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PCR AMPLIFICATION OF MITOCHONDRIAL CYTOCHROME B GENE OF ANIMALS IN MALAYSIA

ABSTRACT

The aim of this study is to acquiring basic data for identifying blood meal species of vector arthropods in Malaysia by amplify and sequence the mtDNA cytb gene of captive and domestic animals. Polymerase Chain Reaction (PCR) using universal primers complementary to the conserved region of the mitochondrial DNA (mtDNA) cytochrome b (cytb) gene fragment, was performed on DNA of blood samples of 27 captive and domestic animals in Malaysia. DNA of hosts was amplified by PCR and the products were visualized on gel electrophoresis. Twenty two sequences were obtained and compared with sequences registered in GenBank using a BLAST program. The percentage of similarity between the study species and GenBank species was in the range of 78 - 100%. Four sequences had no significant similarity and 3 species were mismatched with other species in the range of 78 - 89% similarity. Nine new gene sequences with accession number JQ812112 (Prionailurus bengalensis), JQ812113 (Prionailurus bengalensis), JQ812114 (Tapirus indicus), JQ812115 (Oryx dammah), JQ812116 (Macaca nemestrina), JQ409474 (Panthera tigris 2), JQ409475 (Panthera tigris 1), JQ409476 (Panthera tigris 4) and JQ409477 (Malayan Elephant) had been deposited in GenBank. These results will contribute to develop the GenBank database and can be applied for further epidemiological studies on vector borne diseases in tropical countries including Malaysia.

keywords Cytochrome b gene, Polymerase Chain Reaction, GenBank, Mitochondrial DNA, Arthropods.

1. INTRODUCTION

Majority of pathogens survive in nature by utilizing animals as their vertebrate hosts. The organisms are taken up by arthropod vectors from infected hosts and transmitted either to an intermediary host or directly to a susceptible human host [1]. Some of vector-borne diseases like West Nile virus use an intermediary animal host to serve as a reservoir for the pathogens until susceptible human populations are exposed to the disease. Increased contact between man and wild animals due to environmental changes and deforestation has increased the risk for contracting certain arthropod-borne diseases [2]. Captive animals in zoos and domestic animals may serve as feeding hosts for arthropods. These animals may be harboring pathogens of public health importance. It is important to identify natural hosts of the pathogens as fast and accurate possible for an effective outbreak management and control. If the vertebrate host of a certain disease is known, control measures can be implemented and transmission of disease prevented as early as possible.

In the past, identification of the blood meals of haematophagous arthropods by serological techniques such as the precipitin test, latex agglutination test and enzyme-linked immunosorbent assays (ELISA) were commonly used for a wide variety of purposes [3, 4, 5, 6, 7]. While those techniques continue to provide valuable and insightful data, the identification of many arthropod blood meal sources is limited to only order or family level [8]. A technique is therefore needed to further identify the blood meal sources beyond this level. The availability of DNA sequence data of various vertebrates has opened the door for molecular-based blood meal analysis approaches, such as Polymerase Chain Reaction (PCR) [9, 10, 11]. The alternative method of PCR-based identification of vertebrate host blood meals is more convenient and easier to perform than the previously used serological tests. The most straightforward and specific method to identify blood meals is sequencing of amplified DNA. This approach is ideal since primers can be employed to amplify conserved homologous DNA fragments from diverse potential of vertebrate blood sources [8].

Nowadays, mitochondrial DNA (mtDNA) cytochrome b gene (cytb) is widely used in identifying the species of an organism. The mtDNA contains high proportion of evolutionary-caused nucleotide substitution making it particularly valuable in discriminating

between closely related vertebrates [12]. The availability of a conserved region of the gene in vertebrate also makes mtDNA cytb gene a good candidate for PCR blood meal identification.

The objectives of this study was to acquiring basic data for identifying blood meal species of vector arthropods in Malaysia by amplify and sequence the mtDNA cytb gene of captive and domestic animals. Those gene sequences were then compared with sequences in GenBank and any new sequences found, were deposited in GenBank.

2. MATERIALS AND METHODS

2.1 Collection of blood

Blood was collected from a total of 27 animals (Table 1) comprising of 13 known species of local domestic and captive animals reared in National Zoo, Universiti Kebangsaan Malaysia (UKM) and Wildlife Department. Two to three ml of blood were taken from each animal by veterinarians and other staff using appropriate humane procedure, placed in EDTA anticoagulant tubes and sent to the Institute for Medical Research (IMR). The samples were kept frozen at -20 ˚C until processed further for DNA amplification. Blood samples of 5 of the above animals from UKM and Wildlife Department were also collected as spots on filter paper (Whatman FTA Micro Card, GE Health Care, UK).

2.2 Dilution and extraction of blood

Ten µl of freeze-thawed blood of each species were mixed with 90 µl of sterile double distilled water to produce a 1:10 dilution and used as a template in PCR amplification [13]. Extraction of blood from spotted filter paper was performed by using Harris UNI-CORE Punch (GE Health Care, UK) and QIAcard FTA purification Reagent (GE Health Care, UK), according to the manufacturer's protocol and the resultant samples were used in PCR amplification.

2.3 PCR amplification of cytb gene and gel electrophoresis

DNA of hosts was amplified using PCR with universal primers complementary to the conserved region of mtDNA cytb gene. The primers L14841 (F5'-CCATCCAACATCTCAGCATGATGAAA-3') and H15149 (R5'-CCCCTCAGAATGATATTTGTCCTCA-3') amplified 359 bp of the cytb gene [14]. The PCR was performed using KAPA Blood PCR Kit (Kapa Biosystems Inc. USA) according to the manufacturer's manual. A mixture of 25 µl solution containing 2x Kappa Blood PCR mix, 0.5 µM of each primer and 2.5 µl of diluted blood/blood spot disc was prepared and amplified using an Eppendorf Master Cycler Personal machine (Eppendorf, Germany) with conditions of pre-heating at 95 ^{o}C for 10 min, 35 cycles of consecutive incubations at 94 ^{o}C for 30 s, 52 ^{o}C for 30 s and 72 ^{o}C for 5 min. For each PCR, a negative control containing distilled water instead of DNA was included. Electrophoresis of amplified DNA products were conducted with 1.2% agarose gel in 0.5x electrophoresis buffer and visualized under ultraviolet (UV) light after staining with 2mg/ml ethidium bromide. A 100 bp DNA ladder (Bioron, Germany) was used to estimate sizes of the products.

2.4 DNA purification and DNA sequencing

Each DNA fragment was excised from the gel using sterile, sharp gel cutter and purified using 5 Prime PCR Agarose Gel Extract Mini Kit (Hamburg, Germany) according to the manufacturer's protocol. DNA sequencing in both directions was done in the presence of the ABI PRISM ready reaction big dye terminator cycle sequencing kit (Applied Biosystems, Forster City, California, USA), following the manufacturer's instructions.

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2.5 Gene sequence analysis

The obtained sequences were compared with deposits in GenBank database using the Basic Local Alignment Search Tool (BLAST) program [15].

3. RESULTS AND DISCUSSION

22 samples were successfully amplified but no product was found with 5 despite repeated amplification and gel electrophoresis. Gel electrophoretic patterns of some amplification products are shown in Figure 1. Sharp bands are obtained for twenty-two samples; MT (Malayan tiger) 1-3 (males), MT (Malayan tiger) 4 and 5 (females), WHG (White-handed Gibbon) keke, popeye, pinky, LB (Monkey-wild) 1 & 3, PTM (Pig-tailed macaque) 1 & 2, LTM (Long-tailed macaque), Oryx, PS (Painted stork), ME (Malayan elephant), PM (Python-male), Pf (Python-female) 1 & 2, WB-1 (Malayan tapir), LC (Leopard cat) domestic and wild. Otherwise, five samples; DL (Dusky leaf monkey), ML (monitor lizard) male & female, PTM (pig-tailed macaque) and WB-2 (Malayan tapir) showed smears instead of a sharp band.

Pairwise comparison of the sequences of amplified mtDNA cytb gene fragments with sequences available in GenBank revealed nucleotide similarities ranging from 78 to 100%. Fifteen sequences had exact species match at the highest percent similarity (Table 2). The highest similarity of 3 sequences, i.e. WHG-pinky (White-handed Gibbon), LB1 (monkey-wild) and LB3 (monkey-wild), were mismatched with other species; the exact species match had similarities less than 85% (Table 3). Four sequences did not have any significant similarity; these were PTM-2 (pig-tailed macaque), LTM (long-tailed macaque), WHG (White-handed Gibbon) – keke and popeye. The reason for the not significant match could be due to the too many noise in sequencing result which lead to low complexity or short query sequence after blasting in GenBank database.

Nine new gene sequences were deposited with accession number JQ812112 (Prionailurus bengalensis), JQ812113 (Prionailurus bengalensis), JQ812114 (Tapirus indicus), JQ812115 (Oryx dammah), JQ812116 (Macaca nemestrina), JQ409474 (Panthera tigris 2), JQ409475 (Panthera tigris 1), JQ409476 (Panthera tigris 4) and JQ409477 (Malayan Elephant).

In this study, twenty-seven blood samples were collected comprising of five reptiles, one bird and 21 mammals. Most of the blood samples were taken from species of mammals due to many arthropod borne diseases is closely related with vertebrate mammals [16]. Short fragment of 359 bp of the cytb gene was used to identify the species level of vertebrate animals because of its widest taxonomic representation in nucleotide databases. Despite the relatively short size of the fragment, it provides enough sequence divergence to fulfill the objectives of the study. Furthermore only a pair of primer was used throughout the study to amplify a 359 bp of the cytb gene for all samples. This is in agreement with studies that reported the use of universal primers as complements to conserved region of mitochondrial cytb gene in vertebrates [14].

Throughout the study, five samples showed smears on gel electrophoresis. The failure to obtain sharp bands during gel electrophoresis for these samples could be due to many contributing factors such as excess DNA, sample contamination during preparation or TAE buffer contamination [17]. Other possible reasons might be the improper storage of specimens at -4 ˚C before sending to IMR or not freshly isolated samples or thawing of samples during transportation that can lead to DNA degradation [18].

There were samples that did not have significant similarity or were mismatched with other species. It is assumed that if a species was not found in the databases, the biological significance of the matching result strongly depended on the availability of closely related species, sometimes distantly related taxon, resulting in lower sequence similarity values [19]. In such cases, the observed similarity of the sequence pair exceeded 99% if the corresponding species already in the databases. Therefore, the correct identification of an unknown

sample depends on the availability of the sequence in nucleotide database which is still growing.

Two Python reticulatus samples had 100% homology with two GenBank accessions numbered U69860.1 and AF153066.1; however the subspecies of P. reticulatus is not mentioned in those accessions. The similarity with Malaysian sequence (AY014889.1) in GenBank is between 93 - 99%. In comparing sequences of cytb gene, some study suggested that less than 1% or 2% sequence divergence was typical of phylogeographical units within a species [20]. Furthermore, the highest similarities (97 - 100%) between the subject sequences and query sequences were discovered among the animal species deposited from Indonesia, Malaysia and also from Vietnam. This indicated that phylogenetics of most vertebrate animals from South East Asian regions are closely related to each other e.g. Python spp.

All samples of the Malayan tiger (Panthera tigris jacksoni) have high similarity values (97 - 99%) with corresponding species sequence in GenBank. Based on the cytb gene, P. tigris jacksoni is phylogenetically closely related to three subspecies, i.e. P. t. altaica (Siberian tiger), P. t. sumatrae (Sumatran tiger) and P. t. corbetti (Indochinese tiger). The nucleotide differences between this study's samples and that in GenBank can be attributed to individual variation, especially within species demonstrating large range of distribution [21].

There is close similarity (98 - 99%) between captive of leopard cat (Prionailurus bengalensis) samples with the database entry in GenBank. In comparing sequences of cytb gene, there is 1% similarity difference between domestic and wild leopard cat. The difference is probably caused by single nucleotide substitutions between two sequences which can be attributed to individual variation.

A very high similarity (99%) with GenBank accession obtained for the elephant (Elephas maximus) sample. This is not unexpected as there are only two species of elephants left in the world (http://www.elephant-world.com/types-of-elephants.html).

The Malayan tapir (Tapirus indicus) give 96% similarity with GenBank species is the only old world tapir and is found in this region (http://www.tapirs.org/tapirs/index.html). Its cytb gene sequence is quite different from the other tapir species, T. pinchaque (Mountain tapir) and T. bairdii (Baird's tapir) where the identity is below 89%; the difference is most likely due to different evolution caused by geographical separation.

The Oryx sample of this study displayed high similarity with the accessions of Oryx dammah, Oryx beisa and Oryx gazelle (99%, 97% and 95% respectively) in GenBank. The phylogenetic analysis based on the cytb sequences among laboratory and GenBank Oryx species also demonstrated that genetic materials among those three species are closely phylogenetically related to each other.

The cytb sequence of the painted stock (Mycteria leucocephala) sample is not highly similar (85%) to the corresponding accession in GenBank or to another related species M. cinerea (85%). This is likely due to insufficient information in GenBank. The class Aves of these species is a very species rich taxon and only low percentage of this biodiversity has been studied genetically [19].

Only one (WHG-pinky) of three white-handed Gibbon (Hylobates lar) samples in this study found matches in GenBank; however the match was not to a similar species. The query sequence was mismatched with three different species, Oryzomys couesi, Semnopithecus entellus and Melanomys caliginosus showing low similarity values in the range of 79% - 81%. Distributions of Gibbons are scarce worldwide and their numbers are declining. In the GenBank database, only 36 sequences of the cytb gene of Gibbon species which is less number comparing to that of other species was found.

Although PCR followed by gene sequencing provides significant advantages over contemporary procedures such as serological techniques of blood meal identification of vertebrate hosts, there will be sequences that do not have significant or similar matches in genetic databases. With such wide genetic variation resulting from evolutionary changes, no genetic databases can have sufficient information to cover all known species. The availability of nucleotide sequences will enhance the development of PCR for rapid

detection and identification of animal species. Blood meal studies using the molecular techniques depend on accurate and complete sequence databases such as at the GenBank library. If DNA sequences for potential hosts are not represented in the database, sequenced blood meals may be misidentified.

Since many studies are using mtDNA cytb gene fragment amplified using the primers of Kocher et al (1989), perhaps the mtDNA sequence for this fragment should be greatly expanded in the GenBank. To overcome these limitations, we should optimize training to improve identification of vertebrate hosts and examine larger numbers of local species of mammal, avian and reptile and registered their gene sequences in GenBank. Larger numbers and species of vertebrate hosts should be collected over the long term to obtain more sequence information to be deposited in GenBank.

4. CONCLUSION

These results will contribute to develop the GenBank database and can be applied for further epidemiological studies on vector borne diseases in tropical countries including Malaysia.

5. ACKNOWLEDGEMENTS

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No.	Code	Sample name(scientific name)	Sample curation (source)
1	PTM	Pig tailed macaque (Macaca nemestrina)	EDTA (National Zoo)
2	ME	Malaysian elephant – male (Elephas maximus)	EDTA (National Zoo)
3	DL	Dusky leaf monkey - wild (Trachypithecus obscures)	EDTA (National Zoo)
4	LTM	Long- tailed macaque (Macaca fascicularis)	EDTA (National Zoo)
5	LC (Dom)	Leopard cat (Prionailurus bengalensis)	EDTA (National Zoo)
6	PTM-1	Pig-tailed macaque (montel) (1) (Macaca nemestrina)	EDTA (National Zoo)
7	PTM-2	Pig-tailed macaque (2) (Macaca nemestrina)	EDTA (National Zoo)
8	LC (wild)	Leopard cat (wild caught) (Prionailurus bengalensis)	EDTA (National Zoo)
9	WHG (keke)	White-handed Gibbon (keke-male) sunway (Hylobates lar)	EDTA (National Zoo)
10	WHG (popeye)	White-handed Gibbon (popeye-male) (Hylobates lar)	EDTA (National Zoo)
11	WHG (pinky)	White-handed Gibbon (pinky-male) (Hylobates lar)	EDTA (National Zoo)

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12	LB1	Monkey (wild)-1 (species unknown)	EDTA and FTA card (Wildlife Department)
13	LB3	Monkey (wild)-2 (species unknown)	EDTA and FTA card (Wildlife Department)
14	ML(f)	Monitor lizard (female) (Varanus albigularis)	EDTA (National Zoo)
15	ML(m)	Monitor lizard (male) (Varanus albigularis)	EDTA (National Zoo)
16	oryx	Oryx (Oryx dammah)	EDTA (National Zoo)
17	PS	Painted stork (Mycteria leucocephala)	EDTA (National Zoo)
18	MT1(m)	Malayan tiger 1 (male) (Panthera tigris)	EDTA (National Zoo)
19	MT2(m)	Malayan tiger 2 (male) (Panthera tigris)	EDTA (National Zoo)
20	MT3(m)	Malayan tiger 3 (male) (Panthera tigris)	EDTA (National Zoo)
21	MT4(f)	Malayan tiger 4 (male) (Panthera tigris)	EDTA (National Zoo)
22	MT5(f)	Malayan tiger 5 (male) (Panthera tigris)	EDTA (National Zoo)
23	PM	Python (wild-male) (Python reticulatus)	EDTA (National Zoo)
24	Pf (5/11)	Python (wild-female) (Python reticulatus)	EDTA (National Zoo)
25	Pf (8/11)	Python (wild-female) (Python reticulatus)	EDTA (National Zoo)
26	WB-1	Malayan tapir 1 (Tapirus indicus)	FTA card (from UKM)
27	WB-2	Malayan tapir 2 (Tapirus indicus)	FTA card (from UKM)

Table 1 Name of the local and captive animals used in this study

No.	Code	Genbank species (accession number)	% similarity
1.	PTM1	Macaca nemestrina (DQ355486.1)	97
2.	LC DOM	Prionailurus bengalensis (AB210238.1)	98
3.	LC WILD	Prionailurus bengalensis (AB210238.1)	99
4.	ME	Elephas maximus (AY791141.1)	99
5.	MTf4	Panthera tigris jacksoni (EF179375.1)	99
6.	MTf5	Panthera tigris jacksoni (EF179375.1)	98
7.	MT1(m)	Panthera tigris jacksoni (EU184702.1)	99
8.	MT2 (m)	Panthera tigris jacksoni (EU184691.1)	99
9.	MT3 (m)	Panthera tigris jacksoni (EU184700.1)	99
10.	PF(5/11)	Python reticulates (U69860.1)	100
11.	ORYX	Oryx dammah (AJ222685.1)	99
12.	PM	Python reticulates (U69860.1)	100
13.	WB1	Tapirus indicus (EU224338.1)	95
14.	PS	Mycteria cineria (U72778.1)	85
15.	WB2	Tapirus indicus (EU224338.1)	88

Table 2 Sequences with highest percent similarity that exactly match corresponding species in GenBank

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No.	Identified as	GenBank species (accession number)	% similarity
1.	White-handed Gibbon (pinky)	Oryzomys couesi (DQ185385.1)	81
2.	Monkey (wild) 1	Homo sapiens (AC013437.8)	86
3.	Monkey (wild) 2	Homo sapiens (AC013437.8	89

Table 3 Sequences with highest percent similarities mismatched with other species in GenBank

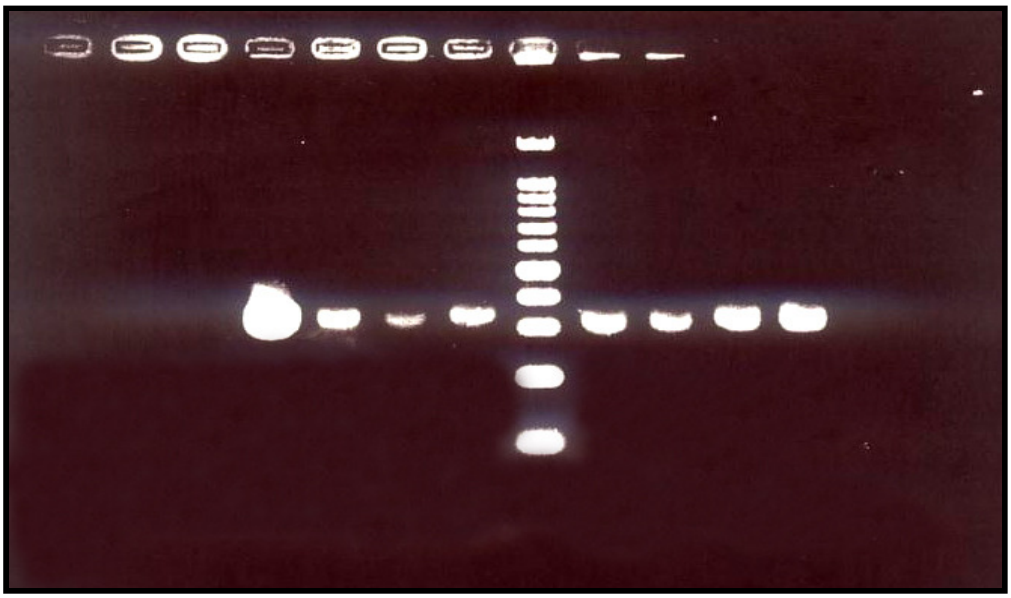

Figure 1

Gel photograph showing clear bands of 359 bp of cytb specific amplification products from local captive and animals. Lane 1: negative control; Lane 2: positive control; Lanes 3 – 9: Animal blood sample (MT1, MT3, LC, PS, oryx, ME and WB1); Lane M: 100 bp marker.

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