ANALYSIS OF DNA POLYMORPHISM WITHIN *Rattus argentiventer* (ROBINSON & KLOSS) POPULATION IN PINANG TUNGGAL PADDY FIELD, PULAU PINANG.

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ABSTRACT

The purpose of this study is to categorise the trapped rice field rat, *Rattus argentiventer* in Pinang Tunggal paddy field into their respective haplotype groups and to investigate if mutation occurs in the VKORC1 gene of *Rattus argentiventer* that was exposed to anticoagulant rodenticide. The study also aims to determine the warfarin residue level in the liver of the rat samples. Prior to haplotype analysis, the DNA of the captured rodents was extracted and the HVR1 of mitochondrial DNA was amplified. Furthermore, Restriction Fragment Length Polymorphism (RFLP) analysis was used in order to investigate the mutation of VKORC1 gene. The rats' livers were also being used to analyse the residue level of warfarin intake using the High Performance Liquid Chromatography (HPLC). The results showed that two haplotype groups among the 58 samples were detected and no mutation was found in the entire trapped rodent. The results also demonstrated that the amount of warfarin level in the liver was at 1.421 min of retention time. This is relatively low in concentration, which in turn assured that there was no mutation detected in the earlier RFLP analysis. In conclusion, low haplotype numbers and no mutation of VKORC1 gene was detected in the trapped *Rattus argentiventer*. Implication of this research is that it prepares as a reference for future studies on molecular profile of *Rattus argentiventer* in Pinang Tunggal paddy field, Pulau Pinang.

ANALISIS POLIMORPHISME DNA DALAM POPULASI *Rattus argentiventer* (ROBINSON & KLOSS) DI SAWAH PADI PINANG TUNGGAL, PULAU PINANG.

ABSTRAK

Kajian ini dijalankan bertujuan mengkategori tikus sawah, *Rattus argentiventer* yang telah ditangkap di sawah padi Pinang Tunggal mengikut kumpulan haplotipnya dan untuk mengenalpasti sekiranya berlaku mutasi pada gen VKORC1 pada tikus sawah yang terdedah kepada racun tikus antikoagulan. Selain itu, kadar sisa warfarin pada sampel hati roden yang telah diaplikasi dengan warfarin turut diuji. Sebelum analisis haplotip dilakukan, DNA daripada roden yang ditangkap telah pun diekstrak dan HVR1 pada mitokondrial DNA telah digandakan. Kaedah analisis *Restriction Fragment Length Polymorphism* (RFLP) telah digunapakai untuk memeriksa kewujudan mutasi pada gen VKORC1 bagi tikus sawah berkenaan, dan sampel hati tikus tersebut turut digunakan untuk menganalisis kadar sisa warfarin yang telah diambil oleh *Rattus argentiventer* dengan mengaplikasi kaedah *High Performance Liquid Chromatography* (HPLC). Menerusi kajian ini, pengkaji telah menemui dua kumpulan haplotip hasil daripada 58 sampel ujikaji dan tiada mutasi dijumpai pada kesemua roden yang ditangkap. Keputusan ujikaji turut menunjukkan kadar sisa warfarin di dalam hati adalah 1.421 minit bagi masa pengekalannya. Masa yang dicatatkan itu merujuk kepada kadar kepekatan yang rendah, sekaligus mengesahkan ketidakhadiran mutasi pada analisis RFLP yang telah dijalankan. Kesimpulannya, jumlah haplotip yang rendah dan ketiadaan mutasi pada gen VKORC1 telah dikenalpasti pada *Rattus argentiventer* yang ditangkap. Implikasi daripada dapatan kajian yang telah dijalankan ini adalah sebagai rujukan kepada kajian akan datang berkenaan profil molekular bagi *Rattus argentiventer* di sawah padi Pinang Tunggal, Pulau Pinang.

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AMOVA	Analysis of Molecular Variance
ARMS-PCR	Amplification Restriction Mutation System – Polymerase Chain Reaction
BP	Base pair
DAD	Diode array detector
DNA	Deoxyribonucleic Acid
EDTA	Ethylene – diamine – tetraacetic acid
GC	Gas Spectrometry
GC / MS	Gas Spectrometry / Mass Spectrometry
HA	Hectare
HPLC	High Performance Liquid Chromatography
HVR1	Hyper Variable Region 1
IPM	Integrate Pest Management
LU	Luminescence Unit
MARDI	Malaysian Agricultural Research and Development Institute
MtDNA	Mitochondrial DNA
PCR	Polymerase Chain Reaction
REBASE	Restriction Enzyme Database
RFLP	Restriction Fragment Length Polymorphism
RM	Ringgit Malaysia
SNP	Single Nucleotide Polymorphism
TAE	Tris – Acetate – EDTA
TBS	Trap Barrier System

UV	Ultraviolet
VKOR	Vitamin K 2,3 Epoxide Reductase
VKORC1	Vitamin K 2,3 Epoxide Reductase Complex 1

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CHAPTER 1

INTRODUCTION

1.1 Introduction

The main culprit identified for the damages of rice field crop in the Southeast Asia is the *Rattus argentiventer* (Wood, 2001). Here in Malaysia, 5% of rice production is considered to be lost due to this damaging pest. Our neighbouring country, Indonesia, face the most destruction in their rice field with more than 17% losses, compared to other countries in the region. This has in turn effect the economic growth of the developing countries because of the funds being allocated to fight against the rodent pests. Furthermore, the rice is considered the staple food for humans and it is taken in a huge amount daily. The demand for rice production is increasing and this has been the national interest. Under the Ministry of Agriculture and Agro – Based Industry,

the rice production in Malaysia is put under the control management of Malaysian Agricultural Research and Development Institute (MARDI). Here, all research regarding the rice is done and makes the production still sufficient to encounter high demand by the nation.

The ecosystem of a paddy field usually consists of insects, birds, owls, snakes and rats. The rice field rat is one of the major pests in the paddy field since it spoils the paddy from the beginning of tillering until ripening stage. This rodent pest population can be controlled in three ways including biological control, chemical control and integrated pest control. In terms of chemical control, the paddy field used in this study had long ago being treated with warfarin in order to reduce the target population which is the *Rattus argentiventer*. Rodenticide comes in three physical appearances such as powder, baits and fumigant gases. In addition to warfarin, other examples of rodenticide that had been used by rice farmers are chlorophacinone, brodifacoum and bromadiolone (Brown *et al.*, 2006).

Warfarin is a form of anticoagulant rodenticide, which was first introduced during 1940s. It is the first generation rodenticides, which interferes the Vitamin K 2,3 epoxide reductase (VKOR) of the Vitamin K cycle in the liver. Inhibition of VKOR by anticoagulant reduces carboxylation of clotting factor and gives way to the coagulation process. Long time application of anticoagulant has led to a phenomenon called anticoagulant resistance in rodents. Mutation in Vitamin K 2,3 epoxide reductase complex 1 (VKORC1) is involved in the resistance to anticoagulants in rats.

These mutations decrease the sensitivity of VKORC1 to warfarin, increasing the efficiency of coagulation process in warfarin-exposed rats (Haniza, 2008).

The deoxyribonucleic acid (DNA) profile of rats is also very important for us to know besides their behaviour. This can be analysed by using numerous genetic markers such as Restriction Fragment Length Polymorphism (RFLP), Single Nucleotide Polymorphism (SNP), Microsatellite and many more (Andreas & Francis, 2005). Profile of the mitochondrial DNA (mtDNA) is also one of the approaches that have been regularly used in molecular studies. MtDNA sequencing usually consists of Polymerase Chain Reaction (PCR) amplification, DNA sequencing reactions, separation using electrophoresis, and data collection and sequence analysis. Once sequencing is done, the haplotype of each rat samples was categorised into their respective group (Haniza *et al*., 2015). Besides, the liver is also further being used to analyse the residue level of warfarin intake by the *Rattus argentiventer* in the rice field.

The rodent trapping activity was held at the rice field located in MARDI Seberang Perai, Pinang Tunggal, Pulau Pinang because this location is well known in Malaysia that produces a huge amount of paddy annually (Singleton, 1994; Department of Agriculture Peninsular Malaysia, 2014) whereas all molecular works was performed in the Animal Biotechnology Laboratory in Faculty of Science and Mathematics situated at Sultan Azlan Shah Campus of Sultan Idris Education University, Tanjung Malim.

1.2 Problem Statement

To date, plentiful efforts have been done by the Malaysian government through their agencies to educate rice cultivators and researchers about the tragic lost and damage caused by rodent, both in health and economic aspects. The significance that come up from this circumstances is how the local farmers can control the expanding amount of rodent population through effective mechanism by means of biological control, chemical control and the integration of both controls. For that, it is important to be familiar with the molecular profile of the *Rattus argentiventer* in conjunction with the ecological parts of this species so that we can contribute to shrink these rodent pest population effectively. In addition, educating rice farmers can in turn increase their rice production and also increase their earnings.

In addition, there had been numerous studies conducted in many other parts of the world to examine the mutation of VKORC1 gene that is caused by rodenticides in the Norway rats, black rats, and many other types of rats but not in Malaysia (Diaz *et al*., 2010; Haniza *et al*., 2015; Kohn *et al*., 2008). Remarkably, given the undoubted field damage and health effects of the rodent pest, the *Rattus argentiventer* have acquired little attention from researchers as molecular data lacks for the species. Study had been conducted long time ago in Malaysia, regarding the physical barrier with multiple capture traps to catch the *Rattus argentiventer* (Singleton & Petch, 1994).

The recorded death cases concerning rodent is at fear nowadays. Rodent, apart from being a pest had make the Malaysian community unease when it brings together hazardous health effect namely Leptospirosis, Hantavirus Pulmonary Syndrome, Rat – Bite Fever and many more (Ministry of Health Malaysia, 2011). William Fletcher first detected the case regarding Leptospirosis in 1925. Due to the cyclic wet and warm weather throughout the year in the region, it favours the outbreak of this endemic disease. The number of reported cases in public hospitals is expanding annually with death toll increasing each year since 2004 with 20 victims to 62 victims in 2009 (Ministry of Health Malaysia, 2011; Jalii & Bahaman, 2004).

According to the paddy statistics 2014 by the Department of Agriculture Peninsular Malaysia, the amount of rice imported from our neighbouring countries was one third of the total amount of rice produced internally. From that, 1,645 hectare (ha) of paddy was destroyed due to disease; attack from animals and insects, and also due to natural disaster such as flooding. The net yield of paddy can be increased if these factors can be minimised. This has been one of our interests to conduct this study whereby we want to play a part in helping hands with the government agencies to boost up the rice production through knowledge on molecular profile of the rice field rats. Besides, the growth of science and technology in Malaysia is really fast, where researchers are encouraged to come up with latest discovery on the field of science and technology. Interestingly enough, as a developing country the research and development industry is really crucial in order to develop our ability to compete with other developed countries in conjunction with Vision 2020 to become a fully developed nation in our very own way. Through our study on the molecular genetics

of the rice field rat, we are looking forward to come up with a base to ease the later studies regarding the molecular profile of the rice field rats in Malaysia.

1.3 Objectives

The objectives of this study are:

1) To categorise the *Rattus argentiventer* collected in the Pinang Tunggal paddy field into their respective haplotype groups.

2) To investigate if mutation occurs in the VKORC1 gene of rice-field rats that is exposed to anticoagulant rodenticide.

3) To determine the liver residue level of the samples that had been treated with warfarin.

1.4 Research Questions

The research questions for this study are:

1) How many haplotypes group of *Rattus argentiventer* exists in the study area?

2) Is there any mutation to the rice field rat after being treated with anticoagulant rodenticide?

3) What is the residue level of the rodenticide in the liver of the rice field rat?

1.5 Limitations

The study aims to examine the molecular profile of the *Rattus argentiventer* in Pinang Tunggal, exclusively. The results obtained were not to be compared to other rodent species and other localities. There are some restrictions that we faced during the study was conducted. During the sample collection, we had set our limit to one trap barrier system in the Pinang Tunggal paddy field. This is due to lack of manpower to set up

the barrier along fifty meters times fifty meters of perimeter. Even so, the sample collected was quite a bunch given that the paddy inside the plot that we locate the trap barrier system is in tillering stage whereby adult rats were in abundance searching for their foods. Other than that, other limitation is in term of the rodent species in study. Given the study area was in the paddy field ecosystem, our sample was limited to *Rattus argentiventer* whilst other rat species and unwanted animals was discarded.

1.6 Thesis Outline

In Chapter 1, researcher introduces briefly about the study as a whole. Here, the background of the study had been introduced as well as the problems that make up the study were stated; the objectives and the research questions were also being declared. In Chapter 2, researcher discussed more about the rodent, their background, the characteristics and some examples of the rodent species. Moreover, researcher elaborates on the paddy and paddy field pest. The pest controls were elaborated such as biological control, chemical control and integrated pest management. The molecular studies of rats from previous researches were also being included. In Chapter 3, the materials and methods being used in this study were detailed; MtDNA for haplotype study, RFLP for mutation study and Liver Residue Analysis. Chapter 4 depicted the results of the studies in the form of diagrams, images and tables. Some of the tables and diagrams were incorporated in the Appendix. The last part, Chapter 5,

researcher discussed the entire study specifically by mean of each studies being conducted previously.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Nature would name human as her number one pest if it has a conscience. Human has caused more destruction, changed the natural landscape more deeply and extensively, destroy more of the other species, and killed the innocent souls. To date, the human population has increased where we have to move into land that was at first occupied by wildlife organism. This causes serious damage to agriculture, wildlife, and natural resources across the world (Kogan & Jepson, 2007).

Other than human, the species most often contribute to a serious amount of damage are recognized to be birds and mammals. Among the mammals, rodent has

been the major vertebrate pest group with numerous efforts to reduce the numbers and damage that is caused by them (Conover, 2002). A range of rodents are potential crop pests. Some rodents are considered as commensal, which rely on human to obtain their food and shelter, while others have appeared from the natural habitat or are introduced escapees. Larger animals such as foxes and owls are common predators which are manipulated to manage these rodent pests (Davies *et al*., 2010).

The prime reason for controlling rodents in agriculture is to prevent or at least to minimise damage to the crops. For that, it is wise enough for us to recognize, measure, and evaluate the economic significance of the damage. For example, in the natural habitat of rice field, the damage by *Rattus argentiventer* that leads to the loss of rice yield is about 5% in Malaysia, while higher loss of 17% was calculated in Indonesia (Singleton & Petch, 1994). There are several aspects should be taken into account in making practical assessments of rodent damage. Firstly, it is all about a perfect timing to initiate the rodent control measures. On top of that, it is important to identify the species responsible for the damage, as different pest species requires different measures to control (Bird, 2003). The control measures of rodents can be basically differentiated into three categories, the biological control, the chemical control which includes the usage of anticoagulant rodenticides and the integrated pest measures.

The size of the rodent population is the most obvious factor affecting the extent of damage. Rodent numbers may vary with the seasonal climates. The amount of rodent will increase as a result of huge amount of food during harvesting time. The second factor to consider is the crop availability and reservoir habitat for the rodents

(Bogdanske *et al*., 2011). As conditions gets better, rodent population expend out of its refuge habitat and crop damage will later spreads to a larger area. The damage that affects yield loss starts from the booting stage onwards (Perkins, 2002). The primary rodent pest in the rice field is the *Rattus Argentiventer*. Other species also occur in small amount as they usually pass by the rice field to get back to their natural habitat which includes oil palm plantations, rubber plantations and farms. However, the effects of damage on yield are difficult to evaluate, as the damage period happens at any time from sowing to harvest.

2.2 Rodents at a Glance

Apart from being the major vertebrate pest group, rat is considered as pet when referred to their beautiful coat colours and textures such as the kangaroo rat. Remarkable characters about the rodent is that most rodent species are small, secretive, nocturnal, and have keen senses of touch, taste and smell (Buckle & Smith, 1994). Latin verb *rodere*, which means to gnaw, shows the main feature that distinguishes rodents from other groups of mammals, which is the possession of two pairs of large incisor teeth. Their teeth are very strong that they can chew the wires, aluminium and even glass. This will help the teeth to be at the right position with appropriate length (Kogan & Jepson, 2007). Rodents also spend a lot of their time to groom themselves, which makes them clean omnivores. They also clean themselves about six times per day. The sizes of rat are usually larger than mouse with lifespan about two to five years only. Nevertheless, since their reproductive ability is fast, their

short lifespan is not a major problem for their abundance in nature (Alfredo & Veronica, 2009).

There are about 64 species of rats documented to date, such as *Rattus rattus, Rattus tanezumi, Rattus tiomanicus, Rattus argentiventer, Rattus norvegicus*, and also *Rattus exulans* (Pages *et al.*, 2011). In this study, we focus on *Rattus argentiventer* as it is the most abundant species that can be found in Malaysia's rice fields (Wood, 2001). More often than not, the male rats are usually called Buck, while female rats are called Doe and they love to live in their own groups of species. Other remarkable character that differ rodent from other pest species is that they have strong memory on their daily experiences. For example the rats have the ability to commit to memory if the route to go back home has changes and also towards the unwanted meals that they feels strange and bring hazardous effect to them (Robinson & Horn, 2007). These distinctive characteristics that lie in this small mammals really make them a unique yet exclusive animal compared to the others.

2.3 *Oryza Sativa* and the Rat

Rice can properly be called the world's most important crop. Over 588 million metric tons of rice harvested each year to feed 1.7 billion people worldwide (Beryl & Molly, 2001). Asians do not feel they have completed a meal unless rice is among the items eaten. Paddy was believed to originate from China, where about 93% of the world's rice production comes from the Asian countries. The Malaysian government had spent a total of RM 1.85 billion in 2011 to import rice from other neighbouring countries