ISOLATION & PCR ANALYSIS OF ITS MITOCHONDRIAL DNA GENOME FROM FRUGIVOROUS CATS

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ISOLATION AND PCR ANALYSIS OF 12S MITOCHONDRIAL DNA GENE FROM FRUGIVOROUS BATS

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ABSTRACT

Keywords: Megachioreptra, mtDNA, PCR

Frugivorous bats or fruit bats form the sub-order Megachioreptra. Cynopterus brachyotis, Balionycteris maculata and Macroglossus minimus are three species of fruit bats from Borneo studied in this research. Whole blood and tissue samples were taken from fruit bats from Matang and stored in -70°C deep freezer. Studies were also done on samples from DNA archive of previous studies on fruit bats from Poring, Sabah. From this, genomic DNA were isolated for PCR and DNA sequencing. The targets for PCR analysis is mitochondrial gene marker 12S. All isolated genomic DNA and PCR products are stored at -20°C for DNA archiving. This molecular research on genetic variations is aimed at studying the phylogeny of the Megachioreptera using the 12S mitochondrial DNA. In this study, out of nineteen megabats samples, ten were successfully processed to obtain genomic DNA which were eventually subjected to PCR analysis.

ABSTRAK

Kata kunci: Megachioreptra, mtDNA, PCR

INTRODUCTION

Species are the basic units of biodiversity. Since much of evolutionary biology focuses on the process of speciation, understanding species limits is clearly a fundamental requirement. In addition, an accurate estimate of species limits is a key factor in improving the accuracy and validity of biodiversity assessment (Puerto et al., 2001). Until recently, species identification were done morphologically and anatomically. This is known as taxonomical research. Advances in science and technology have allowed phylogenetic relationships of species to be elucidated further by the analysis of mitochondrial DNA sequences. MtDNA sequence is also used intensively for phylogeographic studies to identify the genetic differentiation of sets of populations, thus merit special conservation attention (Puerto et al., 2001). This research, however, aims to compare the genetic differences between frugivorous bats of Borneo based on mtDNA in the hope of forming a phylogenetic tree.

Megachiroptera

Bats are vertebrates that fall under the group of mammals. They are covered with fur, give birth and nurse their young. But what makes them unique among mammals is the fact that not only are they perfectly designed for hanging upside-down, but they can also fly. They are the only flying mammals. Bats are not rodents, instead, they are essential in maintaining the stability of the ecosystem (Feldhammer et al., 1999). Here, the study will be on fruit bats which fall under the sub-order, Megachiroptera, the ‘big bats’. Megachiroptera differs from microchiroptera in very distinct divergence. Megachiroptera has large eyes and well-developed visions (Kunz, 1982). The wings and flight anatomy are much less sophisticated than in microchiroptera, and details of dental and cranial anatomy suggests remote relationship at best. Microbats use echolocation, while megabats rely on their keen eyesight. Microbats teeth are designed for prey while megabats teeth grind plant parts as they feed on fruit, flowers, or pollen (Findley, 1993). Suborder Megachiroptera only branch out to one family, which is Pteropodidae (Orn, 1982). In Borneo, there are eighteen species identified from eleven genera which are Cynopterus, Balionycteris, Macrognlossus, Rousettus, Pieropus, Pteropus, Megaderops, Chinorux, Aethalops, Eonycteris and Dyacopterus (Corbett and Hill, 1999). The samples studied are from three species of fruit bats which are Cynopterus brachyotis from different locations, Balionycteris maculata and Macrognlossus minimus. The Borneo Island consists of eight families of bats with 94 species (Payne et al., 1985). To date, there is no molecular data to support the taxonomical records and phylogeny of megachiroptera in Borneo.

Mitochondrial Deoxyribonucleic Acid (mtDNA)

MtDNA is an organel found in the eukaryotic cytoplasm with diameter between 0.5 to 1.0 μm and 0.7 μm in length. Figure 1 shows the human mitochondria with its circular structure. The inner strand is called the light strand (L strand) and the outer strand is the heavy strand (H strand). Both strands consist of 37 genes (Weaver and Hedrick, 1992). MtDNA differs from nuclear DNA in its location, its sequence, its quantity in the cell, and its mode of inheritance. Nuclear DNA has more bases than mtDNA, but mtDNA is
present in many more copies than nuclear DNA. This characteristic of mtDNA is useful in situations where the amount of DNA in a sample is very limited. In recent years, mtDNA has become the most widely used molecular marker in animal systematics, particularly at low taxonomic levels, due to its ease of isolation and interpretation (Avise et al., 1987). Furthermore, due to its smaller effective population size, mtDNA will show lineage coalescence more rapidly than a nuclear marker; thus, a resolved mtDNA gene phylogeny is more likely to represent organismal phylogeny than a tree based on any specific nuclear sequences. (Puerto et al., 2001). Mitochondrial DNA forms a single inherited unit and thus is just one single gene. In this study, gene marker 12S is the target region for genetic analysis. The primers employed to initiate the PCR process are short sequences that exhibit high sequence similarity to regions flanking the target sequence.

![Diagram of human mitochondrial gene](image)

Figure 1: Human mitochondrial gene (Mindell et al., 1998).

**Polymerase Chain Reaction (PCR)**

The use of the PCR in this study allows isolation and examination of genomic DNA sequences using specific oligonucleotide primers to amplify a target region with Taq DNA polymerase (Rapley, 1996). DNA amplification by the PCR undergoes the three stages which are denaturation of the double stranded mtDNA by heating, followed by primer annealing to the target region and thirdly, the primer synthesis to the complementary strand with Taq polymerase. This is repeated for 35 cycles to exponentially increase the number of copies of the target region 12 S. The PCR is well known for being a rapid and versatile routine method for the amplification of defined target DNA sequences (Rapley, 1996).
METHODS AND MATERIAL

Sample collection

A trip was organized to Matang Wildlife Centre on the 22nd of July 2002. The fruit bats were captured using mist nets. The mist nets were set at ground level and across streams. The sites chosen were in separate primary and secondary forests. The mist nets were checked once in few hours, concentrating on bats feeding time which is early morning and late evening.

Captured bats were then identified through morphological characteristics such as; total length (TL), ear (E), tibia (TB), tail (T), testes (TS), weight (W), forearm (FA) and body length (HB). All species identification was based on Payne et al, 1985. Whole blood and muscle tissue samples were collected from chloroform-killed bats. Whole blood samples were drawn from the heart using a sterile syringe and mixed with lysis buffer as anticoagulant. The tissue was mixed in alcohol 80% and stored in liquid nitrogen. These samples were then transferred to a -20°C to -70°C ultrafreezer.

DNA Extraction

Isolation of genomic DNA was done on both blood and tissue samples. Both processes were done using Viogene Kit (Viogene USA Cat GG1001). This is to ensure a faster and uncomplicated result of pure genomic DNA. The isolated genomic DNA were free from protein, tissue, blood, plasma, serum and bacteria.

From blood samples

Isolation of genomic DNA from blood was carried out as follows; 200μl of whole blood sample was pipetted into a 1.5 microcentrifuge tube. This was added with 20μl Proteinase K and 200μl of Buffer EX to the sample. After immediate vortexing, this mixture was incubated at 60°C for 20 minutes to lyse the sample and consequently, at 70°C for 10 minutes to inactivate Proteinase K. Then, 210μl of ethanol 100% was then added to the sample, which was then vortexed before transferring the entire sample from the tube to a Genomic DNA column in a 2ml collection tube and centrifuged at 8000 rpm for 2 minutes. The filtrate was then emptied while 500μl of wash buffer was added before centrifuging again for 2 minutes. This step was repeated again before the genomic DNA column was placed in a new tube. The DNA was eluted with 200μl of preheated ultrapure water and then centrifuged for 2 minutes. The tube containing eluted DNA was then stored at -20°C until used.
From tissue samples

About 30 mg of muscle tissue was cut into small pieces with sterile scalpel blade and placed in a 1.5 ml centrifuge tube and 200 µl of buffer LYS was added. Then, 20µl of Proteinase K was added to the sample which was immediately vortexed. Here, the sample was incubated at 60°C for 3 hours to lyse sample. Then, at 70°C for 20 minutes to inactivate Proteinase K. After that, 200µl Buffer EX was added to the sample, vortexed and incubated again at 70°C for 10 minutes. After that, 210µl of ethanol 100% was added to the sample and vortexed. All the mixture was then transferred to a Genomic DNA column in a microcentrifuge tube and centrifuged at 8000 rpm for 2 minutes. The filtrate was emptied out and 500µl of Wash Buffer was added before centrifuging for 2 minutes. This step was repeated again before eluting the DNA with 200 µl of preheated ultrapure water for 2 minutes. The eluted DNA was stored at -20°C.

Gel electrophoresis

After genomic DNA isolation, the samples were tested for genomic DNA presence by running gel electrophoresis. Agarose gel of 0.5g was mixed with 50 ml of 1X TAE buffer and 1 µl of ethidium bromide (EtBr), which attaches to DNA and fluoresces under UV light from the transilluminator. Five µl of the sample was loaded into the well of the gel with Gel Loading Dye (GLD) which gave anchorage for the DNA. DNA band visualized on the gel was compared to the 1 kb ladder which was also loaded into the gel well. Gel electrophoresis run was also done after every PCR session and after gel extraction.

Polymerase Chain Reaction (PCR)

Samples that show DNA bands on the gel were selected for PCR. Prior to PCR, a master mixture excluding the templates were prepared. This is called the Master Mix. The ration for each substance was stated in Table 1. A tray of crushed ice is prepared and all tubes containing each substance were placed in it, to maintain low temperature. This is to avoid degradation of the substances. One 1.5ml tube was prepared for the master mix. Firstly, ultrapure water was filled into the tube, followed by PCR buffer, MgCl₂, primers, deoxynucleoside triphosphate (dNTP), Taq Polymerase and the template being the last to be mixed in.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Materials for PCR master mix</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Master Mix</td>
</tr>
<tr>
<td></td>
<td>Ultrapure water</td>
</tr>
<tr>
<td></td>
<td>10 x PCR Buffer</td>
</tr>
<tr>
<td></td>
<td>MgCl₂</td>
</tr>
<tr>
<td></td>
<td>Primer 12 SU</td>
</tr>
<tr>
<td></td>
<td>Primer 12 SL</td>
</tr>
<tr>
<td></td>
<td>10mM dNTP</td>
</tr>
<tr>
<td></td>
<td>Taq Polymerase</td>
</tr>
<tr>
<td></td>
<td>Template</td>
</tr>
<tr>
<td></td>
<td>Total</td>
</tr>
</tbody>
</table>
DNA amplification by the PCR undergoes three stages that was repeated for 35 cycles to exponentially increase the number of copies of the target region 12 S. PCR process Table 2 shows the time and temperature for PCR reactions. Table 3 features the primer sequence obtained from the gene bank [http://www.ncbi.nlm.nih.gov:80/entrezviewer.cgi](http://www.ncbi.nlm.nih.gov:80/entrezviewer.cgi).

**Table 2: Temperature and reaction duration for primer 12S in 35 cycles**

<table>
<thead>
<tr>
<th>Phase</th>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Start of denaturation</td>
<td>98</td>
<td>2 minutes</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94</td>
<td>40 seconds</td>
</tr>
<tr>
<td>Start of Annealing</td>
<td>55</td>
<td>45 seconds</td>
</tr>
<tr>
<td>Annealing</td>
<td>72</td>
<td>1 minute</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>7 minutes</td>
</tr>
<tr>
<td>Final Soak</td>
<td>4</td>
<td>∞</td>
</tr>
</tbody>
</table>

**Table 3: Primer 12S and its sequence**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 SL</td>
<td>5' - ACCCGGATCCCTTACCAAAAAAACATCACC - 3'</td>
</tr>
<tr>
<td>12 SU</td>
<td>5' - AAAAAGCTTCAAACCTGATTAGATACCACAT - 3'</td>
</tr>
</tbody>
</table>

**Gel Extraction**

To confirm the result, PCR products were subjected to electrophoresis using 1% agarose gel (30 minutes with 105 volts). Samples that show positive PCR products were subjected to Gel extraction procedure for postamplification purification. Samples showing faint presence of DNA were reamplified using PCR. Gel extraction was done using Viogene Gel extraction Kit. The agarose gel slice of about 50 g containing the DNA was placed in a 1.5 tube and half a ml of Buffer GEX was added and the mixture was incubated at 60 °C for 10 minutes to solubilize the gel slice. After this, the mixture was transferred to a gel extraction column and centrifuged at full speed for 60 seconds. The filtrate was emptied before adding 0.5 ml Wash Buffer I and centrifuged again for 60 seconds. 0.7 ml of wash buffer II was then added and centrifuged for 3 minutes. This step was repeated before adding 30 µl of ultrapure water. The eluted DNA was then stored in -20°C. The purified sample was then checked using electrophoresis analysis to confirm presence of PCR product.
RESULTS

Sample collection

A total of fifteen fruit bats were captured at Matang Wildlife Park. Nine bats were tagged and freed while blood and tissue samples were taken from the other six bats. Two species of bats were identified; *Cynopterus brachyotis* and *Batisnyceteris maculata*. The *C. brachyotis* caught were from both primary and secondary forests while all *B. maculata* were only caught in the secondary forests. Thirteen samples of blood samples from collection to Poring, Sabah collected by previous researchers were also studied. Samples were from two species of bats, *C. brachyotis* and *MacroGLOSSUS minimus*. The total samples that were studied were nineteen samples. Table 4 states total for each species studied.

Table 4: Total of species studied

<table>
<thead>
<tr>
<th>Source</th>
<th>Species</th>
<th>No. of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Matang (Primary Forest)</td>
<td><em>Cynopterus brachyotis</em></td>
<td>3</td>
</tr>
<tr>
<td>Matang (Secondary Forest)</td>
<td><em>Batisnyceteris maculata</em></td>
<td>3</td>
</tr>
<tr>
<td>Poring Collection</td>
<td><em>Cynopterus brachyotis</em></td>
<td>10</td>
</tr>
<tr>
<td></td>
<td><em>MacroGLOSSUS minimus</em></td>
<td>3</td>
</tr>
</tbody>
</table>

DNA Isolation

Genomic DNA were successfully isolated from ten of the nineteen samples. Samples with genomic DNA results were chosen for PCR. The other unsuccessful attempts were due to low sample volume. This is shown in the Figure 2, Figure 3 and Figure 4. Samples 1065 and Sample 1067 in Figure 3 was isolated from muscle tissue samples while the rest of the samples were isolated from blood samples.

Figure 2: Result of genomic DNA isolation

Lane 1: 1 kb ladder
Lane 2: PRO47  Cynopterus brachyotis
Lane 3: PRO54  Cynopterus brachyotis
Lane 4: PRO42  MacroGLOSSUS minimus
Lane 5: PRO62  Cynopterus brachyotis
Lane 6: PRO48  Cynopterus brachyotis
Figure 3: Result of genomic DNA isolation

Lane
1  1 kb ladder
2  PRO50  Cynopterus brachyotis
3  PRO51  Cynopterus brachyotis
4  PRO44  Cynopterus brachyotis
5  1065  Cynopterus brachyotis
6  1067  Balionycteris maculata
7  PRO53  Cynopterus brachyotis

Figure 4: Result of genomic DNA isolation

Lane
1  1 kb ladder
2  1056  Cynopterus brachyotis
3  1057  Balionycteris maculata
4  1061  Cynopterus brachyotis
5  1063  Balionycteris maculata
6  PRO41  Macroglossus minimus
7  PRO46  Cynopterus brachyotis
8  PRO49  Cynopterus brachyotis
9  PRO60  Macroglossus minimus
PCR Analysis

After PCR analysis, ten samples had PCR product. These are 1065, 1067, PRO42, PRO44, PRO47, PRO50, PRO51 and PRO54. The DNA bands were compared to a gene ladder of 1 kb and with 12S primer, where the product size indicates around 500 bp at the 6th band of the gene ladder. As seen on the Figure 4 and Figure 5, the concentration of the non-specific products were quite high. An attempt to use more diluted primers yielded no PCR product. Figure 5 and Figure 6 shows the successful PCR products. In figure 6, samples PRO53 and PRO62 showed negative genomic DNA results but were included in the PCR analysis as negative control.

Figure 5: Results of PCR product

![DNA bands](image)

Lane
1 1 kb ladder
2 1067 *Balionycteris maculata*
3 1065 *Cynopterus brachyotis*
4 PRO44 *Cynopterus brachyotis*
5 PRO47 *Cynopterus brachyotis*
6 PRO48 *Cynopterus brachyotis*
7 PRO50 *Cynopterus brachyotis*

Figure 6: Results of PCR product

![DNA bands](image)

Lane
1 1 kb ladder
2 PRO51 *Cynopterus brachyotis*
3 PRO53 *Cynopterus brachyotis*
4 PRO54 *Cynopterus brachyotis*
5 PRO42 *Macroglossus minimus*
6 PRO62 *Cynopterus brachyotis*
7 PRO60 *Macroglossus minimus*
Table 5 shows the results for both Genomic DNA isolation and PCR analysis. Sample PRO53 and sample PRO62 showed negative result for the Genomic DNA isolation but was subjected to PCR analysis as negative control.

Table 5: The result for the successful samples to produce PCR products

+ve shows sample with PCR product
-ve shows sample with no PCR product
(-) shows sample did not undergo PCR

<table>
<thead>
<tr>
<th>Species</th>
<th>Sample no</th>
<th>Genomic DNA result</th>
<th>PCR result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cynopterus brachyotis</td>
<td>PRO44</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>Cynopterus brachyotis</td>
<td>PRO46</td>
<td>-ve</td>
<td>-</td>
</tr>
<tr>
<td>Cynopterus brachyotis</td>
<td>PRO47</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>Cynopterus brachyotis</td>
<td>PRO48</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>Cynopterus brachyotis</td>
<td>PRO49</td>
<td>-ve</td>
<td>-</td>
</tr>
<tr>
<td>Cynopterus brachyotis</td>
<td>PRO50</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>Cynopterus brachyotis</td>
<td>PRO51</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>Cynopterus brachyotis</td>
<td>PRO53</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>Cynopterus brachyotis</td>
<td>PRO54</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>Cynopterus brachyotis</td>
<td>PRO56</td>
<td>-ve</td>
<td>-</td>
</tr>
<tr>
<td>Cynopterus brachyotis</td>
<td>PRO62</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>Cynopterus brachyotis</td>
<td>1056</td>
<td>-ve</td>
<td>-</td>
</tr>
<tr>
<td>Cynopterus brachyotis</td>
<td>1055</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>Macroglossus minimus</td>
<td>PRO41</td>
<td>-ve</td>
<td>-</td>
</tr>
<tr>
<td>Macroglossus minimus</td>
<td>PRO42</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>Macroglossus minimus</td>
<td>PRO60</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>Balionycteris maculata</td>
<td>1063</td>
<td>-ve</td>
<td>-</td>
</tr>
<tr>
<td>Balionycteris maculata</td>
<td>1057</td>
<td>-ve</td>
<td>-</td>
</tr>
<tr>
<td>Balionycteris maculata</td>
<td>1067</td>
<td>+ve</td>
<td>+ve</td>
</tr>
</tbody>
</table>

Postamplification purification

Purification of PCR product using Viogene Kit was done by gel extraction method. This step, however, was unsuccessful for most of the sample as there was insufficient volume of DNA.
DISCUSSION

In this study, the samples studied were extracted from blood and tissue and were stored in -20°C. The anticoagulant used and the temperature might affect the quality of the samples to yield DNA.

Eventhough the same procedure was applied to all samples during isolation and extraction, on to PCR and gel extraction was the same, some samples showed desired products while some did not. A factor contributing to this outcome could be that most of the blood and tissue samples were collected in very small volumes. This is just as much as a small fruit bat would allow.

PCR

The PCR process was successful. Adjustments have been attempted to the temperature of the annealing phase from 55 °C to 58 °C. This was attempted to enhance the primers to bind to the DNA template, but no bands were observed. Thus, the ideal setting was still carried out according to the protocol used by previous research. As PCR products showed high concentration of non-specific products, the 25 pmol primers were diluted five times but there was no PCR product. At this stage, the PCR was still contaminated with non-specific products and still had to undergo further purification.

Gel Extraction

Gel extraction results show that the DNA bands were blurry as compared to pre-gel extraction. This shows that the DNA concentration is lower at this stage. This is caused by the purification process that is supposed to get rid off non-specific DNAs and PCR reagents. Reamplification through PCR was also carried out but did not yield enough DNA for sequencing. Due to time constraint, this study was unable to proceed to the sequencing phase as the volume of the DNA was insufficient for cycle sequencing.
CONCLUSION

From the total of nineteen samples, ten samples showed positive Genomic DNA isolation and PCR products. From the species *Cynopterus brachyotis*, seven samples showed DNA bands. Two of three samples from *Macroglossus minimus* and one sample from three samples of *Baliomycteris maculata* also showed DNA bands.

As molecular studies on bats have been carried out in previous years, the *Cynopterus brachyotis* is most commonly studies upon. There are nine subspecies of *C. brachyotis* recognized to date. (Salleh *et al.*, 1999). The species *Cynopterus brachyotis* samples were taken from both location; Matang Wildlife Park and from Poring, Sabah. The species *Baliomycteris maculata* samples were only from Matang Wildlife and *Macroglossus minimus* samples were from Poring, Sabah. With the successful isolation and PCR of the 12S mitochondrial DNA, further studies can be continued to obtain the complete sequence of the 12S mitochondrial DNA.

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