POPULATION GENETICS AND DISTRIBUTION OF TWO SYMPATRIC FROG SPECIES IN PENINSULAR MALAYSIA, *Fejevarya cancrivora* (Gravenhorst, 1829) AND *Fejevarya limnocharis* (Boie, 1834)

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(Gravenhorst, 1829) AND *Fejevarya limnocharis* (Boie, 1834)

By

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This piece of work is a token of dedication to my beloved mom,

Khairun Mahmood.

A special dedication…

My late father

Allahyarham Hurzaid Hj. Mohamad Isa

Who passed away on 18th February 2001.

-1st April 2013-

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'On no soul doth Allah Place a burden greater than it can bear'

[Al Baqarah: 286]

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LIST OF SYMBOLS AND ABBREVIATIONS

π	nucleotide diversity
h	haplotype diversity
AMOVA	Analysis of Molecular Variance
bp	Basepair
DNA	Deoxyribonucleic Acid
dNTP	Dinucleotide triphosphate
EDTA	Ethylenediamine tetra-acetic acid
EtBr	Ethidium Bromide
EtOH	Ethanol
IUCN	International Union for the Conservation Nature
kb	Kilobase
mM	Milimolar
mtDNA	Mitochondrial DNA
MW	Mouth Width
OD	Optical density
rpm	rotations per minute
SVL	Snout –Vent Length
TBE	Tris borate EDTA
TD	Tympanum Diameter
TE	Tris EDTA
TL	Tibia Length
UV	Ultraviolet

GENETIK POPULASI DAN TABURAN DUA SPESIES KATAK SIMPATRIK DI SEMENANJUNG MALAYSIA, *Fejevarya cancrivora* (Gravenhorst, 1829) DAN *Fejevarya limnocharis* (Boie, 1834)

ABSTRAK

Satu kajian genetik populasi dua spesies katak simpatrik di Semenanjung Malaysia, *Fejevarya cancrivora* dan *F. limnocharis* telah dijalankan dengan menggunakan segmen D-loop yang berevolusi tinggi. Tinjauan genetik telah dijalankan ke atas enam populasi (26 individu) *F. cancrivora* di kawasan utara Semenanjung Malaysia dan 16 populasi (106 individu) *F. limnocharis* melibatkan kawasan Barat Laut, Barat Tengah dan Timur Semenanjung Malaysia. Secara umumnya, *F. cancrivora* menunjukkan genetik variasi yang sangat tinggi bagi populasi Pulau Langkawi dan ketiadaan variasi bagi populasi Jitra. Pohon filogeni 'Neighbour Joining' (NJ) dan 'Maximum Parsimony' (MP) menunjukkan topologi yang sama dimana populasi Pulau Langkawi membentuk klad tersendiri. Oleh itu, dicadangkan bahawa populasi Pulau Langkawi mencapah jauh dengan kadar mutasi yang tinggi dari haplotip utama disebabkan oleh lanjutan masa pengasingan populasi yang telah mengalami pengecutan (bottleneck) diikuti dengan hanyutan genetik. Bagi spesies *F. limnocharis*, min kepelbagaian haplotip (h) dan kepelbagaian nukleotida (π) secara puratanya adalah rendah dalam semua sampel (h = 0.471 ± 0.27; π = 0.004 ± 0.00005). Pembiakbakaan dalaman dan pencemaran oleh racun perosak telah dicadangkan sebagai punca yang

membawa kepada kepelbagaian genetik yang agak rendah dalam spesies ini. Analisis filogenetik yang dijalankan menggunakan kaedah NJ dan MP gagal menunjukkan sebarang penstrukturan geografi antara populasi pantai timur dan pantai barat. Analisis AMOVA menggabungkan kumpulan barat laut dan barat tengah serta kumpulan timur menunjukkan bahawa kebanyakan varian genetik bertabur di antara kumpulan (Φ= 54.81%) yang menunjukkan bahawa populasi barat tengah adalah lebih berkaitan dengan populasi timur berbanding dengan populasi barat laut. Di samping itu, kajian awal yang menggunakan pendekatan GIS untuk memeta taburan kedua-dua spesies ini di Semenanjung Malaysia telah menunjukkan bahawa kedua-dua spesies berkelompok dan kebanyakannya hanya bertumpu di kawasan utara Semenanjung Malaysia. Kajian yang lebih terperinci terhadap penanda mitokondria DNA dan nuklear DNA ke atas saiz sampel yang lebih besar serta merangkumi seluruh taburan geografi spesies-spesies ini dicadangkan untuk merancang pengurusan pemuliharaan yang efektif. Usaha daripada ahli herpetologi di Malaysia amat diperlukan untuk mengisi jurang pengetahuan yang sedia ada pada kedua-dua spesies ini serta spesies amfibia asal yang lainnya.

POPULATION GENETICS AND DISTRIBUTION OF TWO SYMPATRIC FROG SPECIES IN PENINSULAR MALAYSIA, *Fejevarya cancrivora* (Gravenhorst, 1829) AND *Fejevarya limnocharis* (Boie, 1834)

ABSTRACT

A population genetic study of two sympatric frog species in Peninsular Malaysia, *Fejevarya cancrivora* and *F. limnocharis* was conducted using the highly evolving D-loop segment of mtDNA. The genetic survey was conducted on six populations (26 individuals) of *F. cancrivora* from northern Peninsular Malaysia and 16 populations (106 individuals) of *F. limnocharis* from northwest, central west and east of Peninsular Malaysia. In general, *F. cancrivora* showed very high genetic variation for Pulau Langkawi population and lack of variation for the Jitra population. The constructed Neighbour Joining (NJ) and Maximum Parsimony (MP) phylogenetic trees showed similar topology, with the Pulau Langkawi population forming its own clade. It is suggested that the deep branching of Pulau Langkawi populations with high mutation rates from major haplotypes may be caused by the extension time of isolation of populations which had experienced bottleneck followed by genetic drift. As for *F. limnocharis*, mean haplotype and nucleotide diversity was low over all samples (h = 0.471 ± 0.27; π = 0.004 ± 0.00005). Inbreeding and pollution by pesticides are suggested as the causes which have led to the comparatively low genetic variability detected in this

species. Phylogenetic analysis based on NJ and MP methods failed to show any geographic structuring between the east and west coast populations. AMOVA analysis between northwest group with combined central west and east populations group, showed that the majority of genetic variance was distributed between groups (Φ= 54.81%) which indicated that the central west was more related to the eastern populations than it is to the northwest populations. Additionally, the preliminary use of GIS approach to map the distribution of both species in Peninsular Malaysia revealed that both species were clustered mostly in the northern area of Peninsular Malaysia. More detailed study of mitochondrial DNA and nuclear DNA markers on larger sample sizes, throughout the geographical distributions of these species are suggested in planning an effective conservation management. Efforts from herpetologists in Malaysia are much needed to fill the existing knowledge gap on these species.

CHAPTER 1

INTRODUCTION

1.1   Introduction

Amphibians are unique vertebrates comprising over 6,700 known species (Frost, 2011), found in diverse habitats around the world, except in very high latitudes in the hemispheres, most oceanic islands and the most low-lying, arid regions (Pough et al. 2004; Hillman et al. 2009). Amphibians are recognised as important components of natural ecosystems and they play important roles in the ecological process of the ecosystem. They are the mid-level consumers in the food chain of many tropical ecosystems, meaning that they consume insects, other arthropods, large invertebrates and small vertebrates. Likewise, amphibians are important prey for numerous predators, such as snakes, wading birds, lizards and small mammals in the food web (Ibrahim, 2004).

Amphibians have existed since the Devonian period, nearly 350 million years ago having evolved from lobe-finned fish (Crossopterygia) that used their strong, bony fins to radiate into most habitats on earth (Duellman & Trueb, 1986; Hickman et al. 2006). In doing so, they have acquired spectacular and sometimes unusual physiological, morphological, behavioural, and ecological attributes that form their innovative life histories. Their successful adaptation and resilience contribute to their survival from the past until present. However, sadly even though they had overcome many catastrophes in the recent decades, they are now facing serious threats due to human activities.

Biologists have divided the class Amphibia into three major groups namely Anura (frogs and toads), Caudata (salamanders and newts) and Gymnophiona (caecilians). Among these, the order Anura is the largest and most diverse with approximately 5,100 species. Unfortunately, nearly one-third (32%) of the world's amphibian species are threatened, representing more than 1,800 species (Stuart et al. 2004). Various factors are known to cause the decline including habitat loss, alteration and fragmentation (Fisher & Shaffer, 1996; Davidson et al. 2001; Marsh & Trenham, 2001; Norhayati et al. 2005a), introduction of competitors or predators (Kats & Ferrer, 2003; Norhayati et al. 2005a) over-harvesting (Blaustein & Wake, 1990, Lannoo et al. 1994), climate change (Pounds et al. 1999; Kiesecker et al. 2001; Carey & Alexander, 2003), increased UV-B radiation, chemical pollution (Hayes et al. 2002; Blaustein et al. 2003) and emerging infectious diseases (Daszak et al. 2003). Thus, their continued survival depends on our concern and assistance.

Kiew (1984) found that forest frog species in Malaysia are threatened by extensive logging and development and hence they are susceptible to extinction. To further aggravate the problem, Ibrahim (2004) stated that paddy field frogs are suffering from pesticide pollution and over collecting such that their numbers are declining and dwindling. It is worrisome that if no progress is made in the near future with respect to measures on amphibian conservation, many species will be lost even before they are discovered, recorded and studied.

Malaysia is purported to be an amphibian hotspot and the fourth on the list of countries having the largest amphibian populations in Asia, which currently stands at 218 species (IUCN, 2009). There are 12 families of Anura worldwide and Malaysia harbours seven, namely Bombinatoridae, Megophryidae, Bufonidae, Microhylidae, Ranidae, Dicroglossidae and Rhacophoridae. *Fejevarya limnocharis* and *F.*

cancrivora are two frogs from the Dicroglossidae family commonly found around human habitation, lowland plains and agricultural areas in Malaysia (Ibrahim, 2004). *Fejevarya cancrivora* is also known as 'Crab-Eating Frog' and it is the only Malaysian frog that can tolerate saline habitats (Inger & Stuebing, 1989). According to The World Conservation Union (IUCN) report published in 2009, amphibian status in Malaysia is still under control and there is no extinction report for the two species investigated.

These two frog species always occur sympatrically in paddy fields area. However, *F. cancrivora* is bigger, which allows them to feed on different- sized prey items, thus avoiding competition for food (Ibrahim, 2004; Wong, 2007). The main food items for both species are insects and about 80% of these insects are pests of rice. Hence, these frogs are considered as important biological control organisms in the paddy field habitat (Ibrahim, 2004).

In Malaysia, *F. limnocharis* is utilized for bait in sport fishing or as food for carnivorous aquarium fishes in the ornamental fish industry. In contrast, *F. cancrivora* is exploited for food consumption by Chinese townspeople in Malaysia. They claimed that the meat of this frog is sweet, tender and tasty and even described them as "paddy chicken" (Ibrahim, 2004). Thus, over-harvesting is a potential threat to this species. Since both of these frogs inhabit the paddy field area, another very important threat to their existence is habitat degradation due to pollution by pesticides and chemical fertilizer used on crops. They are also common prey at the tadpole and adult stages to water birds, fishes and snakes. Other possible reasons for their decline are general habitat alteration and loss, urbanization, prolonged drought, habitat fragmentation and habitat modification from deforestation, or logging related activities. Although there is no reported data on the effects of the factors mentioned

above, immediate management steps should be implemented before any of the declining populations reaches extirpation in Malaysia.

O'Brien (1994) and Bowen and Karl (2007) stated that in a holistic management action, genetic information is now recognised as a crucial factor in all biological management programs. According to Schierwater et al. (1994), genetic variability data can reveal information on individual identity, breeding patterns, degree of relatedness and disturbances of genetic variation in populations. Genetic diversity data is also important in the assessment of molecular ecology, such as connectivity of population and characterization of geographic structure (Sivasundar et al. 2001; Barroso et al. 2005). Besides, it can also be utilized to determine the extinction risk of populations (Schierwater et al. 1994).

In addition to the use of molecular data in assessing extinction risk of populations, Geographical Information System (GIS) data can also be applied in the conservation and management of amphibian populations. For the past twenty years, utility and availability of GIS has increased greatly. A major example is the work by Patla and Peterson (2002), which used GIS in addressing amphibian diversity, distribution and habitat in the Yellowstone Lake Basin, United States to conserve and restore amphibian populations. In addition, Ray et al. (2002) also used GIS approach to analyse amphibian's habitat, which is very important in biological conservation.

In Malaysia, limited data is available for both approaches. The genetic variation of amphibians in Malaysia using mtDNA sequencing methods have been reported by Ramlah (2009) and Ramlah et al. (2010). Their studies mainly involved amphibian populations from Borneo and Sarawak with limited samples from Peninsular Malaysia. However, they have provided a foundation for further studies in addressing population subdivision of the Malaysian amphibians. This study was

focused on two important amphibian species in Peninsular Malaysia as models of the application of population genetic studies for conservation; *F. cancrivora* and *F. limnocharis.* While *F. cancrivora* is found in disturbed but generally natural, wild habitats, *F. limnocharis* inhabits paddy field, hence it is vulnerable to pollution by pesticides.

In contrast, to date, there is no reported data on GIS application on amphibian distributions in Peninsular Malaysia. Nevertheless, in this study, the application of the GIS is solely used to map the distribution of two sympatric frog species in Peninsular Malaysia, *Fejevarya limnocharis* and *F. cancrivora* in order to provide better understanding of their occurrence in Peninsular Malaysia.

1.2   Objectives:

With the above considerations, the objectives of this study were:

1) To determine the genetic variation of *F. cancrivora* from Northern Peninsular Malaysia using mitochondrial D-loop gene.

2) To determine the genetic variation of *F. limnocharis* from Peninsular Malaysia based on mitochondrial D-loop gene.

3) To map the distribution of *F. cancrivora* and *F. limnocharis* in Peninsular Malaysia using GIS approach.

CHAPTER 2

LITERATURE REVIEW

2.1   Amphibians in general

Amphibians are found on all continents in the world, except in Antartica (Van der Meijden et al. 2005). To date, over 6,700 known species (Frost, 2011) have been reported and more are being described yearly. The class Amphibia comprised three orders namely Anura (Salientia), Caudata (Urodela), and Gymnophiona (Apoda). There are several distinct characteristics that differentiate each order from the others. Nevertheless, the taxonomic classification of each order is still under debate (Duellman & Trueb, 1986).

Frogs and toads are members of the order Anura, which is further divided into approximately 40 families and more than 5,900 species (Frost, 2011). Generally, anurans are jumping amphibians with the presence of four limbs and no tails (excluding all larval anurans and male of *Ascaphus truei*), which differentiate them from caudates and caecilians (Heyer et al. 1994). The hind limbs are typically larger that are specifically modified for leaping or climbing. Anurans are also unique in terms of their ability to vocalization, and produce an array of sounds from squeaks to barking noises (Miller, 2012). Vocalization is a pivotal component of the reproductive behavior for majority of frogs (Heyer et al. 1994). Unlike the majority of salamanders and caecilians, most anurans display external fertilization, have aquatic eggs and feeding larvae known as tadpoles (Heyer et al. 1994; Miller, 2012).

2.2 Threats to amphibians

Amphibians are thought to be good biological indicators as they are inhabitants of both terrestrial and aquatic environments. Thus, they are the first organism to be exposed to climatic changes and habitat pollution. As a result, amphibian populations have suffered widespread declines and extinctions in recent decades (Kiesecker et al. 2001; Beebee, 2005; Frost, 2011). Stuart et al. (2004) reported that amphibians have become more threatened than birds and mammals.

In the last few years, demand for frog legs for food consumption has increased drastically. The main reason of wild frog exploitation to support this trade is because of its large body (Altherr et al. 2011) and delicious taste (Ibrahim, 2004). In several Asian, African and Latin American countries, frogs are hunted for subsistence or local consumption (Altherr et al. 2011). Some of these countries, are also involved in the commercial trade of frogs and frog products, supplying markets in the European Union (EU) and the United States of America (USA). As a consequence, local frog populations have seriously declined (Lannoo et al. 1994).

Indonesia has been the world's leading exporter of frog legs in the last 20 years followed by China, Taiwan and Vietnam (Altherr et al. 2011). The majority of frogs are caught from their natural habitats on the island of Java - particularly the Crab-eating Frog, *F. cancrivora* (75%) and the Giant Javan Frog, *Limnonectes macrodon* (19%) (Kusrini & Alford, 2006). India and Bangladesh had in the past dominated this frog leg trade until their frog populations collapsed, leading to the reduction of a major natural control agent for agricultural pests and mosquitoes (Abdulali, 1985; Oza, 1990). This unfortunate scenario could just likely impact our local frog populations if no conservation efforts are made in the near future.

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Numerous studies have been conducted around the globe to investigate the effects of pesticides on amphibians. Liu et al. (2011) reported that the broad-spectrum herbicide, butachlor, depresses survival, development, and time to metamorphosis in *F. limnocharis* in subtropical Taiwan. Butachlor is genotoxic to toad and frog tadpoles where it induces DNA strand break in erythrocytes (Geng et al. 2005; Yin et al. 2008). Atrazine, one of the most widely used pesticide in the world, create havoc with the sex attributes of adult male frogs, emasculating three-quarters of them and turning one in 10 into females according to a study by Sanders (2010). Atrazine acts by inhibiting production of testosterone (the male sex hormone) and induces estrogen production (the female sex hormone), resulting in imbalance between these two hormones. As a result, this atrazine-exposed male will possess both chemical castration (demasculinization) and feminization (Hayes, 2005).

Jayawardena et al. (2010) discovered that exposure of propanyl on Common Hourglass Tree Frog (*Polypedates cruciger*), not only affected the survival and growth of tadpoles, but also led to malformation. Exposed tadpoles took a longer period to metamorphose and were smaller in size as compared to tadpoles in natural conditions. The longer periods of metamorphosis and smaller size of adults can have many consequences in nature (Jayawardena et al. 2010) as the ability to escape from predators and defending territories (Bridges, 1999) and survival (Shenoy et al. 2009) are compromised. Other pesticides known to cause devastation in frog populations include carbaryl (Relyea & Mills, 2000; Boone et al. 2004; Relyea, 2006), 2, 4-D butyl ester (Pérez-Coll & Herkovits, 2006) and carbofuran (Bacchetta et al. 2007; Jayatillake et al. 2011). In Malaysia, among the commonly used pesticides in rice fields are propanyl, carbaryl, 2, 4-D butyl ester (Abdul Rani, 2002).

This situation highlights the dire need for conservation. One increasingly popular approach is the generation of genetics data from amphibian populations. Numerous molecular markers have been developed for this purpose (Beebee, 2005). Knowledge on the genetic diversity and population structure is one the fundamental areas for conservation and restoration of species and ecosystem diversity. Conservation of genetic variability is important to the overall health of populations because decreased genetic variability leads to increased levels of inbreeding, and reduced fitness. Amphibians are good models for investigating animal genetic population for several reasons. They have limited mobility, widely distributed in most ecosystems and many are easy to sample because they congregate at specific localities for reproduction (Beebee, 1996). As anurans (frogs and toads) undergo external fertilization, controlled crosses are amenable under laboratory conditions (Beebee, 2005).

2.3   Taxonomy and Species Description

2.3.1   Taxonomic status of *Fejevarya cancrivora*

Fejevarya cancrivora is also known as the Crab-eating Frog, Mangrove Frog, Rice Field Frog and Asian Brackish Frog (Plate 2.1). It was initially called *Rana cancrivora* by Gravenhorst (1829) due to its freshwater crab-eating habit. More recently, it was placed as a member of the genus *Fejevarya* (Iskandar, 1998; Dubois & Ohler, 2000). Currently, the taxonomic classification of this species is represented by the following:

Kingdom: Animalia

Phylum: Chordata

Subphylum: Vertebrata

Class: Amphibia

Order: Anura

Family: Dicroglossidae

Genus: *Fejevarya*

Species: *Fejevarya cancrivora* (Gravenhorst, 1829)

Plate 2.1: *Fejevarya cancrivora* (Gravenhorst, 1829) (Photographed by Shahriza

Shahrudin)

Even though the Crab-eating Frog, *F. cancrivora* is one of the most widely distributed frog species in the Asian region, taxonomic relationships among different populations is still unclear. Several attempts have been made by various researchers (Sumida et al. 2002; Islam et al. 2008; Kurniawan et al. 2010; Kurniawan et al. 2011) to elucidate the taxonomic status of this species using morphological characteristics, allozyme and molecular analysis. Kurniawan et al. (2010) suggested that *F. cancrivora* from Asian populations could be divided into three types, namely, the mangrove, large, and Pelabuhan Ratu/ Sulawesi-types, with the last two types showing the most similarity in morphology. The authors discovered that the mangrove-type was distributed in the Asian mainland and the Philippines, the large-type in Sundaland area and the Pelabuhan Ratu/Sulawesi-type in Pelabuhan Ratu,

Java Island and Sulawesi Island of Indonesia. Kurniawan et al. (2011) proposed the large-type as *F. cancrivora*, the mangrove-type, *F. moodiei*, and the Sulawesi-type, an undescribed species.

2.3.2 Taxonomic status of *Fejevarya limnocharis*

Fejevarya limnocharis is also known as the Asian Grass Frog, Common Pond Frog, Field Frog and Indian Rice Frog (Plate 2.2). *Fejevarya limnocharis* closely resembles *F. cancrivora* in external morphology. However, according to Gravenhorst (1829), *F. cancrivora* is larger than *F. limnocharis*, and the name *F. cancrivora* has been consistently applied to larger individuals in the *F. limnocharis* complex occurring in Java and neighbouring regions (Kurniawan et al. 2010).

As in the case of *F. cancrivora*, the taxonomic status of *F. limnocharis* is also still controversial (Fei et al. 2002). According to Toda et al. (1998), *F. limnocharis* is a species complex of frogs. Kotaki et al. (2008) stated that *F. limnocharis* has been conventionally regarded as a single species because of few morphological differences. However, recent detailed analyses by Dubois & Ohler (2000) have revealed that there is a degree of genetic differentiation within *F. limnocharis*, and therefore it has been proposed that *F. limnocharis* contains several cryptic species.

Sumida et al. (2002) revealed that there is no reproductively isolating mechanism between populations of *F. limnocharis* as shown by hybridization experiments. Nevertheless, phylogenetic tree based on mitochondrial DNA sequences of 12S and 16S rRNA genes discovered three clades of *F. limnocharis* in the East Asian populations. The first clade comprising populations from Japan Islands; the second, Sakishima Island and the third, the Okinawa Island and Taiwan

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populations. Based on crossing experiments and molecular data, Sumida et al. (2002) regarded the Sakishima-Island populations as a distinct species of *F. limnocharis*. Djong et al. (2007) and his colleagues conducted morphological observation, phylogenetic reconstruction of mitochondrial DNA of 16S and cytochrome b genes, backcrossing experiments, histology and spermatogenesis studies among Indonesian, Malaysian, and Japanese populations of *F. limnocharis*. They concluded that the Malaysian population of *F. limnocharis* and *F. multistriata* from China should be designated as a subspecies of topotypic *F. limnocharis*, and that the Japanese population as a distinct species. The current taxonomic classification of this species can be represented as follows.

Kingdom: Animalia

Phylum: Chordata

Subphylum: Vertebrata

Class: Amphibia

Order: Anura

Family: Dicroglossidae

Genus: *Fejevarya*

Species: *Fejevarya limnocharis* (Boie, 1834)

Plate 2.2: *Fejevarya limnocharis* (Boie, 1834) (Photographed by Shahriza Shahrudin)

2.4 Morphology

The frog is unique as it has no tail in the adult but is present in the tadpole. Most frogs have long hind legs, elongated ankle bones, webbed toes, no claws, large eyes and a smooth or warty skin. Besides, they also have a short vertebral column, with no more than ten free vertebrae and a fused tailbone. The general anatomy of frog is shown in Fig. 1.1. Morphological characteristics of *F. cancrivora* and *F. limnocharis* have been described by Berry (1975), Inger and Stuebing (1997), Ibrahim (2004), Kurniawan (2010) and Norhayati (2012a) and the description of each species is given below.
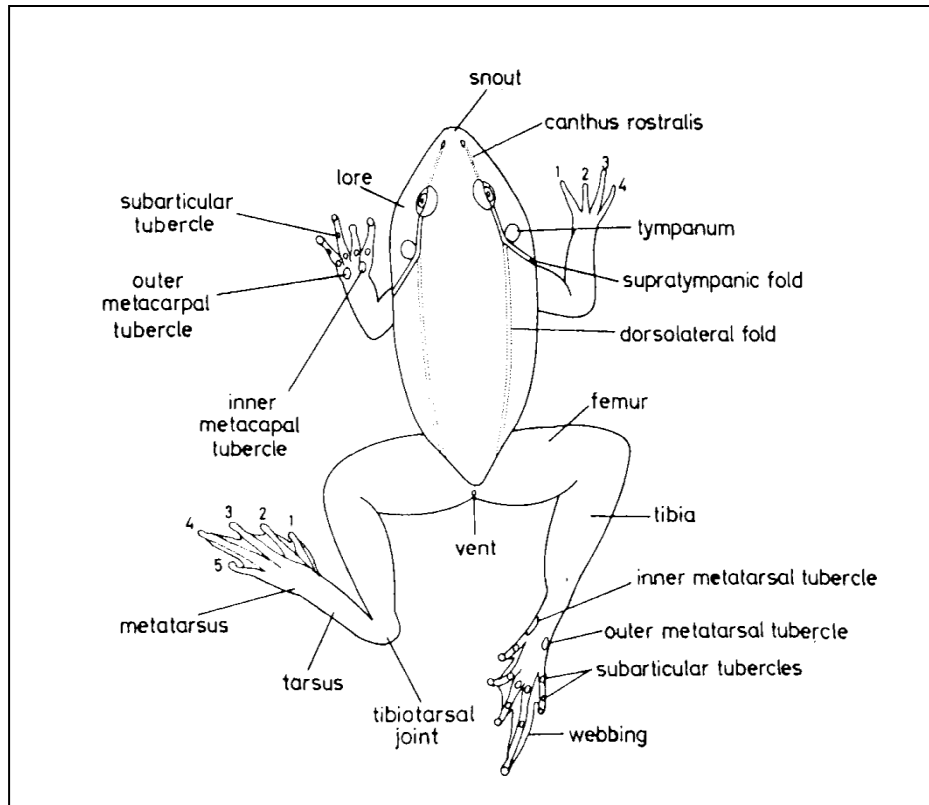
Figure 1.1: General anatomy of frogs (Berry, 1975)

2.4.1 *Fejevarya cancrivora*

Fejevarya cancrivora is a medium-sized frog with a long snout and well-muscled hind limbs (Inger & Stuebing, 1997). The total length of this frog is between 50 mm–85 mm (Inger & Stuebing, 1999), while Berry (1975) gave the snout vent length (SVL) as 50-75 mm. Females are usually larger and their bodies more sturdy and stocky as compared to males. Inger and Stuebing (1999) stated that the total length for female is between 52.9 mm – 82.0 mm, while male is between 51.0 mm to 70.9 mm. Inger and Stuebing (1997) recorded the female to be between 53 and 82 mm in length and male between 51-70 mm. Kurniawan et al. (2010) observed total

length of females and males to range from 58.0–87.5 mm and 59.3–71.2 mm, respectively. Ibrahim (2004) also found many females with mean sizes of 66.0 ± 7.1 mm compared to 61.0 ± 7.1 mm in males.

According to Berry (1975), vomerine teeth are present in this species with its head as broad as or slightly broader than long and the snout is rounded or obtusely pointed. The colour of this frog is grey or brown dorsally with irregular dark markings, often in the form of a 'W'. The skin is irregular with longitudinal ridges on the back and the tympanum is conspicuous about 1/2 to 2/3 of eye diameter (Norhayati et. al. 2012a). The limbs are with dark crossed bars or irregular dark markings. The stomach is creamy white, while the underside of the head is either white or with dark mottling which sometimes extends to the chest area in some individuals (Ibrahim, 2004). Males have black patches under the corner of the jaw on the skin overlying the vocal sacs (Inger & Stuebing, 1997).

2.4.2   *Fejevarya limnocharis*

Fejevarya limnocharis is a small frog with a long narrow head and a slender, oval body (Inger & Stuebing, 1997). According to Berry (1975), snout vent lengths (SVL) of the females are 48-60 mm and 32-50 mm for males. Inger and Stuebing (1997) also reported SVL of 49-58 mm in females and 32- 50 mm for males. Ibrahim (2004) recorded the mean sizes of males as 39.9 ± 2.77 mm and 46.8 ± 4.72 mm in females. Djong et al. (2007) stated that male sizes are between 35.5- 41.7 mm while females between 46.3- 47.9 mm. The total length of this species, however, is smaller than *F. cancrivora*.

Berry (1975) recorded the presence of vomerine teeth in two oblique series between the choanae, a moderate head and snout more or less pointed in *F. cancrivora*. This species is grey or brownish in colour with dark spots or blotches often with a yellow creamy vertebral stripe on the dorsal side. The skin is warty dorsally with distinct tympanum about 3/5 diameter of their eye (Norhayati et. al. 2012a). There is usually a W-shaped marking across the shoulders. The back of the thighs are yellowish in colour while the limbs have dark cross bars (Ibrahim, 2004). The lips are barred brown and white, while the undersides of the female are completely white. Unlike other species, which are difficult to differentiate between the sexes, the presence of a black, M-shaped band across the male throat is the key sex marker in this species (Inger & Stuebing, 1997; Ibrahim, 2004).

2.5 Distribution, Habitat and Biology

2.5.1 *Fejevarya cancrivora*

The crab-eating frog, *F. cancrivora* is a widely distributed frog known to inhabit many parts of Asian region. Its geographic range extends from the coast of Southern China in Guangxi and the north-eastern coast of Hainan Island, China, through to Vietnam, the Andaman and Nicobar Islands (India), Peninsular Thailand, Malaysia, Singapore, the Greater Sundas, the Philippines, and the Lesser Sundas as far as Flores. It has also been intentionally introduced to Sorong and Jayapura, Papua, Indonesia (Kurniawan et al. 2010). In New Guinea, introduced populations are known from the Sorong, Manokwari, Nabire and Jayapura areas of Papua, Indonesia (Frost, 2011). The presence of this species was reported in the Pondicherry Mangroves, Bay of Bengal-India for the first time by Satheeshkumar (2011).

In Malaysia, *F. cancrivora* is usually found in disturbed habitats, cultivated areas, along the coast, lower reaches of large river basins, semi brackish, swampy areas close to the sea or in freshwater swamps beyond tidal influence (Inger & Stuebing, 1989; Norhayati et. al. 2012a). Although it occurs in coastal rice fields in Peninsular Malaysia, the presence of this species from such places in Borneo is not known (Inger & Stuebing, 1997).

Among the amphibian species in Malaysia, *F. cancrivora* is the only frog that can constantly live in saline habitat and tolerate marine environments. Salinity tolerance in this Crab-eating Frog has been conducted by several researchers (Gordon et al. 1961; Schmidt-Nielsen & Lee, 1962; Chew et al. 1972; Wright, 2004). Gordon et al. (1961) and Schmidt-Nielsen and Lee (1962) suggested that the major feature for the high salinity tolerance in the adults of *F. cancrivora* is their osmoregulatory mechanisms. The adults can respond to high external salinities with an increase in internal osmotic concentration, largely due to accumulation of urea (Gordon & Tucker, 1965; Wright, 2004). Gordon et al. (1961) found that adults of *F. cancrivora* can tolerate environmental salinities as high as 28% at 30°C, while the tadpoles tolerated salinities as high as 39% at the same temperature.

Gordon and Tucker (1965) conducted an experiment on salinity tolerance particularly on *F. cancrivora* tadpoles, and the results supported previous study by Pearse (1911) that tadpoles of this species had greater tolerance for high salinities than the adults. Wright (2004) stated that *F. cancrivora* is able to withstand salinity of 75% of seawater which is about 25 parts per thousand (ppt) and higher. The unusually high salinity tolerance of *F. cancrivora* is of great interest to researchers even though this special feature is not unique to them. Balinsky et al. (1972) reported that *Xenopus*

laevis and *Bufo viridis* have also been known to withstand 20 ppt and 26 ppt of salinity, respectively. However, *F. cancrivora* is probably the most tolerant among the known amphibian species (Balinsky et al. 1972).

2.5.2   *Fejevarya limnocharis*

The Rice Frog, *F. limnocharis* is a common and widespread amphibian among Southeast Asian frogs. This species can be found in all Southeast Asian and the rest of the Asian region extending from western Japan, Taiwan, south-western China, the Malay Peninsula, Bangladesh, Nepal, the Philippines, Indonesia, Sri Lanka, and India to Pakistan (Sumida et al. 2002). In China, this species is also distributed throughout a wide range of altitude (2–2000 m) (Fei & Ye, 2001). In Malaysia, the species only exist in disturbed habitats associated with human activities including paddy fields, roadsides, lawns, agricultural fields and football fields (Inger & Stuebing, 1997; Ibrahim, 2004; Norhayati et. al. 2012a).

Similar to *F. cancrivora*, Wu and Kam (2009) observed that *F. limnocharis* tadpoles display higher salinity tolerance compared to tadpoles of most species studied to date. They found that more than 50% tadpoles could survive in 9 ppt salinities for over a month, and a few individuals could survive in 11 ppt salinities for up to 20 days. Tadpoles metamorphosed earlier at a smaller size as salinity increased, suggesting the existence of adaptive developmental plasticity in *F. limnocharis* in response to osmotic stress (Wu & Kam, 2009).

2.6   Status of *Fejevarya cancrivora* and *F. limnocharis* conservation in Asia

Currently, *F. cancrivora* and *F. limnocharis* have been placed under Least Concern category by the International Union for Conservation of Nature (IUCN).

Even though there is no report on the decline of this species, they are facing serious threats due to human activities in their natural habitats, globally. According to Zhigang et al. (2009) over-harvesting, habitat alteration, wood harvest from mangrove forests, human settlement expansion and road kill may threaten the *F. cancrivora* populations. Recently, the global emergence of a chytrid fungus that causes chytridiomycosis in amphibians, *Batrachochytrium dendrobatidis (Bd)* has gained public awareness about this disease (Fisher et al. 2009).

This chytrid fungus was first described in 1998 and 1999 (Berger et al. 1998; Longcore et al. 1999) and is now the known cause of this potentially fatal disease, chytridiomycosis. Up to date, *Bd* has been reported in 36 countries where wild amphibian populations exist (Savage et al. 2011). Skerratt et al. (2007) reported that *Bd* has infected over 350 amphibian species and of these 200 are in decline. In Peninsular Malaysia, infection by this chytrid fungus has been reported in ten species from four families namely Megophryidae, Microhylidae, Ranidae and Rhacoporidae (Savage et al. 2011). Even though there is no data about this infection on *F. cancrivora,* it is still susceptible to the disease through infection by invasive frog species. It has been documented that one of the reasons of *Bd* infection in Peninsular Malaysia is the introduction of non-native amphibians (*Lithobates catesbeianus* and *Holoplobatrachus rugulosus*) in the frog farming industry.

Frost (2011) stated that *F. limnocharis* is adversely affected by pesticide pollution of chemicals used on the crops and falls prey at the tadpole and adult stages to water visiting birds, fishes and snakes. About 10, 000 years ago, rice (*Oryza sativa L.*) was first cultivated in Asia (Vaughan et al. 2008) and grown in paddy fields (Liu et al. 2011). Since paddy field is characterized by both aquatic habitats and dry lands,

it harbours a rich biological diversity (Bambaradeniya & Amarasinghe, 2003). Among them, frogs and toads (anurans) have benefited from the creation of paddy fields, which have become an important breeding habitat (Liu et al. 2011).

However, in order to ensure the good quality of rice produced and enhances rice production; farmers tend to use huge amounts of pesticides and fertilizers. The presence of pesticides in the environment has become a global issue. Field studies have shown that the reproduction, growth and development of wildlife species, invertebrates, amphibians, reptiles, fish, birds and mammals may have been impacted by chemicals that interact with the endocrine system (Khan & Law, 2005). Pesticides at low concentrations may act as blockers of sex hormones, causing abnormal sexual development, abnormal sex ratios and unusual mating behavior. Pesticides can also interfere with other hormonal processes, such as thyroid and its influence on bone. Although, both species are not experiencing conservation threat, determining the genetic structure of these species would be a preemptive measure to arrest any possibility of decline.

2.7   Genetic variation

Genetic variation or diversity is a basic component of biodiversity, forming the basis of species and ecosystem survival. The three major sources of genetic variation are mutation, gene flow and sexual reproduction (Understanding Evolution, 2012). It is well known that a decrease in genetic variation can lead to reduce fitness and lack of adaptability to a changing environment (Allentoft & O'Brien, 2010). Populations of species with low genetic variation are exposed to a higher probability of becoming genetically inbred, with the potential consequence of lowered fitness. In contrast, wide genetic variation helps improve the species ability to survive in a

changing environment, as the chances that some individuals will tolerate a particular change are increased (Martin & Hine, 2000). For instance, the extremely low genetic diversity in *Ranodon sibiricus* (Amphibia: Caudata) was taken to imply an absence of genetic variation for adaptive quantitative characteristic, hence, enhanced risk of extinction due to genetic homogeneity (Chen et al. 2012). Besides the importance of genetic diversity in adaptability to a changing environment, information on genetic diversity also helps in ascertain pedigrees, reconstructing phylogenies and estimating migration frequencies (Nicod et al. 2004; Nguyen et al. 2006). Various types of markers such as mtDNA and nuclear DNA markers are utilised to assess genetic variation and hence address issues of conservation and management of wildlife.

2.7.1   Genetic marker: Mitochondrial DNA (mtDNA) in general

Mitochondria are organelles specialized in energy conservation reactions and appear to have endosymbiotic origin in eukaryotic cells (Gray, 1992). They play an important role in metabolism, apoptosis, illness, and aging (Boore, 1999; Cao et al. 2006). For instance, the formation of adenosine-5'-triphosphate (ATP) by oxidative phosphorylation occurs in these organelles. They also possess their own double-stranded circular mitochondrial DNA (mtDNA), which are inherited independently of the nuclear genome.

Animal mtDNA is a double-stranded, closed circular molecule. This extrachromosomal genome is small and usually ranges from 16-20 kilobase in length (Boore, 1999; Kucuktas & Liu, 2007). MtDNA differs from nuclear DNA in the sense that mtDNA is maternally inherited and non-recombining (Avise, 1994; Nguyen et al. 2006), thus rendering it suitable for tracing maternal lineage. MtDNA also has high rates of nucleotide substitution as compared with nuclear DNA (Moritz,

1987; Pesole et al. 1999) with an evolutionary rate 5 to 10 times faster than the nuclear genes (Avise, 1994; Castro et al. 1998). These characteristics make mtDNA suitable for population level studies.

In general, animal mitochondrion contains only 37 genes; 13 protein-coding genes, two ribosomal RNA genes (rRNAs), and 22 transfer RNA genes (tRNAs) and a noncoding control region known as the D-loop containing the signals for regulation and initiation of mtDNA replication and transcription (Wolstenholme, 1992). Studies of the whole amphibian genome have been conducted by several researches (Roe et al. 1985; Zardoya & Meyer, 2000; Sumida et al. 2001; Zhang et al. 2003; Liu et al. 2005; Ren et al. 2009). To date, the complete mtDNA sequences are available for 26 anuran species (Ren et al. 2009).

Figures 2.1 and 2.2 show the organisation of *F. cancrivora* and *F. limnocharis* genome based on the studies by Ren et al. (2009) and Liu et al. (2005), respectively. The complete nucleotide sequence of mtDNA of *F. cancrivora* is 17,843 bp in length and contains 13 protein-coding genes (ATP6, ATP8, COI–III, ND1–6 and 4L and Cyt b), two ribosomal RNAs (12S and 16S rRNA), and 23 transfer RNAs genes (Ren et al. 2009). In contrast, the complete mtDNA sequence of *F. limnocharis* is 17,717 bp in length containing 13 protein-coding genes, 2 rRNA genes and 23 tRNAs genes and noncoding region (Liu et al. 2005). Although almost all animal mtDNAs encode 22 tRNA genes, the *F. cancrivora* and *F. limnocharis* mtDNA possesses an extra copy of tRNA^{Met}. The genome organization of *F. cancrivora* is identical with *F. limnocharis*, suggesting that the unique gene arrangement occurred in the common ancestor of the genus of these two species.
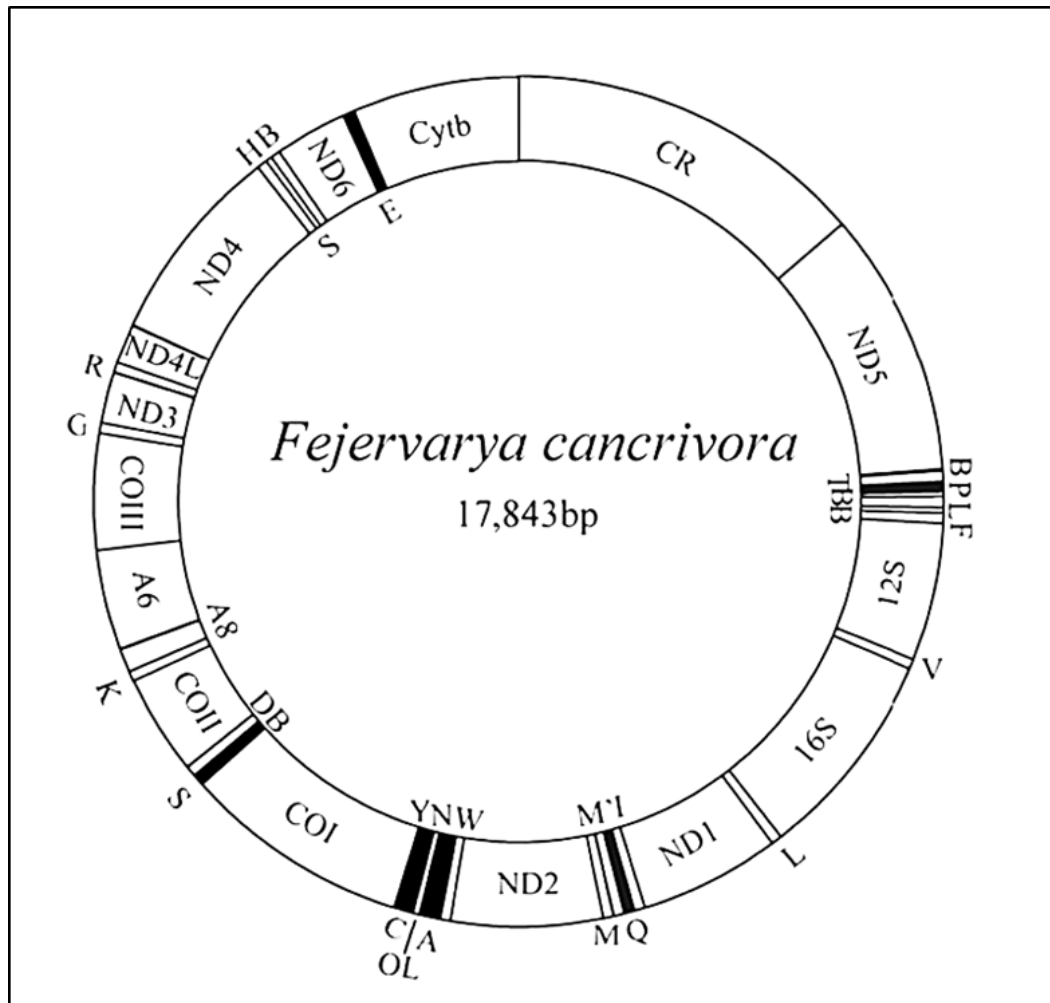
Figure 2.1: The gene organization of the *Fejervarya cancrivora* mitochondrial genome. The protein-coding genes are designated using abbreviations. The tRNA genes are denoted by the single-letter amino acid code. O-L represents the replication origin of the L-strands; and B is the intergenic spacer (Ren et al. 2009).

Abbreviations: A6, ATPase subunit 6; A8, ATPase subunit 8; COI–III, cytochrome c oxidase subunit; CR, control region; Cyt b, cytochrome b; ND1–6 and ND4L, NADH dehydrogenase subunits 1–6.