



Faculty of Resource Science and Technology

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R. ERYTHRAEA AT BAU LIMESTONE AREA USING
MITOCHONDRIAL DNA OF CYTOCHROME OXIDASE I (COI)
GENE**

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GENETIC DIFFERENTIATION AMONG POPULATIONS OF *R. ERYTHRAEA* AT BAU LIMESTONE AREA USING MITOCHONDRIAL DNA OF CYTOCHROME OXIDASE I (COI) GENE

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ABSTRACT

This study focuses in comparing genetic differentiation among populations in one species of anuran from Bau limestone area, *Rana erythraea*. Samples were subjected to PCR-DNA sequencing analysis using cytochrome oxidase I (COI) gene. Result from the sequence variation analysis shows that there is still variation among the frog population studied although the percentage is very low.

Key words: Population of *R. erythraea*, mitochondrial DNA, COI.

ABSTRAK

Kajian ini memfokus untuk membandingkan perbezaan genetik di antara spesies anura daripada kawasan batu kapur di Bau, *Rana erythraea*. Sampel telah dijalankan analisa PCR-penjujukan DNA menggunakan gen sitokrom oksidase I (COI). Keputusan daripada analisis variasi jujukan menunjukkan walaupun peratusan perbezaan diantara populasi katak yang dikaji adalah amat kecil namun variasi tetap ada.

Kata kunci: Populasi *R. erythraea*, mitochondrial DNA, COI.

- 650.
- DNA -- analysis
 - Frog
 - Amphibians -- Borneo
 - Genome

Introduction

One hundred and forty three species of frogs occurred in Borneo with six families, Bombinatoridae, Megophryidae, Bufonidae, Microhylidae, Ranidae and Rhacophoridae (Inger and Stuebing, 1999). Ninety eight species are endemic to Borneo (Inger and Stuebing, 1999). The exact number is unsure because nearly new species are discovered every year. There are two major groups of frogs in Borneo based on their habits and habitats which are group of frogs that are closely associated with human activity and group of frogs that can be found in the forest or the edge of the forest (Inger and Stuebing, 1997).

Rana erythraea or known as 'Green Paddy Frog' is a frog with long, muscular hind limbs and a long pointed snout with a medium-sized (male 50-45 mm; females 50-75 mm) (Inger *et al.*, 1974). It is usually found near human habitation in cultivated or disturb area and developed large population even in town (Alcala, 1995; Inger and Greenberg, 1963). *R. erythraea* have an efficient digestive system thus enable them to survive although in a disturb area (Heng, 1972). The borders of lakes and ponds, open swamps, paddy fields, weed-choked ditches, and sluggish creeks are typical habitats (Inger *et al.*, 1974). *R. erythraea* never moves far from standing or flowing water (Alcala, 1955). This species is widely distributed in Southeast Asia (Inger and Stuebing, 1997).

Frog, which is the most common form of amphibian, indicates variability in colour and pattern and this cause confusion when trying to identify them (Arnold *et al.*, 1978). The taxonomist usually arranged species into group based on their shared feature thus patterns of evolution and relationships become more obvious (Inger and Stuebing, 1997). The choice

of molecular data is crucial for phylogenetic analyses, and molecular studies can now be tailored specifically for particular phylogenetic groups (Lamb and Lydeard, 1994). There is little molecular study of the Bornean frogs. It is important to know the evolutionary relationships among amphibians and genetic structure whether gene flow occurs among population of frogs occurred in the different location. The studies of molecular genetic using the mitochondrial DNA (mtDNA) genome have been done in Bornean riparian frogs (Zainuddin, 1998) but not in this species. However, Roe *et al.* (1984) had completed the nucleotide sequences of the *Xenopus laevis* mtDNA genome.

Mitochondrial DNA (mtDNA) is the most common tool for the study of population variation. This is because it often shows significant variation even among individual organisms (Greenberg *et al.*, 1983). Mitochondrial DNA has been the most common source of information at the DNA (restriction fragment) level for various kinds of population study for a variety good reason (Berry *et al.*, 1991). Beside that, it has an ability to evolve faster than the nuclear genome (Brown *et al.*, 1979). According to Hoelzel (1992), mtDNA are widely used in studies of animal population because of reasons such as maternal inheritance, general conservation of gene order and composition especially among closely related form, rapid rate of sequence divergence especially in vertebrata, small size compared to the nuclear genome and relative ease of isolation and purification.

DNA sequencing was employed to measure variation among population of *R. erythraea*. The introduction of PCR had made the application of sequencing analysis easier when the specific regions of DNA can be amplified (Hoelzel, 1992). PCR is an *in vitro* method for the enzymatic synthesis of specific DNA sequences by using two oligonucleotide primers that

hybridize to opposite strands and flank the region of interest in the target DNA (Erlich, 1992). Oligonucleotide primers or amplimers are short, single-stranded DNA molecules which are complementary to the ends of a defined sequence of DNA template (Newton and Graham, 1994). During a PCR cycle, three distinct event must occur which are the denaturation of the template, primer annealing and DNA synthesis by a thermostable polymerase (Dienffenbach and Dveksler, 1995).

The selection of primer in the PCR is the most important to make sure the quality of the result. This is because small differences in primer efficiency can give rise to large differences in product yield (Mullis *et al.*, 1994). Cytochrome oxidase I (COI) gene which is one of the cytochrome c oxidase complex other than COII and COIII, is a targeted gene that were used in this study. This primer was chosen as my targeted gene because it has been widely used by previous researchers and the results of amplifications were very efficient. Although the subunit II (COII) has been much more widely used for phylogenetic studies than COI (Adkins and Honeycutt, 1994; Ashley and Vaughn, 1995), but the recent studies on anuran have successfully employed COI (Zainuddin, 1998). The study had shown remarkable variation of genetic at population level (Zainuddin, per. comm.). Beside that, according to Adkins *et al.*, (1994), the low-resolution structure of COI is used because it contains a significant amount of information to detect variation between species.

In order to examine the genetic structure of the *R. erythraea*, two sites were sampled. The first site was Fairy Cave while the second site was the Wind Cave. Both studied sites were located at Bau limestone area. The rationale of choosing these different localities was there is a river connected to both locations. Thus, there are possibilities for the frogs to migrate

between each location. This will show whether there are migrations between the two populations.

The goal of my study was to measure variation in mitochondrial DNA of *R. erythraea* populations at two different localities. My hypothesis is there will be no variation among the population. The distance between the two localities is only about 3km. Secondly, how effective the COI gene of mtDNA as genetic marker for frog species at population level.

Materials and methods

Study sites

R. erythraea were collected from different localities in Sarawak, which are Fairy Cave (N 01° 38.133', E 110° 12.140') and Wind Cave (N 01° 24.915', E 110° 08.109') situated at the Bau limestone area (Figure 1). Samples from previous collection, which were collected from UNIMAS peat swamp forest, were also being used as comparison in my laboratory analyses (Table 1). At each locality, frogs were located by headlamp and caught by hand. The captured frogs were placed in a separate plastic bag. The data of the frogs such as location, identification, time of capture and vegetation were recorded. Tissue muscles were stored in 20% DMSO buffer.

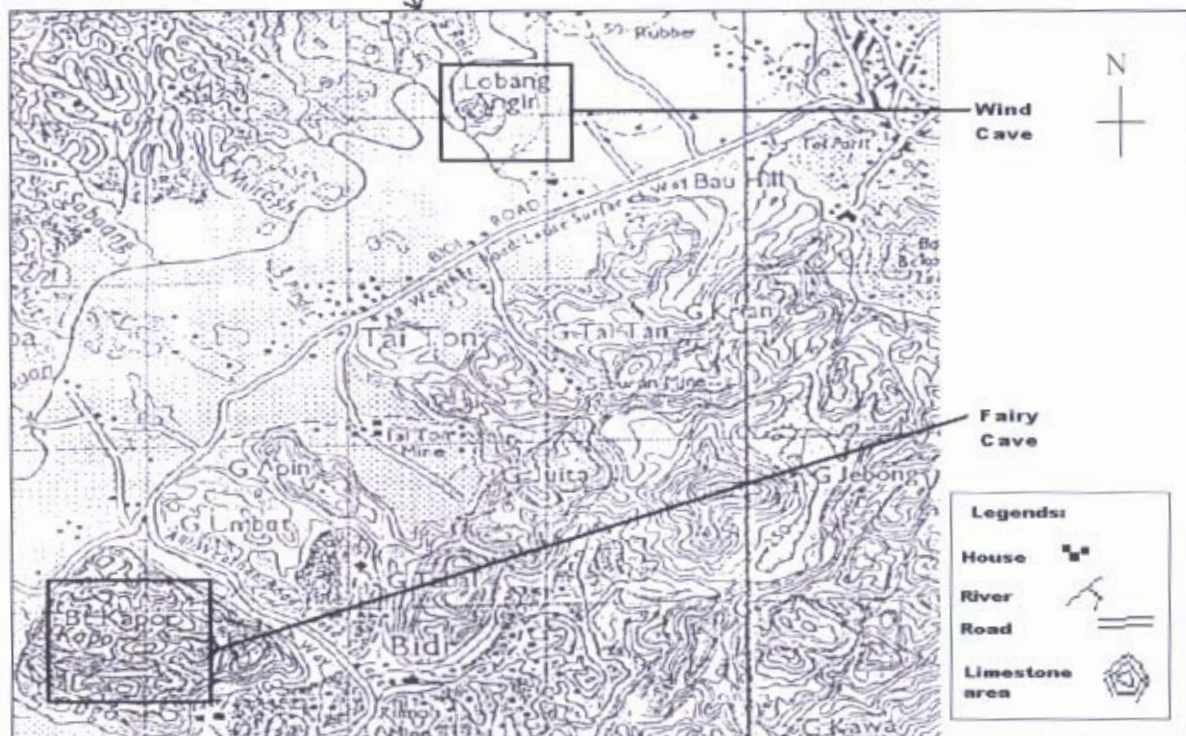


Figure 1: Map of Sarawak (above) and Bau Limestone area (Foh, 1999) (below) showing sampling location.

Table 1: Collected samples and locations.

| Species | Location | Number of frogs |
|---------------------|------------|-----------------|
| <i>R. erythraea</i> | Fairy Cave | 21 |
| | Wind Cave | 2 |
| | UNIMAS | 1 |

Molecular technique

DNA extraction

Total genomic DNA was extracted from muscle tissue taken from the frog hind leg or toe and stored in 20% DMSO using Pure-Gene™ Tissue DNA kit (manufactured by BioSynTech, Subang Jaya). According to the manufacture's protocol, 30mg of tissues were minced and placed into a 1.5ml microfuge tube, which added with 200µl of TL buffer. After that, 25µl of OB protease were added and the mixture was well mixed by vortexing. The mixture was then incubated at 55°C in a shaking waterbath for three hours to affect complete lysis.

After the incubation period, 220µl Buffer BL were added and vortexed to mix before incubated at 70°C for 10 minutes. Then, 220µl absolute ethanol were added and mixed thoroughly by vortexing. The mixture was transferred into a column and centrifuged at 8,000 rpm for 1 minute to bind DNA. The collection tube and flow-through was discarded. Next, the column was placed in a new 2ml collection tube and wash by pipetting with 750µl of wash buffer diluted with ethanol. After that, centrifuged at 8,000 rpm for 1 minute to bind DNA and the column was washed and centrifuged again as above.

The column was then washed with a second 750µl of wash buffer using a new collection tube. After that, centrifuged at 8,000 rpm for 1 minute and the flow-through liquid were discarded. Using the same 2ml collection tube, the column was then dried by centrifuge at maximum speed about 10,000 rpm for 2 minutes and placed into a sterile microfuge tube added with 200µl or 100µl of preheated (70°C) Elution Buffer. The microfuge tube was allowed to stand at room temperature for 3 minutes.

Lastly, the tube was centrifuged at 8,000 rpm for 1 minute to elute DNA from the column. The second elution was repeated with a second 100µl of preheated (70°C) Elution Buffer. The tube was allowed to sit for 3 minutes at room temperature. Finally, the tube was centrifuged at 8,000 rpm for 1 minute to elute DNA from the column. Extraction products were then visualized on electrophoresis system using 1.0% agarose gel containing ethidium bromide (0.08 µg/mL). A 1Kb Plus ladder (Fermentas) was used as a standard marker.

PCR analysis

After isolation, amplification of COI was done by PCR. Four microlitres of extracted DNA was used in a 46µl PCR reaction. The primer used was designed to amplify approximately 550 bp of the selected DNA region. Primers COI-f is a down-stream primer and COI-e is an upper-stream primer designed by Palumbi *et al.*, (1991) were chosen.

This reaction mixture contained 5µl 10x buffer, 3µl MgCl₂, 1µl dNTPs, 2.5µl each of forward and reverse COI primers, 0.25µl Taq polymerase, 4µl template DNA and 31.75µl dH₂O. Samples were spin down at 5000 rpm at 10 seconds before amplified it in the PCR machine.

Cycling conditions were: one cycle of 5 minutes at 96°C, 35 seconds at 95°C, 6 minute at 50.5°C followed by 30 cycles of 1 minute 30 seconds at 72°C, 10 minutes at 72°C and the reaction was left to stand at 4°C. For each sample, 5µl of PCR product were resolved using 1.0% agarose gel containing ethidium bromide (0.08 µg/mL) and run for approximately 1 hour 30 min at 70V. A 100bp Plus ladder (Fermentas) was used as a standard marker.

Samples purification

PCR product obtained was purified using Promega purification kit before sending for sequencing at the First Base Lab. Purification has to be done due to the non-specific product. 45µl of PCR product were placed into PCR tube. Then, 45µl of MemBind solution were added. The mixture was vortexed for a while to mix well. Then, the mixture was transferred into a column and centrifuge at 14,000 rpm for 1 minute. The flow-through was discarded.

After that, 700µl of 80% isopropanol were added to wash the column and centrifuged at 14,000 rpm for 1 minute. The flow-through was discarded. The column was then washed with a second 500µl of 80% isopropanol and centrifuged for 5 seconds and the flow-through was discarded. Using the same 2ml collection tube, the column was then dried by centrifuging at 14,000 rpm for 1 minute and placed into a sterile microfuge tube added with 30µl of dH₂O.

Finally, the tube was centrifuged at 14,000 rpm for 30 seconds to elute DNA from the column. Purify products were then visualized on electrophoresis system using 1.0% agarose

gel containing ethidium bromide (0.08 µg/mL). A 100bp Plus ladder (Fermentas) was used as a standard marker.

Sequence analysis

In this study, CHROMAS (Version 1.45) program was used to display fluorescence-based DNA sequence analysis result. Modification of nucleotide designated as noisy (N) that occur in all DNA sequences samples were done by referring to the best peak that would indicate the correct expected nucleotides (A, C, G or T). CLUSTAL X (1.81) program was then used to align the sequences. Beside that, Phylogenetic Analysis Using Parsimony (PAUP*) program Version 4.0b10 (Swofford, 2001) was also used for sequence variation analysis.

Pairwise sequence divergence to see the transition/transversion ratio and distance matrix were obtained base on distance measure of Kimura 2-parameter model using the PAUP* program Version 4.0b10 (Swofford, 2001). Kimura 2-parameter was used because this model assumes an equal distribution of nucleotide composition and unequal rate of evolution of transition and transversion (Kimura, 1993). Molecular Evolutionary Genetic Analysis (MEGA) program Version 2.1 also used to indicate variable and parsimony site in the DNA sequences.

Phylogenetic analysis

Phylogenetic relationships were estimated with neighbor-joining (NJ) analysis, maximum likelihood analysis using bootstraps methods with heuristic search and maximum parsimony analysis also using bootstraps method with heuristic search. All analysis was done with 1000 replicates using PAUP* Version 4.0b10 (Swofford, 2001). The entire trees

were bootstrapped in order to assess their statistical reliability. Bootstrap proportion of <50% was considered as not supported, proportion between 50% and 70% as weakly supported, and proportions $\geq 70\%$ as well supported (George *et al.*, 1998; Hillis and Bull, 1993). *Limnonectes leporinus* was selected as an outgroup.

Results

DNA extraction

Fifteen samples were successfully extracted (Table 2, Figure 2). Total genomic DNA extracted is about 12Kb in length. Only 10 samples were subjected to gel electrophoresis figure.

Table 2: Samples selected for extraction.

| Species | Location | Number of frogs |
|---------------------|------------|-----------------|
| <i>R. erythraea</i> | Fairy Cave | 12 |
| | Wind Cave | 2 |
| | UNIMAS | 1 |

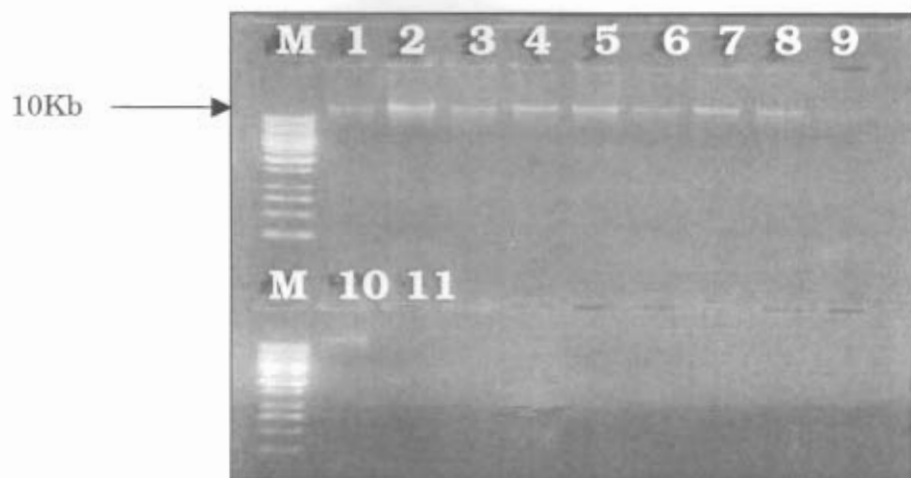


Figure 2: Polaroid picture taken for extracted DNA. [M = 1Kb marker (Fermentas); 1,2,3,4,5,6,7 = samples from Fairy cave; 8,9 = samples from Wind Cave and 10 = samples from UNIMAS; 11= Negative control].

PCR product

Approximately 500bp of the COI were obtained for all specimens (Figure 3). 15 PCR products were obtained. Only five samples subjected to gel electrophoresis figure.

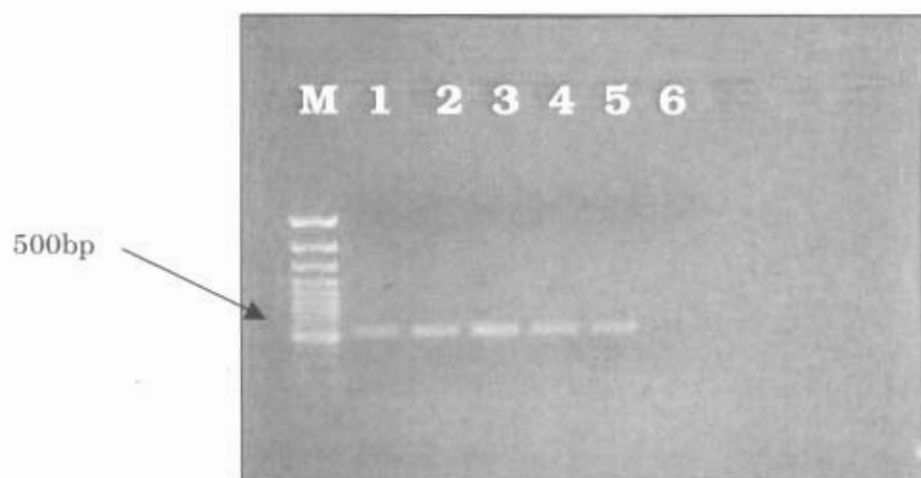


Figure 3: Polaroid picture for PCR product [M = 100bp Plus (Fermentas); 1, 2, 3 = samples from Fairy Cave; 4 = sample from Wind Cave; 5 = samples from UNIMAS; 6 = negative control].

DNA sequencing

Overall, 17 purified samples (individuals) were send for sequencing (Table 3). Out of all samples, only 8 products showed good signal in DNA sequencing. Between 471 and 514 bp of the COI gene for all specimens were obtained (Table 3). The aligned sequences are in Appendix A.

Table 3: Sequencing result for all samples.

| Samples | Length after alignment | Result and comment from First Base Lab |
|----------------|------------------------|--|
| Fairy Cave 52 | 478 bp | Good signal generated |
| Fairy Cave 96 | 511 bp | Good signal generated |
| Fairy Cave 100 | 471 bp | Good signal generated |
| Fairy Cave 104 | 478 bp | Good signal generated |
| Fairy Cave 22 | 513 bp | Good signal generated |
| Fairy Cave 94 | 514 bp | Good signal generated |
| Fairy Cave 99 | 497 bp | Good signal generated |
| Wind Cave 65 | 500 bp | Good signal generated |

Sequence variation analysis

A total of 26 sites were variable (5%) and of those, 12 sites (24%) were phylogenetically informative. In mtDNA, transitional substitutional (purine to purine or pyrimidine to pyrimidine) occur more frequently than transversional substitution (purine to pyrimidine or vice versa). The average of transition and transversion ratio was 0.6838 or 2:1 (Table 4). This ratio is comparable with the *L. leporinus* ratio (2:1) (Zainuddin, 1998).

Table 4: Transition/transversion ratio of Kimura 2-parameter of *R. erythraea* showing within and between population (bold).

| | FC-100 | WC-65 | FC-94 | FC-52 | FC-96 | FC-104 | FC-99 | FC-22 |
|--------|---------------|---------------|--------|--------|--------|--------|--------|-------|
| FC-100 | - | | | | | | | |
| WC-65 | 1.5000 | - | | | | | | |
| FC-94 | 0.6667 | 0.3333 | - | | | | | |
| FC-52 | 0.7500 | 0.8000 | 0.6000 | - | | | | |
| FC-96 | 1.3333 | 1.0000 | 0.6667 | 0.7143 | - | | | |
| FC-104 | 0.6000 | 0.8000 | 1.0000 | 0.5000 | 0.8333 | - | | |
| FC-99 | 0.7500 | 1.0000 | 0.3333 | 0.5714 | 1.3333 | 0.6667 | - | |
| FC-22 | 0.5000 | 0.5000 | 0.0000 | 1.0000 | 0.7143 | 0.4286 | 0.2500 | - |

Among the eight sequences, eight haplotypes were identified (Table 5). These indicate all individual are unique and no haplotypes were shared among localities. This shows that the populations were highly polymorphic. Beside, there is no evidence of local geographical structuring in this species as shown in the distribution of haplotypes.

Table 5: Frequency distribution of mtDNA haplotypes among eight samples of *R. erythraea* from two different localities in Sarawak.

| Haplotypes Localities | FC-22 | FC-52 | FC-96 | FC-94 | FC-99 | FC-100 | FC-104 | WC-65 |
|--------------------------|-------|-------|-------|-------|-------|--------|--------|-------|
| Fairy Cave | I | I | I | I | I | I | I | - |
| Wind Cave | - | - | - | - | - | - | - | I |

Range of sequence divergence was relatively low. Range obtain was between 0.6% (FC-22 versus WC-65) to 2.6% (FC-96 versus FC-52) compare to other study, *R. chalconata* (0.2% to 7.6%), *L. kuhli* (0.2% to 21%) and *L. leporinus* (0.4% to 12.3%) (Zainuddin, 1998).

Table 6: Sequence divergence of Kimura 2-parameter of *R. erythraea* showing within and between population (bold).

| | FC-100 | WC-65 | FC-94 | FC-52 | FC-96 | FC-104 | FC-99 | FC-22 |
|--------|----------------|----------------|---------|---------|---------|---------|---------|-------|
| FC-100 | - | | | | | | | |
| WC-65 | 0.01070 | - | | | | | | |
| FC-94 | 0.01071 | 0.00806 | - | | | | | |
| FC-52 | 0.01508 | 0.01915 | 0.01696 | - | | | | |
| FC-96 | 0.01502 | 0.00809 | 0.01991 | 0.02559 | - | | | |
| FC-104 | 0.01722 | 0.01915 | 0.01269 | 0.01271 | 0.02343 | - | | |
| FC-99 | 0.01508 | 0.00814 | 0.00813 | 0.02347 | 0.01429 | 0.01058 | - | |
| FC-22 | 0.01290 | 0.00610 | 0.01796 | 0.01274 | 0.02405 | 0.02131 | 0.01017 | - |

Phylogenetic analysis

From the analysis, all tree showed similar topology, which indicate all sequences are monophyletic group. Only neighbor-joining (NJ) analysis (Figure 4) was shown since the phylogenetic tree indicates the best topology. From the clades, haplotypes WC 65 diverged much earlier than the rest of haplotypes except for FC 96 as supported by 60% of bootstrap value.

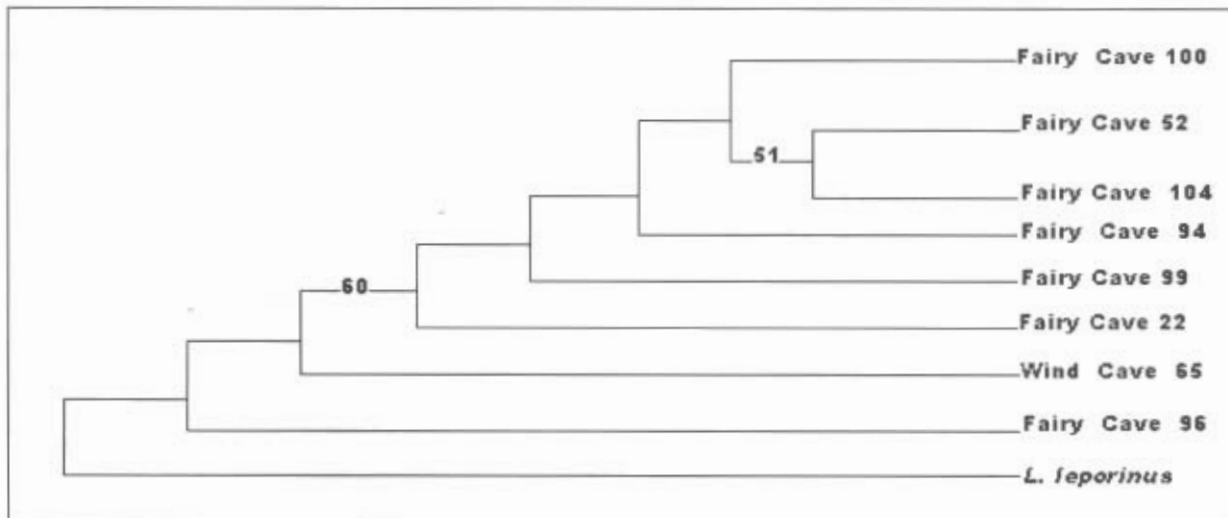


Figure 4: Bootstrap 50% majority-rule consensus tree of neighbor-joining analysis with 1000 replicates. The bootstraps value indicated at the branch. *L. leporinus* from Matang was used as an outgroup.

Discussion

The low observed ratios of transitions (s) to transversions (v) among *R. erythraea* (2:1) are relatively the same with the observed ratios of s:v in the COI region found in the *L. leporinus* (2:1) (Zainuddin, 1998). However, this ratio is consistent with observed ratios of s:v in another cytochrome oxidase subunit' COII in primates species, *Aotus* (5:1 in Ashley and Vaughn, 1995) and among Lepidoptera, *Greya species* (range 2:1 to 6:1 in Brown *et al.*, 1994).

Although samples sizes at each location were small, the distribution of haplotypes within the two locations indicates no local geographical structuring of species. The remarkable levels of intraspecific variability of the COI of *R. erythraea* indicate that the populations of the species are highly polymorphic within and among the population. This result suggested that COI of the Borneon frogs can be a useful genetic marker for further population studies of frogs in this region.

The finding indicated remarkable level of sequence divergence within and between populations, as high as 2.6%. Other studies have revealed substantial levels of sequence divergence in other region of cytochrome oxidase, for instance 6.2% of sequence divergence of COII in *Aotus* (Ashley and Vaughn, 1995) and 6.8% of sequence divergence of COI + COIII within species of *Mytilus* (Hoeh *et al.*, 1996). Comparison of my COI data with *R. chalconata*, *L. kuhli* and *L. leporinus* (Zainuddin, 1998) indicates much lower levels of sequence divergence between populations of frogs. This indicates that population may come from the same population where there is extensive gene flow going on.

Based on the maximum neighbor-joining (NJ) analysis indicates no separation clades of Wind Cave and Fairy Cave population. This showed all haplotypes are phylogenetically related to each other, with less than 50% bootstraps support (1,000 replications) except for Fairy Cave100 with 51% bootstraps support and Wind Cave 65 with 60% bootstraps support. From the clade, haplotype Wind Cave 65 diverse much earlier as supported by 60% of bootstrap value. This may suggest that the haplotype may have first emerged or colonized this area and dispersed to other part of Bau area. Although the Fairy Cave 100 and Wind Cave 65 indicate more than 50% bootstraps support, the value is not strong enough to conclude there is isolation between them. This result suggests that gene flow might exist between Fairy Cave and Wind Cave population and they come from the same population.

Conclusion and recommendation.

The finding suggested that there are variations among *R. erythraea* population in Fairy Cave and Wind Cave. According to the sequence variation analyses, there are still variations among the individuals in although the rates are very low. Besides that, the findings also indicate that a sample from Wind Cave (Wind Cave 65) show that it is an ancient haplotype than the other individuals. It can be seen from the phylogenetic analyses which show that the individual evolves earlier than the other. Thus, this show that COI gene of mtDNA is a good genetic marker for frog species at population level. In order to obtain good result, more samples are needed for further analyses.

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