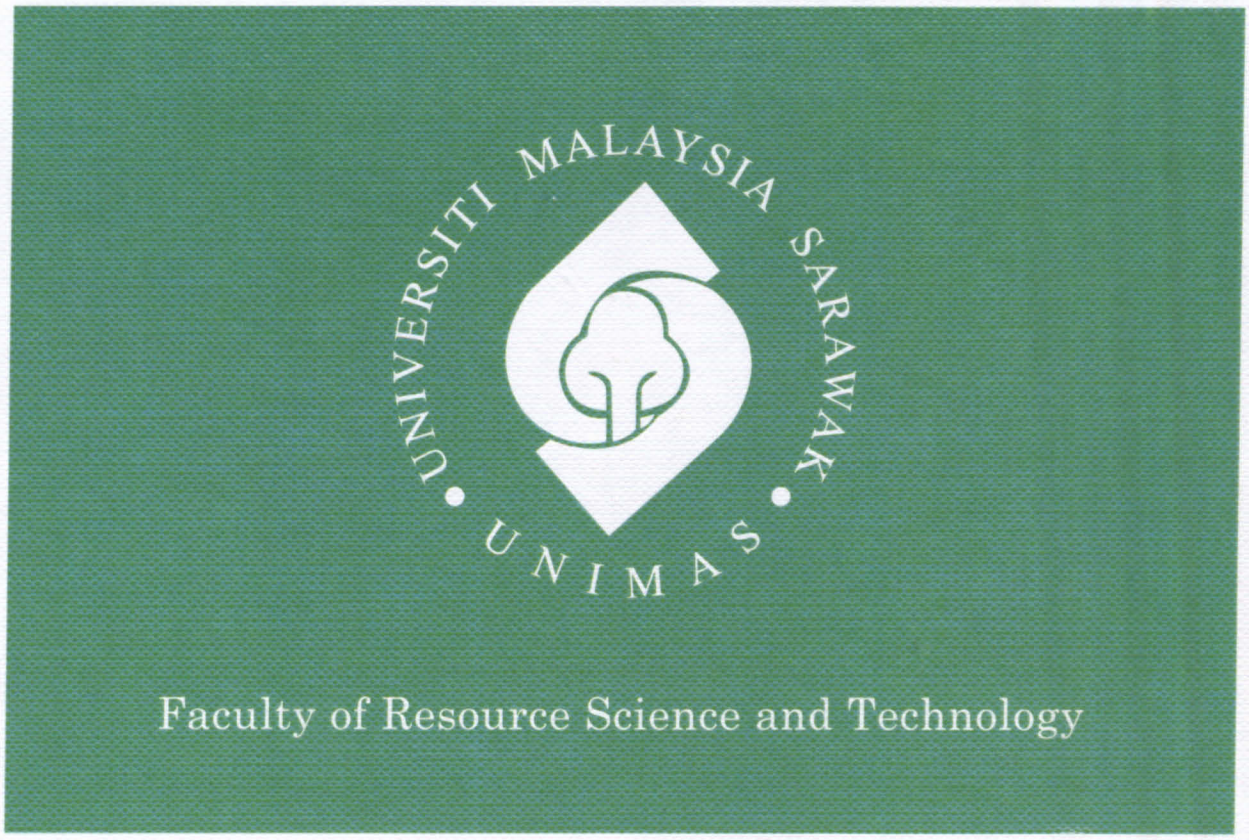

ISOLATION AND CHARACTERIZATION OF PARTIAL FRAGMENT OF MITOCHONDRIAL GENOME *(tRNA-Gln' - tRNA-Lys')* OF RASBORA *SARAWAKENSIS*

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Bachelor of Science with Honours
(Resource Biotechnology)
2017

Isolation and Characterization of a Partial Fragment of Mitochondrial Genome (*tRNA-Gln' - tRNA-Lys'*) of *Rasbora sarawakensis*

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A Thesis Submitted in Partial Fulfilment of the Requirement of the Degree of Bachelor of Science with Honours (Resource Biotechnology)

Supervisor: Dr Chung Hung Hui

Resource Biotechnology Programme
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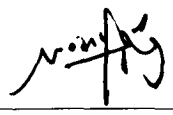
Student's signature  Supervisor's signature: _____

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List of Abbreviations

BLAST	Basic Local Alignment Search Tool
bp	Base pair
COI	Cytochrome c Oxidase subunit I
COII	Cytochrome c Oxidase subunit II
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotides
EtBr	Ethidium Bromide
mtDNA	Mitochondrial genome
ND2	NADH dehydrogenase subunit 2
nDNA	Nuclear DNA
NCBI	National Center for Biotechnology Information
PCR	Polymerase Chain Reaction
spp.	Species
TAE	Tris-Acetate Ethylenediaminetetraacetic Acid
Taq	*Thermus aquaticus*
T_m	Melting temperature
UV	Ultraviolet

Isolation and Characterization of Partial Fragment of Mitochondrial Genome (*tRNA-Gln' to tRNA-Lys'*) of *Rasbora sarawakensis*

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ABSTRACT

Rasbora sarawakensis is well known as Sarawak Rasbora that belongs to family *Cyprinidae*. This fish is significant for ornamental purposes and as molecular tools to study the phylogenetic relationship among the other *Rasbora spp.* In order to determine the phylogenetic relationship, mitochondrial genome is being targeted for analysis. In this study, our purpose is to isolate and characterize a partial fragment of mitochondrial genome *(tRNA-Gln'-tRNA-Lys')* of *R. sarawakensis*. The collection of *R. sarawakensis* was conducted at Matang Wildlife Park and acclimatized at 26 °C, 12 hours dark and light photoperiod. DNA extraction was conducted using CTAB method and the fragment of mitochondrial genome was amplified through PCR. Four pairs of gene specific primer were designed for PCR amplification of this fragment based on the conserved sequences from multiple alignment of three selected freshwater fishes. PCR amplification had generated four fragments of PCR amplicons in which their length are close to the expected size during primer design. The PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Germany) and was analyzed through AGE. Subsequently, the sample was sent to the First BASE Company for sequencing. The E-value and blast identity obtained for all of the four fragments are 0.0 and more than 80% respectively. Sequence that is obtained was aligned to other previously isolated fragment to generate complete mitochondrial fragment for *R. sarawakensis*. Phylogenetic analysis revealed high bootstrap support between *R. sarawakensis* and other closely related *Rasbora spp.* This study serves as a platform for further understanding in the population genetic of *Rasbora spp.* that might be useful in future conservation effort.

Keywords: Mitochondrial genome, *Rasbora sarawakensis*, Phylogenetic analysis

ABSTRAK

Rasbora sarawakensis terkenal sebagai Sarawak rasbora yang tergolong dalam famili Cyprinidae. Ikan ini penting untuk tujuan hiasan dan sebagai alat molekul untuk mengkaji hubungan filogenetik dengan rasbora spp. yang lain. Genom mitokondria dipilih untuk analisis bagi menentukan hubungan filogenetik. Tujuan kajian ini adalah untuk mengasingkan dan mencirikan sebahagian genom mitokondria (tRNA-Gln'- tRNA-Lys') R. sarawakensis. Pengumpulan R. sarawakensis telah dijalankan di Matang Wildlife Park dan aklimasi dalam 26 ° C, 12 jam gelap dan cahaya photoperiod. Pengekstrakan DNA menggunakan kaedah CTAB dan serpihan genom mitokondria telah dipenggandakan melalui PCR. Empat pasang gen primer telah direka untuk PCR berdasarkan domain terpelihara dalam 'multiple sequence alignment' tiga ikan air tawar yang dipilih. PCR menghasilkan empat serpihan amplicons dengan saiz dekat dengan saiz yang dijangka semasa mereka primer. Produk PCR dibersihkan dengan QIAquick PCR Purification Kit (QIAGEN, Jerman) dan dianalisis melalui AGE. Selepas itu, sampel dihantar ke Syarikat First BASE untuk sequencing. Nilai-E dan identiti BLAST diperolehi bagi kesemua empat serpihan adalah 0.0 dan lebih daripada 80%. Sequence yang diperolehi telah align dengan serpihan yang diasingkan sebelum ini untuk menjana serpihan mitokondria yang lengkap untuk R. sarawakensis. Analisis filogenetik mendedahkan sokongan bootstrap yang tinggi antara R. sarawakensis dan rasbora spp. yang berkait rapat. Kajian ini berfungsi sebagai platform untuk pemahaman lanjut dalam populasi genetik rasbora spp. yang mungkin berguna dalam usaha pemuliharaan masa depan.

Kata kunci: Genom mitokondria, Rasbora sarawakensis, analisis filogenetik

1.0 Introduction

1.1 Background

Over 1000 identified species of freshwater fish fauna are being found in India subcontinents and Southern Eurasia including Indonesia, Malaysia, Philippines, Pakistan and Thailand (Kottelat, 1999). Most of these identified fishes are *Rasbora spp.* which are found to be significant for ornamental purposes. *Rasbora spp.* are species-rich as well as their size varies from small to medium (Tang *et al.*, 2010). They are found to have variable morphological characteristic, such as the colour and present of lateral stripe along the side of its body. Genus *Rasbora* composed of small minnow fishes under order *Cypriniformes*, family *Cyprinidae* and subfamily of *Rasborinae*.

Previously, traditional taxonomists use multiple morphological traits to delineate species and morphological characteristic to explore the monophyletic grouping and species relationship (Dasmahapatra & Mallet, 2006). However, Hebert *et al.* (2003) argued that this classification method is not precise enough to identify organism and classify them into group. Besides, cryptic diversity issues may exist and this lead to characterization of two or more distinct species into one single species due to the similarity in morphology (Trontelj & Fiser, 2009). Hence, molecular classification including DNA barcoding and study of mitochondrial genome (mtDNA) have been employed to increase the preciseness and accuracy in species classification. For example, Ward and co-workers have utilized *COI* gene which can be found on the mtDNA as barcode to classify 207 species of Australian marine fish in 2005 to resolve the species classification by based on the *COI* sequencing or barcoding (Ward *et al.*, 2005).

mtDNA is the genetic material which is found in mitochondria. Most of the mitochondria contain between 1 and 10 copies of mtDNA (Phillips *et al.*, 2014). mtDNA is organized as a

closed, circular and double-stranded DNA molecule. However, according to Moraes *et al.* (2002), instead of closed circular, the mtDNA of mammalian can be arranged as uncircular monomers and uncircular dimers as well. mtDNA consists of heavy chain and light chain. Chinnery and Hudson (2013) recorded that these chains can be distinguished by the composition of nucleotide: Guanine (G) rich in heavy chain and Cytosine (C) rich in light chain. In animal, the 37 genes that coded by mtDNA including 13 polypeptides, 2 ribosomal RNAs and 22 transfer RNAs (Chinnery & Hudson, 2013). Besides, the gene arrangement in mitochondrial is highly conserved, thus, significant insights into the evolution of organisms may result from the comparisons of gene arrangement in mtDNA (Boore, 1999). mtDNA is inherited from the mother (maternal inheritance) which make it useful in the study of phylogenetic relationship. Due to this characteristic, mtDNA have been selected in most of the phylogenetic study as it can be used to trace whether the organisms or the species originated from same ancestor. Furthermore, the evolution rate of mtDNA are around 10-fold faster compared to nuclear DNA, thus it provides a better view in population genetics study.

However, the molecular studies in Malaysia are inadequate to classify and identify the *Rasbora* taxonomy as *Rasbora's* genus makes up a very homogenous group in term of taxonomy. Since genus *Rasbora* possessed no distinguishable features that can differentiate them from other member under family *Cyprinidae*, thus, their species are significantly hard to differentiate. Cryptic diversity issue may exist as traditional taxonomists study the phylogenetic relationship based on *Rasboras* morphological trait, thus, the phylogenetic relationships of *Rasbora spp.* still remain uncertain. In order to resolve the ambiguity of the *Rasbora's* taxonomy, this research aim to isolate and characterize the partial fragment of mtDNA and understand the phylogenetic

relationship of *Rasbora sarawakensis* with other closely related species that might be useful for conservation effort in future.

1.2 Objectives

The objectives of this research are to:

1. To isolate the partial fragment of mitochondrial genome *(tRNA-Gln' to tRNA-Lys')* of *Rasbora sarawakensis.*
2. To characterize the partial fragment of mitochondrial genome *(tRNA-Gln' to tRNA-Lys')* of *R. sarawakensis.*
3. To understand phylogenetic relationship of *R. sarawakensis* with closely related *Rasbora spp.*

2.0 Literature Review

2.1 Background and Biology of *Rasbora spp.*

2.1.1 Family *Cyprinidae*

Cyprinidae is the largest family of freshwater fishes comprise of about 220 genera and 2420 species (Nelson, 2006). It is highly distributed in Africa, North America and Eurasia. Roberts (1989) recorded that in western Borneo, there are about one third of all freshwater fish was being categorized into this family. Due to the Cyprinids' large-scale of dispersion in the area of South East Asia, they form the most prominent freshwater fish for now (Nelson, 2006). Besides, most of the Cyprinids including gold fish, common carp and *Danios* play an important role in aquatic fish industry due to their ornamental value (Refer to Figure 2.1). However, there are lack of morphological characteristics which can be used to differentiate them from the other family of fishes.

Howes (1991) found that there are two major lineages under the family *Cyprinidae*, Leuciscines and Cyprinines. Principally, the presence and absence of barbels and nature of pharyngeal dentition have been recruited for the dichotomy recognition between Leuciscines and Cyprinines (Howes, 1991). This two are then subdivided respectively into sister tribes which are Danionines and Leuciscines; Barbines and Tincanes (Howes, 1991). However, according to Howes (1991), barbels occurred in other cyprinids as well, thus it cannot serve as synapomorphic. Synapomorphy is a shared trait or character that used to differentiate a clade from other organisms and it is shared within the member of a monophyletic group. Since the polarity of these features has still to be evaluated, therefore the monophyly of the group is remaining doubt.

Within *Cyprinidae*, *Danioninae* are one of the subfamily that are rich in species and poorly understood. In 1991, three major groups within *Danioninae* including rasborin lineage, barilin group and an unnamed group is being identified by Howes (Liao *et al.*, 2010). Liao *et al.* (2010) recorded that danionins and rasborins are the two major species-rich groups within *Danioninae*. Danionins are those absence of dark supra-anal pigment and subpeduncular streak and it covers mostly in the genera *Danio* and *Devario* (Liao *et al.*, 2010). Meanwhile, rasborin are those with dark supra-anal pigment and subpeduncular streak, it composed of *Rasbora* and related genera such as *Trigonostigma, Rasboroides* and *Boraras* (Liao *et al.*, 2010). Genus *Rasbora* is paraphyletic in both molecular and morphological analyses in which species of *Rasbora* are genetically more closely related to the member under *Trigonostigma, Rasboroides* or *Boraras* compared to *Rasbora spp.* (Liao *et al.*, 2010).

Figure 2.1 Example of economically important cyprinids fishes. (Adapted from *Google source*. (n.d.). Retrieved August 10, 2016, from https://www.google.com/search?q=cyprinidae&source=lnms&tbm=isch&sa=X&ved=0ahUKEwiVpe_Ko-XTAhUFSo8KHVgwCfgQ_AUICigB&biw=1366&bih=662)

2.1.2 Genus *Rasbora*

Genus *Rasbora* composed of small minnow fishes under order *Cypriniformes*, family *Cyprinidae* and subfamily of *Rasborinae*. According to Nelson (2006), various members of this genus are important in the biological research, as food fish and as aquarium fish. *Rasboras* are restricted to freshwater habitats such as great rivers, small streams, ponds and lakes, and rarely occur in brackish water. Due to the incapability of acclimation to the sea, freshwater fishes can only produce their offspring at the connections of freshwater habitats (Miya & Nishida, 2014).

It is highly distributed around India subcontinents, Southern Eurasia including Indonesia, Malaysia, Philippines, Pakistan and Thailand. In Sarawak, most of the *Rasbora spp.* are endemic to Borneo such as *Rasbora einthovenii*. According to Muchlisin *et al.* (2012), there are *Rasbora spp.* that are endemic in Indonesia as well for example *Rasbora tawarensis*. *R. tawarensis* only occur in the Lake Laut Tawar, Indonesia and thus it is endemic to this lake (Muchlisin *et al.*, 2012). National show fish guides & technical information (1980) recorded that the temperature of water for *Rasbora spp.* should be maintained in a range of 22 °C to 27 °C. It is better if the water is well aerated, acidic and peaty.

Rasbora spp. are species-rich as well as their size varies from small to medium (Tang *et al.*, 2010). *Rasbora spp.* have a variety of morphological characteristics, for instant the colour of the stripe that present on its lateral body. However, *Rasbora spp.* are homogenous in group as their morphological characteristics are almost similar to one another thus, it is hard to differentiate among themselves (Refer to Figure 2.2). According to National show fish guides & technical information (1980), *Rasbora's* body is elongated and slim. It has oblique mouth, symphyseal knob (small hard lump on the lower lip) and three rows of pharyngeal teeth present within the mouth. Besides, the horizontal stripes that are normally black, blue or grey in colour

extend from the snout, operculum or midway along the flank to the caudal peduncle (National show fish guides & technical information, 1980). In some case, the stripe edged with gold or red colour. For the young *Rasboras*, pre-caudal spot or blotch is present instead of lateral stripe (National show fish guides & technical information, 1980). This blotch obscured by the lateral stripe when the fish grow bigger and older. National show fish guides & technical information (1980) recorded that *Rasboras* carry no barbels except for *Rasbora elanga* that has a tiny pair.

Figure 2.2 Example of *Rasbora spp.* which possess almost similar morphological characteristics. (Adapted from *Google source*. (n.d.). Retrieved August 12, 2016, from https://www.google.com/search?q=rasbora &source=lnms&tbm=isch&sa=X&ved=0ahUKEwjJl56wpOXTAhWMsY8KHfGqBhYQ_AUIBigB&biw=1366 &bih=662)

2.1.3 *Rasbora sarawakensis*

Rasbora sarawakensis is well known as Sarawak Rasbora. It is a ray-finned freshwater fish that classified under the phylum Chordata, class *Actinopterygii*, family *Cyprinidae*, subfamily *Rasborinae* and genus *Rasbora*. *R. sarawakensis* is native to West Kalimantan of Indonesia and Borneo. In Sarawak, it was found in Sungai Sarawak and Batang Kayan. *R. sarawakensis* mostly

inhabits in slow-moving forest streams that are shaded by dense rainforest canopy (Nyanti & Jongkar, 2007). It is a schooling species by nature that live in a group of 8 to 10 fish.

R. sarawakensis is a small freshwater fish with an average size range from 4.5 cm to 5 cm. Males *R. sarawakensis* usually display more attractive colouration compared to female which are dull in colour and this help them to compete with one another for female attention. The body of *R. sarawakensis* is light brown in colour and become paler towards the anal contour. It carries a blue lateral stripe along the side of its body and extends from the operculum to caudal peduncle. Their fins are clear, yellowish and their caudal moderately forked with slightly round lobes (Refer Figure 2.3).

Figure 2.3 *Rasbora sarawakensis*. (Adapted from *Fish identification: Find species*, from Choy, H. W. (n.d.). Retrieved August 15, 2016, from http://www.fishbase.org/photos/UploadedBy.php?autoctr=3458&win=uploaded)

2.2 Taxonomy Classification

Taxonomy is the science, laws and principles of classification. It consists of two major subdisciplines which are nomenclature and identification (Madigan & Martinko, 2006). The purposes of taxonomy included to classify all the existing organic diversity of the earth, to provide much of the information to construct the history of life, to study the differences between one groups of organism with another and to reveal the numerous interesting evolutionary phenomena (Russell, 2013). According to Russell (2013), all organisms are organized into taxonomic categories by relatedness. Thus, taxonomy is the basis for the comparisons of all known organisms. Through taxonomy, an unknown organism can be group according to the shared features with the known organisms. Besides, taxonomy is extended to include the phylogeny which is study the relatedness and the evolutionary history of the particular organism (Hagen, 2012).

The system of classifying organism into Kingdom, Phylum, Class, Order, Family, Genus and Species was developed by Carl Linnaeus in year 1758. According to Russell (2013), in this new framework of binomial nomenclature, the genus and species of any particular organism become its scientific name. The scientific name that assigned to each organism must be universal, unique and show stability in the science of taxonomy (Russell, 2013). The traditional taxonomic classification identify and classify organism based on their morphological features. However, the differences of morphological across many closely related species are not significant. For example, the members under *Cyprinidae* including *Rasboroides, Boraras, Trigonostigma* have almost similar morphological features as *Rasbora spp.* (Mayden *et al.*, 2007).

Today, taxonomists use multiple morphological traits that being supplemented with DNA based information to delineate species. Hence, genetic and molecular techniques such as

study of mitochondrial genome and DNA barcoding are used in classification and identification of the organism in the modern taxonomy. According to Fogelstrom (2015), DNA barcoding that uses a short DNA sequences (*COI*) that originate from the mtDNA is introduced by Paul Hebert in 2003 as the rapid tools to identify species at molecular level. For instance, Hebert and co-workers used *COI* as DNA barcodes to distinguish 260 species of birds in 2004 and they found that the identification process of species are straightforward (Hebert *et al.*, 2004).

Thus, by studying the mtDNA, we can further understand the phylogenetic relationship and evolution of species more efficiently compared to the traditional taxonomic classification. Besides, mtDNA can lead to the discovery of cryptic species within what had previously been thought to be single morphological based species and correctly identified them (Dasmahapatra & Mallet, 2006). For example, by using the *COI* gene that originate from mtDNA as barcode, Hebert and his colleagues have been 100% successful in correctly identifying the subsequent species based upon the analysis of a single individual from each of 200 closely allied species of lepidopterans in 2003 (Hebert *et al.*, 2003).