Biology and Evolution*, 30(4), 772–780.
- Kerns, E. H., Rourick, R. A., Volk, K. J., & Lee, M. S. (1997). Buspirone metabolite structure profile using a standard liquid chromatographic-mass spectrometric protocol. *Journal of Chromatography B: Biomedical Sciences and Applications*, 698, 133-145.
- Kim, R. O., Kim, B. M., Hwang, D. S., Au, D. W. T., Jung, J. H., Shim, W. J., & Lee, J. S. (2013). Evaluation of biomarker potential of cytochrome P450 1A (CYP1A) gene in the marine medaka, *Oryzias melastigma* exposed to water-accommodated fractions (WAFs) of Iranian crude oil. *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology*. 157(2), 172–182.
- Kinoshita, M., Kani, S., Ozato, K. & Wakamatsu, Y. (2000). Activity of the medaka translation elongation factor 1 $\alpha$ -A promoter examined using the GFP gene as a reporter. *Developmental Growth and Differentiation*, 42, 469-478.
- Kinoshita, M., Murata, K., Naruse, K., & Tanaka, M. (2009). Medaka Biology, Management, and Experimental protocols. Iowa: Wiley-Blackwell. 444 p.
- Kinoshita, M. (2015). Application Note No. 1 Genome Editing and Creating Mutant Strains in Medaka-Application of MultiNA TM-36A Life Science Genome Editing and Creating Mutant Strains in Medaka-Application of MultiNA TM. 36, 1–12.
- Kubota, A., Iwata, H., Tanabe, S., Yoneda, K., & Tobata, S. (2005). Hepatic CYP1A induction by dioxin-like compounds, and congener-specific metabolism and sequestration in wild common cormorants from Lake Biwa, Japan. *Environmental Science and Technology*, 39(10), 3611–3619.
- Kumar, V. & Majumdar, D. (2016). Pharmacogenomics – The New Trend for Personalized Medicine. *Indian Journal of Pharmaceutical and Biological Research (IJPBR)*, 4(1), 39–49.
- Lin, V., & Majumdar, D. (2016). Indian Journal of Pharmaceutical and Biological Research (IJPBR) Pharmacogenomics – The New Trend for Personalized Medicine. *Drug Responses*, 4(1), 39–49.
- Latimer, J., & Zheng, J. (2003). The sources, transport, and fate of PAH in the marine environment, in: P.E.T. Douben (Ed.), PAHs: an ecotoxicological perspective, John Wiley and Sons Ltd, New York.

- Lee, B.-Y., Park J. C., Kim, M.-S., Choi, B.-S., Kim, D.-H., Lim, J.-S., Yum, S., Hwang, U.-K., Gyoung Nah, J., Lee, J.-S. (2020) The genome of the Java medaka (*Oryzias javanicus*): Potential for its use in marine molecular ecotoxicology. *Marine Pollution Bulletin* 154, 111118.
- Lee, M. L., Novotny, M. V., & Bartle K. D. (1981). Analytical chemistry of polycyclic aromatic compounds. Academic Press. New York.
- Leggieri, L. R., De Anna, J. S., Cárcamo, J. G., Cerón, G. A., Darraz, L. A., Panebianco, A., & Luquet, C. M. (2019). Gills CYP1A of *Oncorhynchus mykiss* as a sensitive biomarker of crude oil pollution in freshwater environments. *Environmental Toxicology and Pharmacology*, 67, 61–65.
- Lin, T.C., Chang, F.H., Hsieh, J.H., Chao, H.R. & Chao, M.R. (2002). Characteristics of polycyclic aromatic hydrocarbons and total suspended particulate in indoor and outdoor atmosphere of a Taiwanese temple. *Journal of Hazardous Materials*, A95, 1-12.
- Magtoon, W. & Termvidchakorn, A. (2009). A revised taxonomic account of ricefish *Oryzias* (Beloniformes; Adrianichthyidae), in Thailand, Indonesia and Japan. *The Natural History Journal of Chulalongkorn University*, 9, 35-68.
- Mansuy D. (1998). The great diversity of reactions catalyzed by cytochromes P450. *Comparative Biochemistry and Physiology*, 121C,5.14
- Means J. C. (1998). Compound-specific gas chromatographic/mass spectrometric analysis of alkylated and parent polycyclic aromatic hydrocarbons in waters, sediments, and aquatic organisms. *Journal of AOAC International*, 81, 657–672.
- Millemann, R.E., Birge, W.J., Black, J.A., Cushman, R.M., Daniels, K.L., Franco, P.J., Giddings, J.M., McCarthy, J.F., & Stewart, A.J. (1984). Comparative acute toxicity of aquatic organisms of components of coal-derived synthetic fuels. *Transactions of the American Fisheries Society*, 113, 74–85.
- Menzie, A.C., Potocki, B.B., & Santodonato, J. (1992). Environmental Science and Technology, 26, 1278–1284.
- Mohamat-Yusuff, F., Sarah-Nabila, A. G., Zulkifli, S. Z., Azmai, M. N. A., Ibrahim, W. N. W., Yusof, S., & Ismail, A. (2018). Acute toxicity test of copper pyrithione on Javanese medaka and the behavioural stress symptoms. *Marine Pollution Bulletin*, 127, 150–153.
- Moore, M.N. (1979). Cellular responses to polycyclic aromatic hydrocarbons and phenobarbital in *Mytilus edulis*. *Marine Environmental Research*, 2, 225-263.
- Moore, M.N. & Farrar, S.V. (1985). Effects of polynuclear aromatic hydrocarbons on lysosomal membranes in molluscs. *Marine Environmental Research*, 17, 222-224.

- Mussolino, C., & Cathomen, T. (2012). "TALE nucleases: Tailored genome engineering made easy," *Current Opinion in Biotechnology*, 23(5), 644–650.
- Naruse, K., Shima, A., Matsuda, M., Sakaizumi, M., Iwamatsu, T., Soeroto, B. & Uwa, H. (1993). Description and phylogeny of rice fish and their relatives belonging to the suborder Adrianichthyoidei in Sulawesi, Indonesia. *Fish Biology Journal, Medaka*, 5, 11-15.
- Naruse, K., Chisada, S., Sasado, T., & Takehana, Y. (2016). Medaka as model animal and current status of medaka biological resources, 2(1), 31–34.
- Nebert, D.W., & Karp, C.L. (2008). Endogenous function of the aryl hydrocarbon receptor (AHR): intersection of cytochrome P450 1 (CYP1)-metabolized eicosanoids and AHR biology. *Journal of Biological Chemistry*. 283, 36061-36065.
- Nelson DR, Koymans L, & Kamataki T. (1996). P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics*, 6(1), 1-42.
- Oh, S. M., Byung, T. R., Ha, R. K., Choi, K. & Chung, K. H. (2009). Molecular cloning of CYP1A gene and its expression by benzo(a)pyrene from Goldfish (*Carassius auratus*). *Environmental Toxicology*, 24(3), 225-234.
- Oliveira, M., Gravato, C., & Guilhermino, L. (2012). Acute toxic effects of pyrene on *Pomatoschistus microps* (Teleostei, Gobiidae): Mortality, biomarkers and swimming performance. *Ecological Indicators*, 19, 206–214.
- Provati, E., Genovese, P., Lombardo, A., Magnani, Z., Liu, P.-Q., & Reik, A. (2012). Editing T cell specificity towards leukemia by zinc finger nucleases and lentiviral gene transfer. *Nature Medicine*, 18 (5), 807-815.
- Pennisi, E. (2013). The CRISPR Craze. *Science*, 341, 833-836.
- Rand, G. M., Wells, P. G., & McCarthy, L. S. (1995). Introduction to Aquatic Toxicology. In G.M. Rand, Fundamentals of Aquatic Toxicology. 2nd ed.; Rand, G., Ed.; Taylor & Francis. Washington. pp. 3-67.
- Reynaud, S. & P. Deschaux. (2006). The effect of polycyclic aromatic hydrocarbons on the immune system of fish: a review. *Aquatic Toxicology*, 77: 229-238.
- Rewitz, K. F., Styris, B., Løbner-Olesen, A., & Andersen, O. (2006). Marine invertebrate cytochrome P450: Emerging insights from vertebrate and insect analogies. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 143(4), 363–381.
- Richardson, K. L., Lopez Castro, M., Gardner, S. C., & Schlenk, D. (2010). Polychlorinated biphenyls and biotransformation enzymes in three species of sea

- turtles from the baja California peninsula of Mexico. *Archives of Environmental Contamination and Toxicology*. 58(1), 183–193.
- Rigaud, C., Eriksson, A., Krasnov, A., Wincent, E., Pakkanen, H., Lehtivuori, H., Ihalainen, J., & Vehniäinen, E. (2020). Retene, pyrene and phenanthrene cause distinct molecular-level changes in the cardiac tissue of rainbow trout (*Oncorhynchus mykiss*) larvae, part 1 – Transcriptomics. *Science of the Total Environment*, 746, 141161.
- Roberts, T.R. (1998). Systematic observations on tropical Asian medakas or rice fishes of the genus *Oryzias*, with descriptions of four new species. *Ichthyological Research*, 45, 213-324.
- Roy, N. K., Candelmo, A., Dellatorre, M., Chambers, R. C., Nádas, A., & Wirgin, I. (2018). Characterization of AHR2 and CYP1A expression in Atlantic sturgeon and short nose sturgeon treated with coplanar PCBs and TCDD. *Aquatic Toxicology*, 197, 19–31.
- Sadek, C.M., & Allen-Hoffmann, B.L. (1994). Suspension mediated induction of hepatic CYP1A-1 expression is dependent on the Ah receptor signal transduction pathway. *The Journal of Biological Chemistry*, 269(50), 31505-31509.
- Sarasquete, C. & Segner, H. (2000). Cytochrome P4501A (CYP1A) in teleostean fishes. A review of immunohistochemical studies. *In Science of the Total Environment*, 247, 313-332.
- Sakaki, T., Shinkyo, R., Takita, T., Ohta, M., & Inouye, K. (2002). Biodegradation of polychlorinated dibenzo-p-dioxins by recombinant yeast expressing rat CYP1A subfamily. *Archives of Biochemistry and Biophysics*. 401(1), 91–98.
- Sapranaukas, R., Gasiunas, G., Fremaux, C., Barrangou, R., Horvath, P., & Siksnys, V. (2011) “The *Streptococcus thermophilus* CRISPR/Cas system provides immunity in *Escherichia coli*,” *Nucleic Acids Research*, 39(21), 9275–9282.
- Segal, D., & Meckler, J. (2003). Genome Engineering at the Dawn of the Golden Age. *Annual Review of Genomics Human Genetics*, 14, 135-58.
- Segner, H. (1998). Isolation and culture of teleost hepatocytes. *Comparative Biochemistry and Physiology*, 120A, 71-81.
- Sette, C. B., Pedrete, T. D. A., Felizzola, J., Nudi, A. H., Sco, A. D. L., & Wagener, A. D. L. R. (2013). Formation and identification of PAHs metabolites in marine organisms. 91, 2–13.
- Shi, X., He, C., Zuo, Z., Li, R., Chen, D., Chen, R., & Wang, C. (2012). Pyrene exposure influences the craniofacial cartilage development of *Sebastiscus marmoratus* embryos. *Marine Environmental Research*, 77, 30–34.



- Song, G., Jia, M., Chen, K., Kong, X., Khattak, B., Xie, C. & Mao, L. (2016). CRISPR/Cas9: A powerful tool for crop genome editing. *The Crop Journal*, 4(2), 75-82.
- Stegeman, J. J. & Hahn, M. E. (1994). Biochemistry and molecular biology of monooxygenases: Current perspectives on forms, functions, and regulation of cytochrome P450 in aquatic species. *Aquatic toxicology: molecular, biochemical and cellular perspectives* D. C. Malins, Ostrander, G.K., Lewis Publishers 87-206.
- Sugio, A., Yang, B., Zhu, T., & White, F. F. (2007). "Two type III effector genes of *Xanthomonas oryzae* pv. *oryzae* control the induction of the host genes OsTFIIAgamma1 and OsTFX1 during bacterial blight of rice.," *Proceedings of the National Academy of Sciences of the United States of America*, 104, (25), 10720–5.
- Sun, L., Zuo, Z., Chen, M., Chen, Y., & Wang, C. (2015). Reproductive and transgenerational toxicities of phenanthrene on female marine medaka (*Oryzias melastigma*). *Aquatic Toxicology*, 162, 109–116.
- Takasu, Y., Kobayashi, I., Beumer, K., Uchino, K., Sezutsu, H., & Sajwan, S., et al. (2010). Targeted mutagenesis in the silkworm *Bombyx mori* using zinc finger nuclease mRNA injection. *Insect Biochemistry and Molecular Biology*, 40, 759-765.
- Takehana, Y., Zahm, M., Cabau, C., Klopp, C., Roques, C., Bouchez, O., Donnadiou, C., Barrachina, C., Journot, L., Kawaguchi, M., Yasumasu, S., Ansai, S., Naruse, K., Inoue, K., Shinzato, C., Scharl, M., Guiguen, Y., Herpin, A. (2020). Genome sequence of the euryhaline Javafish Medaka, *Oryzias javanicus*: A small aquarium fish model for studies on adaptation to salinity. *G3: Genes Genomics Genetics* 10, 907-915.
- Tuan, T., Kaminishi, Y., Funahashi, A., Mohamed, E. A. H. & Abeer, A. I. (2014). cDNA cloning, characterization and expression of cytochrome P450 family 1 (CYP1A) from Javanese medaka, *Oryzias javanicus* by environmental conditions. *African Journal of Biotechnology*, 13(18), 1898-1909.
- Van Veld, P. A., Vetter, R. D., Lee, R. F. & Patton, J. S. (1987). Dietary fats inhibits the intestinal metabolism of the carcinogen benzo[a]pyrene in fish. *The Journal of Lipid Research*, 28, 810-817.
- Varanasi U, Nishimoto M, Reichert WL, Le Eberhart B-T. (1986). Comparative metabolism of benzo[a]pyrene and covalent binding to hepatic DNA in English sole, starry flounder, and rat. *Cancer Research*, 46, 3817–3824.
- Wang, Y., Zhang, W., Dong, Y., Fan, R., Sheng, G., & Fu, J. (2005). Quantification of several monohydroxylated metabolites of polycyclic aromatic hydrocarbons in urine by highperformance liquid chromatography with fluorescence detection. *Analytical and Bioanalytical Chemistry*, 383, 804-809.

- Whitehead, A., Dubansky, B., Bodinier, C., Garcia, T.I., Miles, S., Pilley, C., Raghunathan, V., Roach, J.L., Walker, N., Walter, R.B., Rice, C.D., & Galvez, F. (2012). Genomic and physiological footprint of the Deepwater Horizon oil spill on resident marsh fishes. *Proceedings of the National Academy of Sciences*, 109, 20298–20302.
- Williams, R., Meares, J., Brooks, L., Watts, R., & Lemieux, P. (1994). Priority pollutant PAH analysis of incinerator emission particles using HPLC and optimized fluorescence detection. *International Journal of Environmental Analytical Chemistry*, 54, 299-314.
- Williams, T. D., Lee, J., Sheader, D. L. & Chipman, J. K. (2000). The cytochrome P450 1A gene (CYP1A) from European flounder (*Platichthys flesus*), analysis of regulatory regions and development of a dual luciferase reporter gene system. *Marine Environmental Research*, 50, 1-6.
- Wolinska, L., Brzuzan, P., Wozny, M. and Gora, M. (2011). Preliminary study on adverse effects of phenanthrene and its methyl and phenyl derivatives in larval zebrafish, *Danio rerio*. *Environmental Biotechnology*, 2011, 26-33.
- Woo, S., Yum, S., Park, H. S., Lee, T. K. & Ryu, J. C. (2009). Effects of heavy metals on antioxidants and stress-responsive gene expression in Javanese medaka (*Oryzias javanicus*). *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology*, 149(3), 289-299.
- Woo, S. J., & Chung, J. K. (2020). Cytochrome P450 1 enzymes in black rockfish, *Sebastes schlegelii*: Molecular characterization and expression patterns after exposure to benzo[a]pyrene. *Aquatic Toxicology*, 226(August 2019), 105566.
- Yang, J., Zhao, H., & Ming, K. (2017). Toxic effects of polybrominated diphenyl ethers (BDE 47 and 99) and localization of BDE-99 – induced cyp1a mRNA in zebra fish larvae. *Toxicology Reports*, 4, 614–624.
- Yeh, Y. C., Kinoshita, M., Ng, T. H., Chang, Y. H., Maekawa, S., Chiang, Y. A., Aoki, T., & Wang, H. C. (2017). Using CRISPR/Cas9-mediated gene editing to further explore growth and trade-off effects in myostatin-mutated F4 medaka (*Oryzias latipes*). *Scientific Reports*, 7(1), 1–13.
- Yusof, S., Ismail, A., Koito, T., Kinoshita, M., & Inoue, K. (2012). Occurrence of two closely related ricefishes, Javanese medaka (*Oryzias javanicus*) and Indian medaka (*O. dancena*) at sites with different salinity in Peninsular Malaysia. *Environmental Biology of Fishes*, 93(1), 43–49.
- Yusof, S., Ismail, A., & Rahman, F. (2013). Distribution and localities of Java medaka fish (*Oryzias javanicus*) in Peninsular Malaysia. *Malayan Nature Journal*. 65(3), 38-46.

- Yusof, S., Ismail, A., & Alias, M. S. (2014). Effect of glyphosate-based herbicide on early life stages of Java medaka (*Oryzias javanicus*): A potential tropical test fish. *Marine Pollution Bulletin*, 85(2), 494-498.
- Zapata-Pérez, O., Gold-Bouchot, G., Ortega, A., López, T., & Albores, A. (2002). Effect of pyrene on hepatic cytochrome P450 1A (CYP1A) expression in nile tilapia (*Oreochromis niloticus*). *Archives of Environmental Contamination and Toxicology*, 42(4), 477-485.
- Zhang, Y., Wang, C., Huang, L., Chen, R., Chen, Y., & Zuo, Z. (2012). Low-level pyrene exposure causes cardiac toxicity in zebrafish (*Danio rerio*) embryos. *Aquatic Toxicology*, 114, 119-124.
- Zhao, Y., Wang, X., Lin, X., Zhao, S., & Lin, J. (2017). Comparative developmental toxicity of eight typical organic pollutants to red sea bream (*Pagrosomus major*) embryos and larvae. *Environmental Science and Pollution Research*, 24(10), 9067-9078.

