EVOLUTION OF BREEDING MODE IN BORNEAN FROGS

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Evolution of Breeding Mode in Bornean Frogs

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This thesis is submitted in partial fulfillment of the requirement for the degree of Bachelor of Science with Honors in Animal Resource Science and Management

Department of Zoology
Faculty of Resource Science and Technology
UNIVERSITI MALAYSIA SARAWAK
2013
DECLARATION

I hereby declare that this Final Year Project Report 2013 is based on my original work except for quotations and citations, which have been dully acknowledged also, declare that it has not been or concurrently submitted for any other degree at UNIMAS or other institutions of higher learning.

__________________________
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<th>Definition</th>
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</thead>
<tbody>
<tr>
<td>°C</td>
<td>Celcius</td>
</tr>
<tr>
<td>µl</td>
<td>Microliter</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>milliMol</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium Chloride</td>
</tr>
<tr>
<td>CTAB</td>
<td>Cetyl-trimethyl-ammonium Bromide</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium Bromide</td>
</tr>
<tr>
<td>%</td>
<td>Percentage</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Rotation Per Minute</td>
</tr>
<tr>
<td>mtDNA</td>
<td>Mitochondrial Deoxyribonucleic Acid</td>
</tr>
</tbody>
</table>
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Evolution of Breeding Mode in Bornean Frogs

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ABSTRACT

Bornean frogs used different types of lifestyles as they choose suitable places to breed in order to fulfill the needs of their young. Thus, they have different types of breeding modes which related to their specific microhabitat. Mitochondrial DNA of partial 16S rRNA gene was used in inferring the genetic study of Bornean frogs’ families. The study was aimed to determine the evolution of breeding mode in families of Bornean frogs based on phylogenetic and breeding mode study. A total of 526 bp of 16S rRNA gene from 24 samples were analyzed using molecular technique approach (PCR-direct sequencing). Character coding based on previous study data was used in constructing trees by using the Neighbor-Joining (NJ), and Maximum parsimony (MP). The phylogenetic trees topologies based on 16S rRNA gene show no pattern of each relationship of the breeding mode of frogs’ family. Although partial gene 16S rRNA was used to study amphibian at a specific level, however 16S rRNA is not a suitable gene marker to highlights the breeding mode. Besides that, the tree produced from character coding based on previous study does not have enough characters to infer the representative species. This study should be enhanced in future in order to infer the phylogeny of Bornean frogs based on DNA and breeding mode by adding samples, characters, and breeding mode.

Keywords: Breeding mode, 16S rRNA gene, character coding, Neighbor-Joining (NJ), Maximum Parsimony (MP).

ABSTRAK


Kata kunci: Jenis pembiakan, gen 16S rRNA, pengekodan aksara, Neighbor-Joining (NJ), Maximum Parsimony (MP).
CHAPTER I: INTRODUCTION

1.0 Introduction

Herpetofauna can be divided into two classes which are amphibians (frogs) and reptiles. Frogs are among the most familiar animals that occur in the world and it is easy to find and recognize. They were also classified under the Order Anuran (Amphibian) which have the characteristic of growing without tail when adult (Inger and Stuebing, 2005). Their distinctive features among other taxa including no tail, short (often stocky bodies), long hind legs and short front ones, large bulging eyes and very wide mouth (Inger and Stuebing, 2005; Garbutt and Prudenta, 2006). Haas and Das (2012) stated that Bornean frogs belong to eight families namely Bomminatoridae, Bufonidae, Ceratobatrachidae, Dicroglossidae, Megophryidae, Microhylidae, Ranidae and Rhacophoridae that consists of 166 species. Each of the Bornean frogs’ families is divided into groups of related species or genera.

Most of the Bornean frogs are confined either to forests or to forests edges (Inger and Stuebing, 2005). All species of frogs have three phases of life cycles which includes aquatic larval, terrestrial juvenile and adult that requires different needs in dispersal capacity used and ecology (Inger and Voris, 2001). The developmental site and extent of geographical range influence the breeding mode of frogs. Ponds are pools that are separated from the stream beds, water-containing logs, tree holes or buttress tanks. Streams
on the other hand are divided into two types which are moderate and strong current (Inger and Voris, 2001).

Mitochondrial DNA (mtDNA) is widely used molecular markers for phylogenetic studies due to its simple genomic structure (Avise, 2004). Mitochondrial DNA is essential for normal mitochondrial function as it has higher stability and occurs in higher numbers of copies than nuclear DNA (Prusak et al., 2005). Mitochondrial 16S rRNA genes have the suitable characteristic requirements for a universal DNA barcoding marker in amphibians. The 16S primer pair is universal for amphibians as it reflected by the high number of amphibian 16S sequences in GenBank and has been selected as standard markers for phylogeny reconstruction in amphibians (Vences et al., 2005). Thus, the universality of primers 16S is suitable to analyze various higher level representatives of these phylogenetic study.

1.1 Research Objectives

The objectives of the study are as the following:

i. To construct the phylogenetic tree of Bornean frogs that have different breeding mode based on 16S rRNA gene.

ii. To construct breeding mode tree of Bornean frogs representatives species via Inger and Voris (2001).

iii. To determine whether the evolution of the breeding mode are congruent with their phylogenetic relationship.
CHAPTER II : LITERATURE REVIEW

2.0 General Introduction

Bornean frogs’ families consist of Bombinatoridae, Bufonidae, Ceratobatrachidae, Dicroglossidae, Megophryidae, Microhylidae, Ranidae and Rhacophoridae (Haas and Das, 2012). Frogs are amongst the most familiar animals as they occur everywhere in the world and easy to recognize. They are classified under the order Anuran (Amphibian) that grows without tail when reaching adult (Inger and Stuebing, 2005). Their distinctive features includes no tail, a short (often stocky body), long hind legs and short front ones, large bulging eyes and very wide mouth (Inger and Stuebing, 2005; Garbutt and Prudenta, 2006). Most of the frogs are forest dwellers, and live on one of the lifestyle that includes lifetime stream bank sitter, stream bank sitter (only adult), stream breeder, and forest wanderer (Inger and Stuebing, 2005).
2.1 Breeding Mode, Habitat and Distributions

Frog search for suitable places to breed in order to fulfill the needs of their young to survive. Thus, they have developed different breeding modes that are related to their specific microhabitat. Some species of Bornean frogs breed in standing water such as ornamental pools, temporary rain-filled depressions, drains and flooded rice fields (Inger and Stuebing, 2005). According to Inger and Voris (2001), pond breeder are classified to species that breeds at temporary or permanent pools separated from the stream which includes water-containing logs, tree holes or buttress tanks. As for stream breeder, the breeding can take place at any portion of the stream with both slow and strong current such as riffles and torrents.

The effect of topography on distribution of frogs influenced the breeding mode of a species. Species that developed in ponds have wider geographical distributions than the species that developing in streams (Inger and Voris, 2001). However, some species of frogs also undergoes direct development or non-feeding larvae. According to Preininger et al. (2012), frogs of the genus Staurois are famous for displaying a variety of visual signals which includes foot flagging and they live exclusively along fast-flowing and clear water rainforest streams. Besides that, most stream-side anuran in Borneo are absent in streams with silt bottoms that are lacking riffles and torrents, but are known to breed in clear, turbulent water (Inger and Voris 1993). The reoccurring stream assemblages and habitat specific adaptations brings the heterogeneity of riparian habitats in pristine rainforests (Keller et al., 2009). The evolution of frogs as they adapt to the conditions of environment developed different types of breeding modes which relates to their microhabitat.
Hero (2006) stated that frogs have five types of breeding mode, which include terrestrial breeders, ephemeral breeders, isolated pond breeders, streamside pond breeders, and stream breeders. Terrestrial breeders do not depend on water because they can breed in the forest litter. Ephemeral breeders depend on ephemeral ponds which are usually small and only hold water for few days following the heavy rains, relying on subsequent rains to hold water for several weeks at a time. Isolated pond breeders are species where its tadpole lives in semi-permanent and permanent isolated forests ponds that hold water for extended periods without needing replenishing rains, have plenty of time to grow and develop, so tadpoles may stay in the water for much longer periods (Hero, 2006). On the other hand, streamside pond breeders breed in pond that are only connected to streams during floods, during which the pond may be invaded by fish or other potential stream predators. The tree frogs are the examples that are adapted to breed in this type of habitat (Inger and Stuebing, 2005).
2.2 Mitochondrial DNA and 16S rRNA Gene

Mitochondrial DNA has many advantages as molecular marker as it evolves faster than nuclear DNA (Brown et al. 1982). Different regions of the mitochondrial genome evolve at different rates (Saccone et al. 1991) allowing suitable regions to be chosen for the question under study as mitochondrial DNA is maternally inherited in most species. A significant genetic distance of *Limnonectes kuhlii* which separate Bornean frogs’ population from the continent and Java has been indicated by the mitochondrial DNA for the stream breeders (Inger and Voris, 2001). Species and variants of organism identification by DNA barcoding have great appeal as a universally applicable tool. Mitochondrial 16S rRNA genes have the suitable characteristic requirements for a universal DNA barcoding marker in amphibians (Vences et al., 2005). 16S rRNA gene also used for establishing distant relationships because of their high content of information, conservative nature, and universal distribution (Lane et al. 1985). Arifin et al. (2011) found high genetic variation that may eventually support the recognition of new species by estimating the phylogenetic relationships among all six recognized species of the genus *Staurois* based on 16S rRNA gene.
CHAPTER III : RESEARCH METHODOLOGY

3.0 Tissue Samples Collection

The samples of frogs with 21 individuals from six families of Bornean frog were collected from Gunung Gading National Park, Mulu National Park, Universiti Malaysia Sarawak, Sebangkoi Recreational Park, Bako National Park, Kinabalu Park, and Taman Tuaka Miri. The collected samples were preserved in ethanol and stored at -20°C. The samples were identified through their morphological characteristics and body measurements using the keys provided by Inger and Stuebing (2005).
Figure 1.0: Geographic location of each sampling sites retrieved from Google Earth. Fresh tissue samples were collected from Gunung Gading National Park, Universiti Malaysia Sarawak, and Sebangkoi Country Park. Preserved tissue samples were obtained from Bako National Park, Kinabalu Park, Gunung Mulu National Park, Taman Tuaka Miri, Universiti Malaysia Sarawak, and Niah National Park.
Table 1.0: Description of Bornean frog’s representative species samples according to their breeding mode. Breeding mode: p = pond, rain pool, tree cavity; s = low gradient stream; st = moderate to high gradient stream.

<table>
<thead>
<tr>
<th>Family</th>
<th>Representative Species</th>
<th>Field ID</th>
<th>Breeding Mode</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bufonidae</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Ansonia spinulifer</em></td>
<td>GNP43</td>
<td>st</td>
</tr>
<tr>
<td></td>
<td><em>Ansonia spinulifer</em></td>
<td>GNP84</td>
<td>st</td>
</tr>
<tr>
<td></td>
<td><em>Duttaphrynus melanostictus</em></td>
<td>SCP39</td>
<td>p</td>
</tr>
<tr>
<td></td>
<td><em>Duttaphrynus melanostictus</em></td>
<td>TT2</td>
<td>p</td>
</tr>
<tr>
<td><strong>Dicroglossidae</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Limnonectes kuhlii</em></td>
<td>SCP35</td>
<td>s</td>
</tr>
<tr>
<td></td>
<td><em>Occidozyga laevis</em></td>
<td>UE174</td>
<td>p</td>
</tr>
<tr>
<td><strong>Megophridae</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Leptobrachella mjobergi</em></td>
<td>MBSI45</td>
<td>st</td>
</tr>
<tr>
<td></td>
<td><em>Megophrys nasuta</em></td>
<td>MBSI92</td>
<td>s</td>
</tr>
<tr>
<td></td>
<td><em>Megophrys nasuta</em></td>
<td>MBSI43</td>
<td>s</td>
</tr>
<tr>
<td><strong>Ranidae</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Hylarana baramica</em></td>
<td>KS36</td>
<td>p</td>
</tr>
<tr>
<td></td>
<td><em>Hylarana baramica</em></td>
<td>NNP04</td>
<td>p</td>
</tr>
<tr>
<td></td>
<td><em>Hylarana raniceps</em></td>
<td>GNP26</td>
<td>p/s</td>
</tr>
<tr>
<td></td>
<td><em>Huia cavitypanum</em></td>
<td>MBSI155</td>
<td>st</td>
</tr>
<tr>
<td></td>
<td><em>Huia cavitypanum</em></td>
<td>MBSI171</td>
<td>st</td>
</tr>
<tr>
<td></td>
<td><em>Staurois guttatus</em></td>
<td>GNP03</td>
<td>s</td>
</tr>
<tr>
<td><strong>Rhacophoridae</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Polypedates colletti</em></td>
<td>BNP005</td>
<td>p</td>
</tr>
<tr>
<td></td>
<td><em>Polypedates leucomystax</em></td>
<td>KS17</td>
<td>p</td>
</tr>
<tr>
<td></td>
<td><em>Rhacophorus angulirostris</em></td>
<td>RZ53</td>
<td>s</td>
</tr>
<tr>
<td></td>
<td><em>Rhacophorus angulirostris</em></td>
<td>RZ51</td>
<td>s</td>
</tr>
<tr>
<td><strong>Microhylidae</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Chaperina fusca</em></td>
<td>MHQ126</td>
<td>p</td>
</tr>
<tr>
<td></td>
<td><em>Chaperina fusca</em></td>
<td>MHQ122</td>
<td>p</td>
</tr>
</tbody>
</table>
3.1 DNA Extraction using CTAB Protocol

The DNA extraction was following CTAB (Cetyl-trimethyl-ammonium Bromide) buffer manual extraction protocol. 1-2 cubic millimeters of frog tissue was grinded with 100µl CTAB to obtain the DNA. Then, the grinded skin was deposited into 1.5 µl microcentrifuge tube contain 600µl of one times reaction CTAB, and 10µl of Proteinase K was added inside the tube. Next, the samples were incubated inside water bath with temperature of 65˚C for several times. After completely lyses, 600µl of CIA (Chloroform Isoamly-Actate) was added in and shakes for a few minutes. Then, the samples were centrifuged at 13 000 rpm for 20 minutes. After that, the upper layer of the supernatant was pipetted without touching the middle layer and was transferred to a newly labeled tube. An equal volume of absolute ethanol (EtOH) was added into the tube and was inverted. The mixture in the tube was centrifuged again at 13 000 rpm for 20 minutes. Next, the present of the pellet was observed on the bottom of tube. The excess ethanol was discarded. An equal volume of 70% cold ethanol was added together with 25 µl 3M NaCl and the mixture was centrifuged at 13 000 rpm for 20 minutes. The EtOH was discarded and the DNA pellet was dried under room temperature for several minutes. The final DNA pellet was re-dissolved in 50 - 100µl of water (ddH₂O). The tube was placed at 4˚C overnight to fully dissolve the DNA into the solution.
3.2 Gel Electrophoresis

The electrophoresis was done by using 1% of agarose gel to visualize the product of the DNA extraction. Casting tray was set on along with its comb. 50 ml of 1x TAE buffer solution was added into the 0.5 g of agarose powder and were mixed well before heating in the microwave oven for 1 minute. After the mixture is fully dissolved, it was poured into the casting tray. 2 µl of ethidium bromide (EtBr) was added into the solution. Then, the comb was inserted into the casting tray to create wells. The gel was cooled for 30 minutes, and the comb was taken out slowly to avoid any damages done to the gel. Gel was inserted into the tank which filled with of TAE buffer. 2 µl of sample was mixed with 2 µl of loading dye on a parafilm by adjusting the micropipette to 4 µl. The mixture was resuspended for several times before it can be pipette into each well. 2 µl of 1kb DNA ladder was pipette into the first well and then the electrophoresis was run at 100V. The gel was visualized under UV transilluminator to detect the present of band.
3.3 DNA Amplification - Polymerase Chain Reaction (PCR)

The standard PCR protocol was used to amplify the primers targeting partial 16S ribosomal RNA which is approximately 580 base pair fragment. The amplification of DNA was carried out by using thermal cycler (Bioer GenePro) machine. The primers sequence that was used in this study is listed in Table 2.0.

Table 2.0 : List of primers for partial gene 16S rRNA that is used (Palumbi et al., 1991).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Direction</th>
</tr>
</thead>
<tbody>
<tr>
<td>16 sar-L</td>
<td>‘5-CGCCTGTTTATCAAAAACAT-3’</td>
<td>Forward</td>
</tr>
<tr>
<td>16 sbr-H</td>
<td>‘5-CCGGTCTGAACTCAGATCACGT-3’</td>
<td>Reverse</td>
</tr>
</tbody>
</table>

The master mix was prepared early before the amplification process. The master mixed was prepared according to the number of samples and includes a reaction for negative control. The preparation of PCR master mix is shown in Table 3.0 for one time reaction and the amplification was done by using the PCR profile is shown in Table 4.0.

Table 3.0 : Master mixed preparation for one time reaction.

<table>
<thead>
<tr>
<th>Component</th>
<th>1× reaction (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5× reaction buffer</td>
<td>5.0</td>
</tr>
<tr>
<td>15 mM MgCl₂</td>
<td>1.5</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>0.5</td>
</tr>
<tr>
<td>16 sar-L (forward)</td>
<td>1.0</td>
</tr>
<tr>
<td>16 sbr-H (reverse)</td>
<td>1.0</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>14.3</td>
</tr>
<tr>
<td>Template DNA</td>
<td>1.5</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>0.2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>25.0</strong></td>
</tr>
</tbody>
</table>
Table 4.0: PCR profile

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-denaturation</td>
<td>96</td>
<td>5 minutes</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>96</td>
<td>45 seconds</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>50</td>
<td>1.30 minutes</td>
<td>30</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>1.30 minutes</td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>72</td>
<td>5 minutes</td>
<td>1</td>
</tr>
<tr>
<td>Soak</td>
<td>4</td>
<td>∞</td>
<td></td>
</tr>
</tbody>
</table>

The results of the PCR product was determined by gel electrophoresis and visualized on an ultraviolet transluminator. All products were visualized on 1.5% agarose gel in 1x TAE buffer and stained with ethidium bromide (EtBr).
3.4 Purification of PCR Product

According to Grewe et al. (1993), once PCR product has been obtained, the next stage is purification prior to DNA sequencing. The purification of the amplified DNA was continued according to Promega Purification Kit manual protocol. The purification processes have 3 steps which are binding DNA, washing and elution. There were approximately 23 µl of PCR product, therefore, more than equal volume of membrane binding solution was added into the PCR product. The minicolumn was inserted into the collection tube, and then, the PCR products were transferred into minicolumn assembly and were incubate at room temperature for 1 minute. After that, the minicolumn assembly was spin at 14000 rpm for 1 minute. The flow through was discarded and the minicolumn was reinserted into the collection tubes. 700 µl of Membrane Wash Solution was added into the minicolumn and was spin at 14 000 rpm for 1 minute. The flow through was discarded and the minicolumn was reinserted into the collection tubes. 500 µl of Membrane Washing Solution Nuclease was added into the minicolumn and was spin at 14 000 rpm for 1 minute. The flow through was discarded and the minicolumn was reinserted into the collection tubes. The collection tube was emptied and the assembly was recentrifuged for 2 minutes to allow any residual of ethanol to vaporize. For the process of elution, the minicolumn was carefully transferred to a clean 1.5 µl microcentrifuge tube. Then, 50 µl of nuclease free water was added and was incubated at room temperature for 1 minute. The assembly was centrifuge at 14 000 rpm at 2 minutes and the minicolumn was discarded. The purification product was visualized under ultraviolet transilluminator. The electrophotometer was used to check whether the samples are enough to proceed for DNA sequencing. Finally, the purified product was sent for sequencing to First Base Sequencing Laboratory.