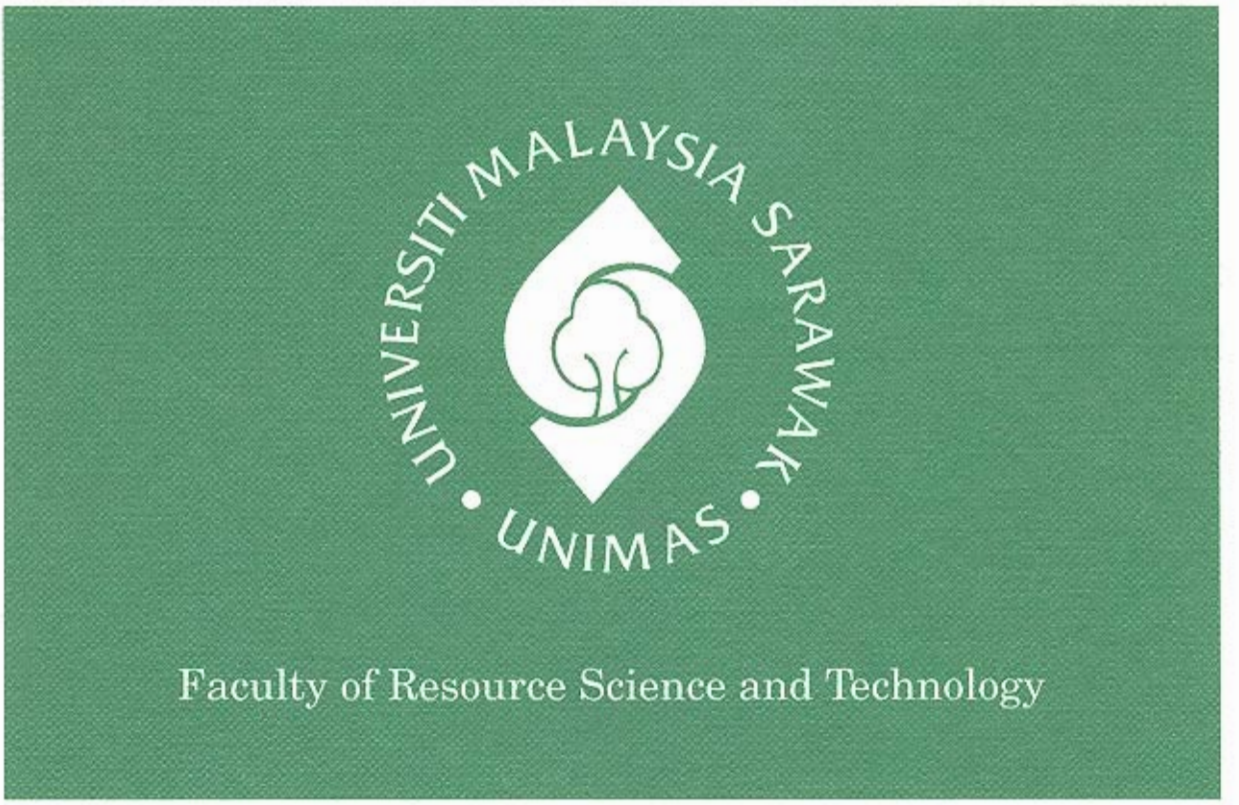

MOLECULAR SYSTEMATIC OF BORNEAN FANGED FROGS (GENUS *LIMNONECTES*) USING MITOCHONDRIAL DNA SUBUNIT 16S rRNA GENE

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Bachelor of Science with Honours
(Animal Resource Science and Management)
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This project is submitted in partial fulfillment of the requirements for the degree of

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UNIVERSITI MALAYSIA SARAWAK
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DECLARATION

No portion of the work referred to in this dissertation has been submitted in support of an application for another degree of qualification of this or any other university or institution of higher learning.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

MOLECULAR SYSTEMATIC OF BORNEAN FANGED FROGS (GENUS *LIMNONECTES*) USING MITOCHONDRIAL DNA SUBUNIT 16S rRNA GENE

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ABSRACT

The taxonomic classification and systematics among members of genus *Limnonectes* had been argued and debated for the past decades. Members of this genus which were previously assigned to the genus *Rana* exhibit a number of remarkable sexual dimorphic characteristics correlated to their breeding system. This study of partial mitochondrial ribosomal 16S gene from 14 individuals of four Bornean fanged species is an attempt to construct the phylogeny and further trying to resolve the classification of this poorly understood group. DNA sequences from a total of 14 individuals from 4 species of this genus with approximately 530 base pairs were analyzed using MEGA program. Maximum Parsimony and Neighbour-Joining analysis resulted in recognition of three species groups within the clade and support the monophyly of this genus.

Keywords: Systematics, genus *Limnonectes*, 16S gene, Parsimony analysis, Neighbour-Joining.

ABSTRAK

Pengkelasan taksonomi dan sistematik di kalangan ahli dalam genus Limnonectes telah diragui dan dibahas selama beberapa dekad kebelakangan ini. Ahli dalam genus ini yang dahulunya dikategorikan dalam genus Rana memiliki beberapa ciri perbezaan morfologi berdasarkan jantina yang menarik serta berkait rapat dengan sistem pembiakan mereka. Kajian menggunakan gen 16S yang merupakan sebahagian daripada mitokondria ke atas 14 individu genus Limnonectes Borneo ini adalah bertujuan untuk membina filogeni dan seterusnya cuba untuk mengklasifikasi genus yang kurang difahami ini. Jujukan DNA daripada 14 individuda empat dalam genus ini yang merangkumi lebih kurang 530 pasangan bes telah dianalisa menggunakan program MEGA. Analisis Maximum Parsimony dan Neighbour-Joining menunjukkan wujudnya tiga kumpulan spesies dalam genus Limnonectes serta menyokong teori monofiletik untuk genus ini.

Kata kunci: Sistematik, genus Limnonectes, gen 16S, analisis Maximum Parsimony, Neighbour-Joining.

1.0 INTRODUCTION

The genus *Limnonectes* has been recently split from the genus *Rana*. Although members of this genus show much variation in size, skin texture, webbing and breeding habits, they shared a great number of phenotypic similarities (Kiew, 1978, 1984; Inger, 1954, 1958, 1966). They are all terrestrial; some wandering widely through forests and others spend their entire lives on stream banks. As a group, they are among the most abundant of frogs in Bornean forests and every area of mature forest has three to five of these species (Inger and Stuebing, 2005).

Most of the fanged frogs were previously placed in the subgenus *Limnonectes* by Dubois (1987, 1992) and further partitioned among three species group, the *grunniens* group, the *kuhlii* group and the *microdiscus* group. This classification though, was not based on systematic analysis of characters (Inger, 1996) and was often quetioned. Morphological and molecular studies (Emerson and Berrigan, 1993; Emerson *et al.*, 2000) support the monophyly of *Limnonectes* and its recognition as a genus but do not support other grouping in Dubois's (1992) classifications.

Mitochondrial 16S ribosomal rRNA gene was utilized for this study due to some of its characteristics. This gene is fairly conserved in sequence and secondary structure and because it amplifies larger DNA fragment compared to the 12S gene, it is slightly more useful in phylogenetic construction. Furthermore, the structural domains of the 16S gene are highly conserved in mammals and amphibians and suitable to study level of divergence at the species level (Roe *et al.*, 1984).

So far, there is very little research on molecular systematic of genus *Limnonectes* done in Borneo to study the systematic of these interesting and unusual ranid frogs.

The aims of this study are to generate phylogenetic tree of Bornean fanged frogs and further analyzing the evolutionary pattern of relationships among individuals in this genus to aid in attempt to resolve the classification of this poorly understood genus using 16S rRNA gene.

2.0 LITERATURE REVIEW

2.1 General Overview of Anurans

Anurans, literally meaning 'without tail' in reference to the form of adults have evolved and diversified into at least 3500 species found throughout the world today, with more still being discovered every year (Tyning, 1990). In Borneo at least 143 species has been discovered which occurred in six families namely Bombinatoridae, Megophryidae, Bufonidae, Microhylidae, Ranidae and Rhacophoridae with 98 species are endemic to Borneo (Inger and Stuebing, 1999). The family Ranidae is one of the most speciose groups of living frogs (Duellman and Trueb, 1986). Presumed to have had an African origin sometime in early Tertiary (Savage, 1973), ranid currently show areas of high species richness in both Southeast Asia and Africa. The Southeast Asian species are of special interest as they occur throughout the Malay Archipelago-a region with well-studied and particularly complex biogeographic history (Emerson *et al.*, 2000).

2.2 Sexual Dimorphic Features of Genus *Limnonectes*

The voiceless species of genus *Limnonectes* or also known as the fanged frogs from the family Ranidae is characterized by a number of remarkable sexually dimorphic features, correlated to their breeding system (Emerson and Berrigan, 1993). The fanged frogs that were previously assigned to the genus *Rana* comprise mainly of nocturnal, moderately to very large terrestrial frogs. This genus is present on Borneo with 12 species, thus forming one of the most diverse genera of Bornean anurans (Malkmus *et al.*, 2002). In contrast to the genus *Rana*, species of fanged frogs exhibit secondary sex characters or sexually dimorphic features which comprise enlargement of both width and length of heads, hypertrophied jaw muscles in

both sexes, enlargement of the bony mandibular projection into long teeth-like structures or odontoid processes, lacking of vocal sacs and well-developed nuptial pads (Inger and Stuebing, 1997; Emerson *et al.*, 1992, 1993 and 1998; Orlov, 1997) except for *Limnonectes kuhlii* which does not have nuptial pads on its first fingers (Inger, 1966). Despite several differences in morphological characteristics, many members of this genus show great phenotypic similarity (Kiew, 1978, 1984; Inger, 1954, 1958, 1966) and even recognizing individual species has proven to be a challenge (Emerson *et al.*, 2000).

2.3 Distribution and Characteristics of the Fanged Frogs

The fanged frogs could be found along streams in Borneo rainforest (Emerson and Inger, 1992) and they usually do not wander far from water. This genus also does not exhibit specialized mode of reproduction and lack of parental care (Emerson, 2001). However there are exceptions such as in the cases of *Limnonectes finchi* and *L. palavanensis* in which they were found away from water source (Malkmus *et al.*, 2002) and the male of these two species provide parental care by attending the eggs after they are laid on land and carried the tadpoles on their backs after they hatch (Inger and Voris, 1988). Emerson *et al.* (2000) stated that extreme size of these fanged frogs is a common phenomenon in island species and some of the fanged species are island endemics. Most *Limnonectes* species lack vocal sacs (Emerson and Berrigan, 1993). Although members of this genus lack of advertisement calls in Borneo, Emerson and Ward (1998) stated that calling has been reported in populations from Peninsula Malaysia and Vietnam. There were some studies done to examine the other characteristics of these fanged frogs. For example, a study done by Emerson (2001) on macroevolution of historical contingency in the fanged frogs of Southeast Asia to examine correlation between fang size of this genus and their sexual dimorphism. This study concluded that the diversity in

size and sexual dimorphism that has evolved among the fanged species is probably due to a combination of evolutionary pressures.

2.4 Systematics of *Limnonectes*

Previously, most fanged frogs were placed in the subgenus *Limnonectes* and further partitioned among three species groups, the *kuhlii* group, the *grunniens* group and the *microdiscus* group (Dubois, 1987, 1992). Dubois also recognized other three subgenera in the genus *Limnonectes* which are *Hoplobatrachus*, *Fejervarya* and *Bourettia*. This classification is however, was not based on systematic analysis of characters (Inger, 1996). A previous systematic study of genus *Limnonectes* using a combination of morphological and behavioural characters to assess phylogenetic relationships among 19 genera was done by Emerson and Berrigan (1993). The result of this study confirmed that the fanged frogs and their relatives constitute a monophyletic group. Another recent systematic study on this genus (Emerson *et al.*, 2000) noted the monophyly of genus *Limnonectes* and presence of species groups within the clade. Overall, both systematic studies done support the monophyly of genus *Limnonectes* and its recognition as a genus but do not support other groupings in Dubois's (1992) classifications.

2.5 Use of Molecular Data in Systematic studies

Before the presence of molecular studies, taxonomist usually arranged species based on their shared features thus patterns of evolutions and relationships become more obvious (Inger and Stuebing, 1997). Today, the techniques from molecular biology have been widely and enthusiastically adopted by many systematists. Their use in systematic rests on the premise that a researcher can assess and compare the structure of genes among individuals, species or

higher taxa through examination of molecular structure of proteins and other compounds that are only a few steps removed from the gene (Zug, 1993).

2.6 Mitochondrial DNA

Mitochondrial genes have been employed extensively in evolutionary studies because of their uniparental mode of inheritance, high rate of evolution and relative simplicity of enzymatic amplification using universal primers (William *et al.*, 1995). As stated by Avise (1994), mitochondrial deoxyribonucleic acid (DNA) is an intraspecific and micro-evolutionary marker that has been employed extensively in molecular genetic as an informative guide to phylogenetic relationships among higher animal taxa including amphibians. Palumbi (1996) noted that although ribosomal genes in animal mitochondrial DNA are highly conserved, they span a region that includes enough variation to be phylogenetically useful at species level and below.

2.7 Mitochondrial DNA 16S rRNA Gene

The 16S gene of mitochondrial DNA evolves more slowly than the mitochondrial genome as a whole (Palumbi, 1996). This gene is very often used to study the phylogenetic relationships among different level of taxa because of the broad spectrum of phylogenetic analysis of this gene, especially in vertebrates (Wang *et. al.*, 2000). In addition, Emerson and Ward (1998) stated that from 37 mitochondrial genes, 16S gene is one of the genes widely used by researcher besides 12S gene, cytochrome c oxidase complex (COI, COII, COIII) and cytochrome b. A complete nucleotide sequence of *Xenopus laevis* mitochondrial genome studied by Roe *et al.* (1984) indicate that the overall structural domains of the 16S rRNA gene are highly conserved in mammals and amphibians and suitable to study level of divergence at

the species level. Palumbi (1996) also noted that the 16S rRNA gene is slightly more useful in phylogenetic construction because it amplifies larger DNA fragment compared to the 12S gene and there is enough variation in some species to be useful in population as well as species level studies. Figure 1 below shows the entire structure of animal mitochondrial DNA.

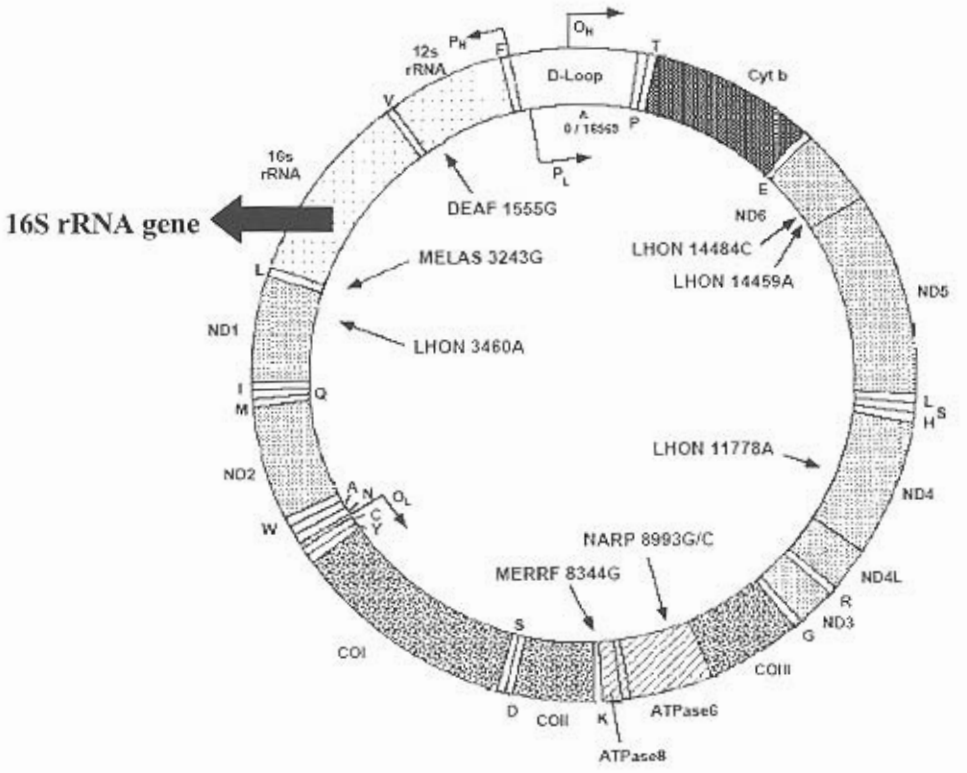

Figure 1: Map of mitochondrial DNA and location of 16S rRNA gene (source: Anon, 2004)

3.0 MATERIALS AND METHODS

3.1 Sample collection

For this project, muscle tissues from collections of genus *Limnonectes* from previous research were used. Tissue samples with a total number of five species were collected from three different sites which are Bau, Matang and Santubong. Besides using preserved tissue samples, fresh specimens were collected at Matang Wildlife Centre. Matang Wildlife Centre is located about 31 km respectively from Kuching at the western boundary of Kubah National Park (Hazebroek and Abang Kashim, 2000). Figure 2 shows the location of the study site.

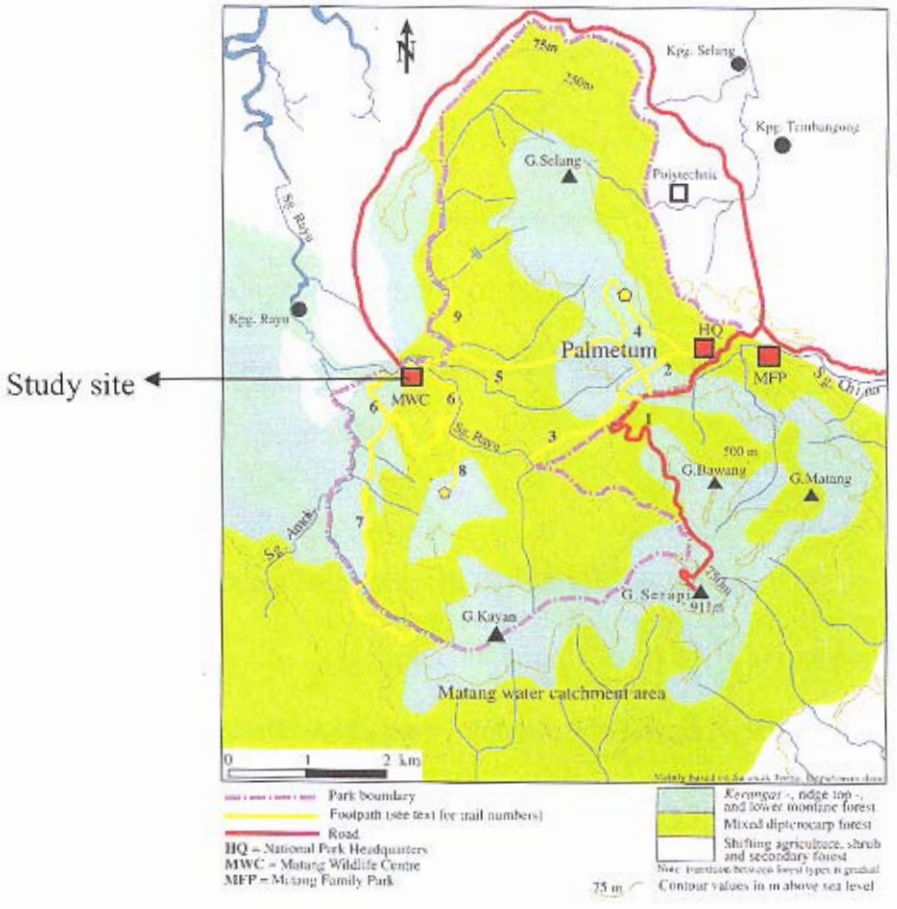

Figure 2: Map showing location of the study site (source: Hazebroek and Abang Kashim, 2000)

Frogs were located using headlamp and caught by hand. All data such as identification, location, time of capture and vegetation were recorded. The tissue muscles were placed into cryogenic vial containing Dimethyl sulfoxide ($DMSO_4$) buffer and then stored frozen at minus 20°C to avoid any possible contamination on tissue samples. Table 1 shows the ID number of species of which the preserved tissue samples were taken and their locality.

Table 1: List of *Limnonectes* species and locality.

Species	Field ID No.	Locality
Limnonectes paramacrodon	18, 1194	Samarahan
Limnonectes kuhlii	1168, 1172, 1165, 1171, 1173	Santubong
Limnonectes malesianus	1121	Matang
Limnonectes leporinus	1237, 1213, 1233, 1239, 1242, 1211,	Bau, Matang
Rana sp	23	Bau

3.2 Isolation of mitochondrial DNA

Genomic DNA was extracted from tissue samples using Genispin™ Tissue DNA kit (BST Techlab, Selangor, Malaysia). Yielding of genomic DNA includes centrifugation which is a process to separate DNA within tissue sample. High concentration of DNA obtained from this process is necessary for good PCR results.

3.3 Polymerase Chain Reaction (PCR) amplification

PCR was performed in 25µl reaction containing 2.5 µl of 10X reaction buffer (10 mM Tris-HCl, pH 8.0; 50 mM KCl; 0.1% [w/v] gelatin; 1%Triton X-100), 1.5 µL of $MgCl_2$, 1.0 µM each of the primers, 0.5 µM dNTPs (deoxynucleosides triphosphates) mix, 1-4µL of template DNA, 0.2µL of *Taq* DNA polymerase (Promega) and sufficient sterile deionized water to add the reactions up to the above volume. The DNA samples were processed in a DNA Thermal

Cycler (BIOMETRA) with a program through 30 cycles. Table 2 shows the 16S primers sequences used for this study:

Table 2: Sequences for 16 S primers (Palumbi, 1996)

Primer	Sequence Information	Direction	Sequence
16S	16Sar-L	Forward	5'-CGCCTGTTTATCAAAAACAT-3'
	16Sbr-H	Reverse	5'-CCGGTCTGAACTCAGATCACGT-3'

Optimized PCR parameters from previous research by Suhailah (2003) with slight modifications were used for this study. Table 3 below shows the parameter for 30 cycles of PCR:

Table 3: PCR parameter for 30 cycles

Phase	Optimized Temperature	Duration
Initial denaturation	94°C	5 minutes
Denaturation	94°C	45 seconds
Annealing	54°C	1 minute
Extension	72°C	1 minute 30 seconds
Final extension	72°C	7 minutes

3.4 Gel Electrophoresis

Visualizations of PCR and extraction products were done on agarose gel using electrophoresis method (Palumbi *et al.*, 1991). PCR and extraction products were run on agarose gel stained by 0.8µl of ethidium bromide (EtBr) for about 45 minutes and then visualized through UV radiation transilluminator. The presence of standard size marker is essential to determine the fragment size of both extraction and PCR products.

3.5 Purification of PCR product

Good PCR products were purified using GeniSpin™ PCR Purification Kit and the protocol provided by BioSynTech Sdn. Bhd. Purified products were then sent for sequencing.

3.6 Sequence analysis

CLUSTAL X 1.81 program (Thompson *et al.*, 1997) was used for multiple alignments of the selected DNA sequences together with CHROMAS 1.45 program (McCarthy *et al.*, 1997) where nucleotides designated as noise (N) were corrected and placed with the respective nucleotide (A, T, C, or G). Sequence variation analysis and statistics for nucleotide compositions were computed using MEGA 2.1 program (Kumar *et al.*, 2001).

3.7 Sequence Divergence

Pairwise distance using Kimura-2-parameter (Kimura, 1980) model was calculated in MEGA 2.1 program to generate the evolutionary distances of *Limnonectes* species studied. These distances usually measured by the number of nucleotide or amino acid substitutions between a pair of sequences. Evolutionary distances are fundamental for molecular evolution study and are useful for phylogenetic construction and estimation of divergence time. Kimura-2-parameter model used is based on assumption that the nucleotide frequencies are all equal throughout the evolutionary process. Calculated pairwise distance will be used in phylogenetic construction using Neighbour-Joining method.

3.8 Phylogenetic Analysis

3.8.1 Maximum Parsimony Analysis

Maximum Parsimony method for phylogenetic construction lies within the principle of producing the topology of a tree with the minimum tree length. Tree length refers to the sum of minimum possible substitution over all sites. All possible topology for this analysis were searched using the Close-Neighbour-Interchange (CNI) algorithm. This algorithm reduces searching time by first producing a temporary tree by topological distance and then examining all topologies that are different from the temporary tree by topological distance. Parsimony-informative characters were utilized for constructing phylogenetic tree using this Maximum Parsimony method with bootstrap test (Felsenstein, 1985) of 1000 replicates to test the reliability of produced tree.

3.8.2 Neighbour-Joining Analysis

This method (Saitou and Nei, 1987) uses the pairwise distances calculated using Kimura-2-parameter together with bootstrap test of 1000 replicates to test the reliability of an inferred tree. The given branch length indicates the rate of divergence for the respective species in the study.

For both analyses, sequences from the Gene Bank were used:

(i) Accession No. AY 322294 – *Rana erythraea*

(ii) Accession No. AY680268 – *Bufo melanostictus*

together with SP13 (*Rana* sp) sequence to add in an outgroup for the analysis.

4.0 RESULT

4.1 DNA extraction

Two extractions were done for a total number of 15 individuals from 4 species of *Limnonectes* using GeneSpin™ Tissue DNA. Both extractions produced good yields of DNA. Some modifications were done to the GeneSpin™ Tissue DNA kit protocol where the volumes of the second Elution Buffer were reduced from 200µL to 150µL. Table 4 shows the ID number of *Limnonectes* species which were successfully extracted. Figure 3 and 4 shows the Gel electrophoresis for Extraction 1 and 2.

Table 4: ID number of extracted individuals and species.

Extraction	ID number	Species
Extraction 1	1121	*Limnonectes malesianus*
	1168	*L. kuhlii*
	1172	*L. kuhlii*
	1213	*L. leporinus*
	1233	*L. leporinus*
	1237	*L. leporinus*
	1239	*L. leporinus*
	23	*Rana* sp
Extraction 2	18	*L. paramacrodon*
	1165	*L. kuhlii*
	1171	*L. kuhlii*
	1173	*L. kuhlii*
	1194	*L. paramacrodon*
	1211	*L. leporinus*
	1242	*L. leporinus*

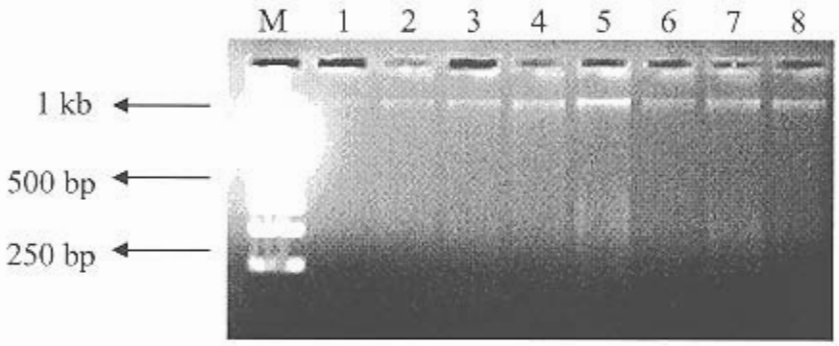

Figure 3: Gel electrophoresis for Extraction 1. Lane 1 *Limnonectes malesianus*. Lane 2 *L. kuhlii*. Lane 3 *L. kuhlii*. Lane 4 *L. leporinus*. Lane 5 *L. leporinus*. Lane 6 *L. leporinus*. Lane 7 *L. leporinus*. Lane 8 *Rana* sp. M represents GeneRuler™ 1kb DNA Ladder (Fermentas) as a standard size marker.

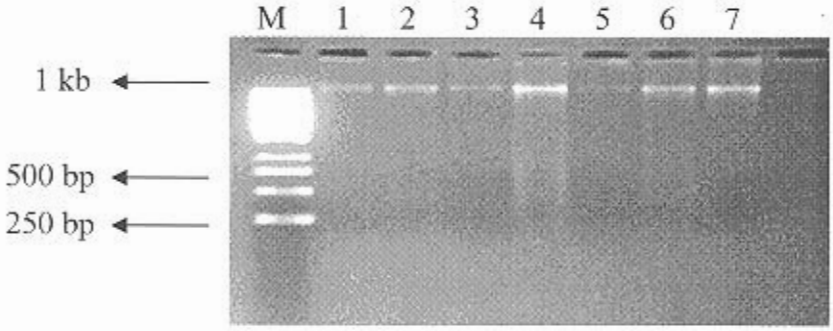

Figure 4: Gel electrophoresis for Extraction 2. Lane 1 *L. paramacrodon*. Lane 2 *L. paramacrodon*. Lane 3 *L. kuhlii*. Lane 4 *L. kuhlii*. Lane 5 *L. kuhlii*. Lane 6 *L. leporinus*. Lane 7 *L. leporinus*. M represents GeneRuler™ 1kb DNA Ladder (Fermentas) as a standard size marker.

4.2 Polymerase Chain Reaction (PCR) amplification and purification of amplified PCR products

Overall, 21 PCR amplifications were done in a 25µL and 50µL reaction of PCR mixture. All 15 samples were amplified using 12S primers and 16S primers. Amplifications using 12S primers were unsuccessful even after some modifications made to the PCR cycle parameters, concentration of magnesium chloride and volume of respective reagents in reaction mixture. Amplifications using 16S successfully produced good yield of DNA products with

approximately 500 base pairs long. All successfully amplified samples were then purified using GeniSpin™ PCR Purification Kit and sent for DNA sequencing. Figure 5 shows PCR amplification for respective samples.

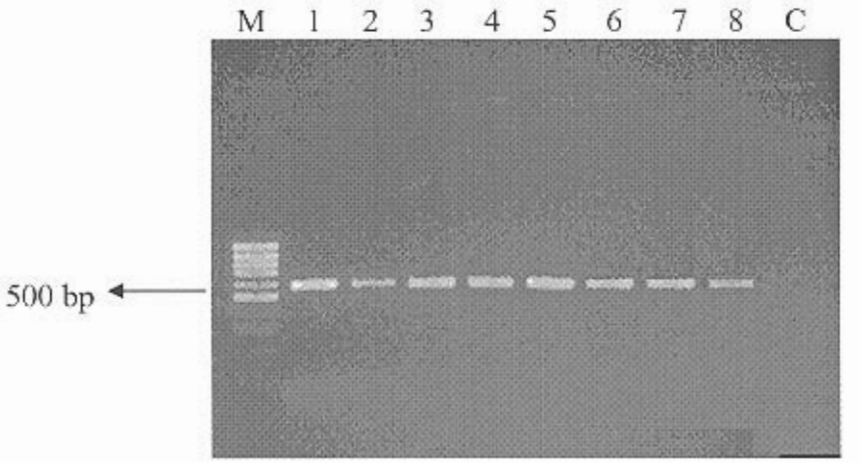

Figure 5: Gel electrophoresis for PCR amplification using 16S primers. Lane 1 *Limnonectes paramacrodon*. Lane 2 *L. kuhlii*. Lane 3 *L. kuhlii*. Lane 4 *L. leporinus*. Lane 5 *L. leporinus*. Lane 6 *L. malesianus*. Lane 7 *L. leporinus*. Lane 8 *L. leporinus*. M represents GeneRuler™ Low Range DNA Ladder (Fermentas) as a standard size marker. C represents the negative control.

4.3 DNA Sequencing

All 15 samples sent for sequencing were successfully sequenced. The sequences were aligned using CLUSTAL X 1.81 program (Thompson *et al.*, 1997) and CHROMAS 1.45 program (McCarthy *et al.*, 1997) before undergo further analysis.

4.4 Sequence Analysis

Sequence analysis were obtained using MEGA 2.1 program by Kumar *et al.* (2002). The sequences ranges from 512 to 515 base pairs long with a total of 76.78% (410/534) of conserve sites, 20.22% (108/534) of variable sites, 14.23% (76/534) of parsimony-informative

sites, 5.99% singleton sites, 61.80% nonsynonymous substitution sites and 11.61% synonymous substitution sites. The nucleotide compositions for all sequences were also analyzed and presented in Table 5. Overall, the sequences assessed constitute higher frequencies of Adenine (A) and Thymine (T) compared to Cytosine (C) and Guanine (G).

Table 5: Nucleotide composition for 14 sequences assessed. Values are given in percent (%) except the total.

Species	Nucleotide Composition (%)				
	T(U)	C	A	G	Total
SP6 *L. kuhlii* Santubong	23.5	24.7	30.9	21.0	**515**
SP9 *L. kuhlii* Santubong	23.3	24.7	30.9	21.0	**514**
SP7 *L. kuhlii* Santubong	23.4	24.8	31.0	20.9	**513**
SP10 *L. kuhlii* Santubong	23.4	24.8	31.0	20.9	**513**
SP8 *L. kuhlii* Santubong	23.4	24.8	31.0	20.9	**513**
SP1 *L. leporinus* Bau	24.4	24.0	31.4	20.1	**512**
SP12 *L. leporinus* Matang	24.3	24.1	31.3	20.2	**514**
SP4 *L. leporinus* Matang	24.4	24.0	31.4	20.1	**512**
SP3 *L. leporinus* Matang	24.4	24.0	31.4	20.1	**512**
SP5 *L. leporinus* Bau	24.5	24.1	31.3	20.0	**514**
SP2 *L. leporinus* Bau	24.4	24.0	31.4	20.1	**512**
SP11 *L. paramacrodon* Samarahan	25.5	23.5	31.1	19.8	**514**
SP14 *L. paramacrodon* Samarahan	25.8	23.4	30.9	19.9	**512**
SP15 *L. malesianus* Matang	25.1	23.7	30.2	21.0	**514**
Average	**24.3**	**24.2**	**31.1**	**20.4**	**513.1**

4.5 Sequence Divergence

Table 6 shows the estimate value of divergence between species of *Limnonectes* studied calculated using distances data only from a total of 507 sites. Gaps and missing data for this analysis were treated using complete deletion option. The highlighted values are the range of divergence for the respective species. The range of divergence within respective species of *Limnonectes* studied is 0 to 0.012 meanwhile the range of divergence between those respective species is 0.099 to 0.146.