DETECTION OF 9-BP DELETION IN COII/tRNALys INTERGENIC REGION OF MITOCHONDRIAL DNA AMONG MURUT ETHNIC GROUP

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by

NAJAH FARAHIN NADIAH BINTI AZMAN

Dissertation submitted in fulfilment of the requirements
for the degree of
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CERTIFICATE

This is to certify that this dissertation entitled "**Detection of 9-bp Deletion in COII/tRNALys Intergenic Region of Mitochondrial DNA Among Murut Ethnic Group**" is a bona fide record of research work completed by Najah Farahin Nadiah binti Azman from October 2024 until Februrary 2025 under my supervision. I have reviewed this dissertation and, in my opinion, kit meets the acceptable standards of scholarly presentation. It is fit and adequate in both scope and quality to be submitted as part of the requirement for the degree of Bachelor of Science in Forensic Science (Honours).

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DECLARATION

I hereby declare that this dissertation is my original work, with the exception of the citations that are properly acknowledged. I also declare that it has not previously been or simultaneously submitted, in its entirety, for any degrees at Universiti Sains Malaysia or any other institution. I grant Universiti Sains Malaysia permission to use this dissertation for teaching, research or promotional purposes.

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Najah Farahin Nadiah binti Azman

Date: 28/02/2025

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LIST OF ABBREVIATIONS

A	Adenine
ATP	Adenosine Triphosphate
bp	Base Pair
C	Cytosine
COII	Cytochrome C Oxidase Subunit II
DCM	Dilated Cardiomyopathy
ddH_2O	Double-distilled Water
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotide Triphosphates
dsDNA	Double-stranded DNA
EDTA	Ethylenediaminetetraacetic Acid
EtBr	Ethidium Bromide
G	Guanine
gDNA	Genomic DNA
HCC	Hepatocellular Carcinoma
min	Minutes
mtDNA	Mitochondrial DNA
Na_2EDTA	Disodium Ethylenediaminetetraacetate
nDNA	Nuclear DNA
PCOS	Polycystic Ovary Syndrome
PCR	Polymerase Chain Reaction
rCRS	Revised Cambridge Reference Sequence
RNA	Ribonucleic Acid
rRNA	Ribosomal RNA
SNP	Single Nucleotide Polymorphism
ssDNA	Single-stranded DNA
T	Thymine
Taq	*Thermus Aquaticus*
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
Tm	Melting Temperature

tRNA	Transfer RNA
tRNALys	Lysine tRNA Gene
UV	Ultraviolet
UV-Vis	Ultraviolet-Visible Spectroscopy

LIST OF SYMBOLS

-	Range
%	Percent
°C	Degree Celsius
A	Absorbance
g	Gram
min	Minutes
mL	Millilitre
n	Number of samples
ng	Nanogram
p	Probability
pmol	Pico molecule
rpm	Revolutions per minute
Tm	Melting temperature
V	Volt
µL	Microlitre

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PENGESANAN PEMADAMAN 9-bp PADA KAWASAN INTERGENIK COII/tRNALys DNA MITOKONDRIA DALAM KALANGAN ETNIK MURUT

ABSTRAK

Pemotongan 9-pasangan bes (bp) dalam bahagian intergenik *COII/tRNALys* bagi DNA mitokondria (mtDNA) telah menjadi penanda penting untuk pengelasan haplogroup B dalam kajian kumpulan populasi, terutamanya di Asia Tenggara. Namun, tiada maklumat genetik mengenai pemotongan 9-bp yang sedia ada bagi kumpulan etnik pribumi di Sabah, termasuk etnik Murut, disebabkan kekurangan kajian genetik populasi yang dilakukan terhadap kumpulan rentan ini. Objektif kajian ini adalah untuk mengisi jurang penyelidikan dengan menyiasat kehadiran atau ketiadaan pemadaman 9-bp dalam kawasan intergenik *COII/tRNALys* mtDNA dalam kalangan etnik Murut. Seramai 100 individu Murut yang tidak mempunyai hubungan kekeluargaan telah menyertai kajian ini dan DNA mereka diamplifikasi dengan PCR menggunakan primer khusus bagi menghasilkan jalur DNA berukuran 211 bp untuk individu tanpa penghapusan 9-bp, dan jalur DNA berukuran 202 bp menunjukkan kehadiran penghapusan 9-bp. Dapatan kajian menunjukkan bahawa 45% individu Murut dikenal pasti mempunyai pemotongan 9-bp, manakala 55% tidak mempunyai pemotongan tersebut. Keputusan ini mencadangkan bahawa individu Murut mempunyai peratusan penghapusan 9-bp yang lebih tinggi berbanding populasi lain seperti Papua New Guinea dan Han Malaysia. Kesimpulannya, penentuan bahagian intergenik *COII/tRNALys* dapat memberikan maklumat tentang struktur haplogroup dalam kalangan individu Murut kerana pemotongan di bahagian ini digunakan untuk mengesahkan kehadiran haplogroup B.

DETECTION OF 9-bp DELETION IN COII/tRNALys INTERGENIC REGION OF MITOCHONDRIAL DNA AMONG MURUT ETHNIC GROUP

ABSTRACT

The 9-base-pair (bp) deletion in the COII/tRNALys intergenic region of the mitochondrial DNA (mtDNA) has been an important marker for haplogroup B classification in population group studies especially in Southeast Asia. However, no genetic data from 9-bp deletion was available for indigenous groups in Sabah including the Murut ethnic group due to the lack of population genetic studies done in these groups. The objective of this research is to fill in the research gap by investigating the presence or absence of 9-bp deletion of the COII/tRNALys intergenic region of mtDNA among the Murut ethnic group. A total of 100 unrelated Murut individuals participated in this research and were PCR amplified using a specific primer to generate 211 bp for absence of 9-bp deletion and a DNA band at 202 bp indicates the presence of 9-bp deletion. The finding shows 45% of Murut individuals were identified with 9-bp deletion and 55% were absence of 9-bp deletion. This outcome suggests that the Murut individual shows a considerably higher percentage of 9-bp deletion than other populations such as Papua New Guinea and Malaysian Han. In conclusion, typing of the COII/tRNALys intergenic region was able to provide information about the haplogroup structure among Murut individuals because the deletion at this region was used to confirm for haplogroup B.

CHAPTER 1

INTRODUCTION

1.1 Introduction

The mitochondrial DNA (mtDNA) is made up of 16,569 bp of a double-stranded circular DNA (Anderson et al., 1981) that produces DNA independently of nuclear DNA. The mtDNA contains 37 genes in total: 13 polypeptide genes encode for essential components for the electron transport chain, 2 encode for mitochondrial rRNA and 22 encode for tRNA genes which are used for protein synthesis (Prasun, 2019). The mtDNA offers significant advantages over nuclear DNA due to several factors. Firstly, since the mtDNA is inherited maternally, it does not undergo recombination during transmission (Prasun, 2019). In addition to that, the mtDNA sequence has a higher mutation rate than nuclear DNA, thus the discrimination between separated populations is possible (Ludes & Keyser-Tracqui, 2005). The mtDNA is also less effected than nuclear DNA due to the abundance of mtDNA (Naue et al., 2024), which makes it suitable for analysis of degraded samples.

The COII/tRNALys intergenic region is is located at position 8270 and 8289 of the mtDNA, in a non-coding region of 25-bp lengths (Anderson et al., 1981). Typically, individuals carry two tandem repeats of “CCCCCTCTA” at the COII/tRNALys intergenic region (Anderson et al., 1981). However, the presence of only one tandem repeat shows that the individual indicates a length polymorphism, specifically a 9-bp deletion in the COII/tRNALys intergenic region (Wrischnik et al., 1987). The 9-bp deletion was first discovered among Asians by Wrischnik et al. (1987) which was used to examine and understand genetic relationships between human populations.

The study of mtDNA genetic markers provides insight into a population's evolutionary history, aiding in the analysis of its maternal origins and migration patterns (Jia et al., 2022). Additionally, it can reveal the genetic relationships between different populations (Jia et al., 2022). Therefore, different markers in the human mtDNA are used to classify humans into specific haplogroups (Prasun, 2019).

The 9-bp deletion is a marker to define several haplogroups including B, B6, T2b15 and T2f (van Oven & Kayser, 2009) but, the 9-bp deletion at the COII/tRNALys intergenic region is mostly associated with haplogroup B (Derenko et al., 2012). Since haplogroup B is commonly observed in mainland Southeast Asia (20.6%) and Island Southeast Asia (15.5%) (Derenko et al., 2012). Thus, studying of the 9-bp deletion among the Murut in Nabawan, Sabah can enhance understanding of genetic relationships of the Murut ethnic group in Southeast Asian populations.

1.2 Problem Statement

The 9-base-pair (bp) deletion in the COII/tRNALys intergenic region of the mitochondrial DNA (mtDNA) has been an important marker for haplogroup B population group studies in Southeast Asia (Derenko et al., 2012). Based on the literature search, there was a lack of research done on indigenous population groups that populated Sabah, particularly the Murut ethnic group. Hence, this study aims to fill in the research gap by investigating the presence of 9-bp deletion of the COII/tRNALys intergenic region of mtDNA among the Murut ethnic group located in Nabawan, Sabah.

1.3 Significance of the Study

The study of the 9-bp deletion in the COII/tRNALys intergenic region of the mtDNA holds significance in several areas, particularly in population genetic study and clinical studies. Deletion at the COII/tRNALys intergenic region is well known to play as a role marker for classification of the haplogroup B in population genetic study (Derenko et al., 2012). Derenko et al. (2012) haplogroup B found commonly in Southeast Asia with a frequency of 20.6% in mainland Southeast Asia and 15.5% in Island Southeast Asia. This finding was backed up by previous research, the Austro-Asiatic population from Southeast Asia are commonly associated with the 9-bp deletion (Thangaraj et al., 2005). Thus, this marker is important to examine the genetic relationships between human populations, for example, understanding the migration and ancestry of the Southeast Asia population.

In addition to that, the 9-bp deletion also has clinical relevance. The 9-bp deletion is a risk factor for Hepatocellular Carcinoma (HCC) concluding that the presence of a 9-bp deletion showed a significant association with developing HCC (Jin et al., 2012). Another study showed that many women diagnosed with Polycystic Ovary Syndrome (PCOS) showed frequent presence of 9-bp deletion in the V region than the controls (Moosa et al., 2022). Moreover, one study reported that the 9-bp deletion has been associated with dilated cardiomyopathy (DCM) and certain neurological disorders, likely due to the dysfunctions in ATP generation by the mitochondria (Komandur et al., 2011).

Hence, this study aims to analyse the presence of 9-bp deletion at the COII/tRNALys intergenic region among 100 unrelated Murut individuals, with the potential to contribute to both population group study and clinical studies.

1.4 Objectives

1.4.1 Main Objective

To investigate the presence of 9-bp deletion among the Murut ethnic group.

1.4.2 Specific Objectives

i. To amplify COII/tRNALys intergenic region in the Murut ethnic group using PCR.

ii. To detect 9-bp deletion in the Murut ethnic group using agarose gel electrophoresis.

iii. To compare the frequency of 9-bp deletion in the Murut ethnic group with other population groups.

CHAPTER 2

LITERATURE REVIEW

2.1 Murut Ethnic Group

The Sabah state is home to up to 40 ethnicities (Hoh et al., 2022), where the Murut (6.6%) are one of the main ethnic groups of the Sabah Aborigines other than the Kadazan/Dusun (36.0%), Bajau (29.3%) and other Bumiputera (34.2%) (*Department of Statistics Malaysia*, n.d.)(Tey Nai Peng et al., 2021). Approximately 82% of the indigenous Murut people live in Keningau, Tenom and Nabawan in Sabah as in Figure 2.1, regions that are characterised as hard to reach out and inaccessible (Tey Nai Peng et al., 2021). The Murut ethnic group have historically lived in geographically isolated areas like interior valleys (Majid Cooke & Johari, 2019). This isolation, along with their resistance to colonial and external influences was considered encroachment into their social, economic, cultural and political lives (Fernandez, 1999), has led to the preservation of their distinct cultural practices and the genetic traits of the Murut people.

Relating to that, geographical isolation could lead to restricted gene flow due to physical barriers separating the populations (Choudhuri, 2014). Consequently, isolated populations like the Murut tend to have restricted gene flow with other groups, therefore having a higher degree of genetic differentiation from other populations (Slatkin, 1987; Jobling et al., 2013).

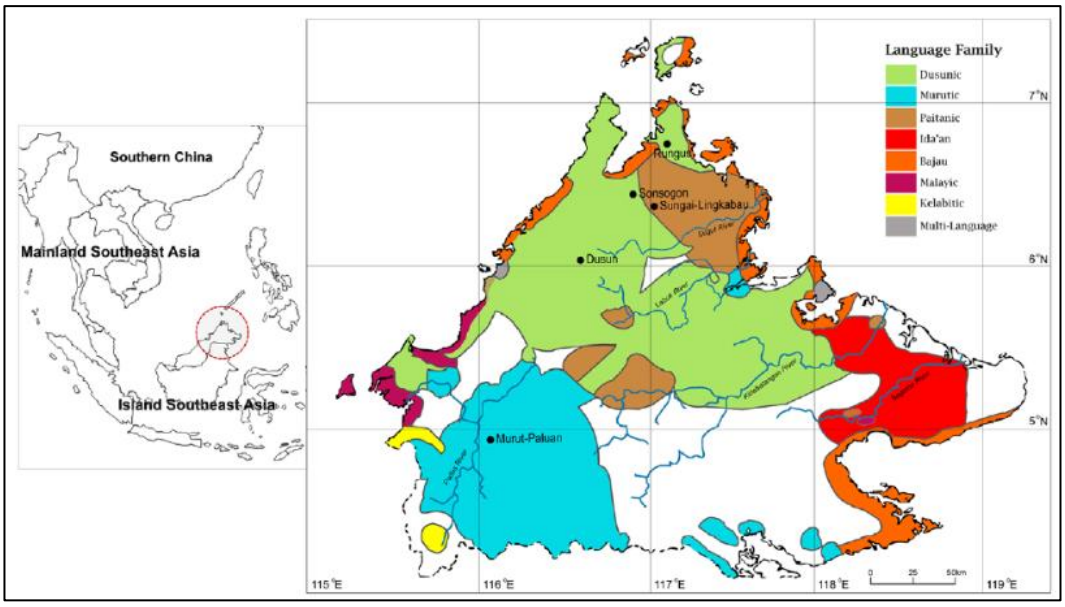

Figure 2.1 Location of Murut ethnic group distribution in Sabah (cyan colour) (Yew et al., 2018).

2.2 Mitochondrial DNA

The genomic mtDNA is a circular double-stranded molecule that is 16,569 bp in length(Anderson et al., 1981) obtained from the mitochondria, an organelle in the human eukaryotic cell. The mitochondria are a semi-autonomous organelle, that undergoes the replication, translation and transcription of its own DNA, independently of nuclear DNA (Anderson et al., 1981). There are approximately 2 – 10 copies of mtDNA in each mitochondrion, and each eukaryotic cell has at most 1000 mitochondria per cell, as compared to nuclear DNA, which only has 2 copies per cell (Sultana & Sultan, 2018).

The nucleotide positioning of the mtDNA is numbered according to the Anderson Reference Sequence (Anderson et al., 1981), where it was first completely sequenced based on its precise locations in the mtDNA. This detailed mapping has allowed the mtDNA to be characterised into 2 strands, heavy strand (rich in purines – nucleotide bases A and G) and light strand (rich in pyrimidines – nucleotide bases C and T) Anderson et al. (1981). These strands encompass 2 main regions which are the coding region, used for coding genes and the non-coding region, which is used for replication and transcription (Yan et al., 2019). However, the Anderson Sequence had some minor errors, and the new revised version is called the revised Cambridge Reference Sequence (rCRS) (Andrews et al., 1999). The mtDNA comprises 37 total genes with 13 encode for essential elements for the electron transport chain, 2 encode for mitochondrial rRNA and 22 encode for tRNA genes used in protein synthesis (Prasun, 2019). The structure of the mtDNA can be referred to Figure 2.1.

The mtDNA is inherited maternally and does not undergo recombination, therefore the mtDNA lacks genetic variation from sexual reproduction (Mishra & Chan, 2014). The paternal DNA is degraded during fertilisation from selective destruction,

inactivation or dilution (Yan et al., 2019). This results in maternal relatives from several generations can serve as reference samples for forensic mtDNA samples (Amorim et al., 2019). Additionally, the uniparental inheritance can also be used to trace maternal lineages back in time (Amorim et al., 2019).

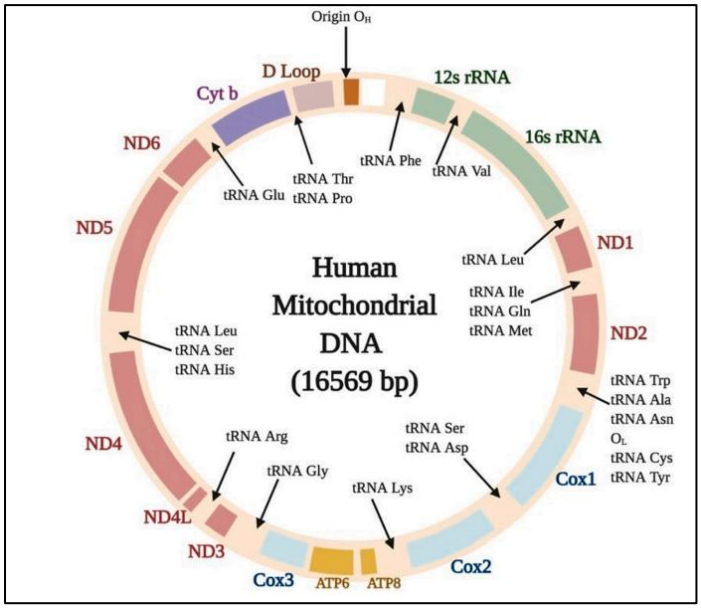

Figure 2.2 Structure of genomic mitochondrial DNA (Morton et al., 2021).

2.2.1 Mutations in Mitochondrial DNA

Referring to the rCRS, any other variations from the rCRS are considered a mutation, or in other words, polymorphisms (Sultana & Sultan, 2018). There are many types of polymorphisms in the mtDNA, which are: nucleotide substitutions, insertions and deletions. Nucleotide substitutions can be further divided into two; namely transitions (substitution of a pyrimidine with a pyrimidine or substitution of a purine with a purine) or transversion (substitution of a pyrimidine with a purine or vice versa). These mutations found in the mtDNA are either inherited maternally or slowly acquired throughout one's life (Lawless et al., 2020).

Lack of histone protection has made the mtDNA exposed to free radicals which cause the mtDNA is more susceptible to mutations compared to nDNA (Yan et al., 2019). This is because the mtDNA is located in the mitochondrial matrix, which acts as the main source for the reactive oxygen species, exposing the mtDNA to oxidative stress and making it more susceptible to damage (Shokolenko et al., 2009). In addition to that, the lack of mtDNA repair mechanisms also lead to a higher mutation rate of the mtDNA compared to the nDNA (Amorim et al., 2019). Most of these mutations were found in the non-coding region of the mtDNA as opposed to the coding region (Parsons et al., 1997).

The first human mtDNA mutation was described in 1988 (Holt et al., 1988) and has led to more and more associations between mtDNA mutations and mtDNA diseases. For example, mtDNA mutations have been linked with diabetes, Alzheimer's disease and cancer (Yan et al., 2019).

2.2.2 A 9-bp deletion in COII/tRNALys intergenic region of Mitochondrial DNA

A deletion is defined as a type of mutation that involves the loss of one or more base pairs from a segment of DNA. Deletion can involve either a deletion of a single nucleotide or deletion of an entire chromosome. The deletion was proposed to have been formed either during replication or repair of the mtDNA (Lawless et al., 2020).

The 9-bp deletion is a type of length polymorphism that involves a deletion of 9 base pairs at the non-coding region between cytochrome c oxidase subunit II and tRNALys (Giuliano et al., 2024). There are usually 2 copies of "CCCCTCTA" observed in the nucleotide positions 8270 – 8289 (Anderson et al., 1981), referred to Figure 2.2.

8221 attaattccc ctaaaaatct ttgaaatagg gcccgtattt accctatagc acccccctcta
8281 ccccctctag agcccactgt aaagctaact tagcattaac cttttaagtt aaagattaag

Figure 2.3 COII/tRNALys intergenic region according to the rCRS of the mtDNA showing two tandem repeats of "CCCCCTCTA" between the nucleotide positions 8270 – 8289 (Anderson et al., 1981).

The 9-bp deletion in this region was first identified in Asian descent by Cann & Wilson (1983) and since his time, this deletion has been observed among populations from East Asia (Wrischnik et al., 1987), South Asia (Thangaraj et al., 2005), Southeast Asia (Redd et al., 1995), Australia and Papua New Guinea (Wrischnik et al., 1987) and Polynesia and Melanesia (Merriwether et al., 1999). Figure 2.3 shows the distribution of the 9-bp deletion among the Asian and Pacific population (Redd et al., 1995).

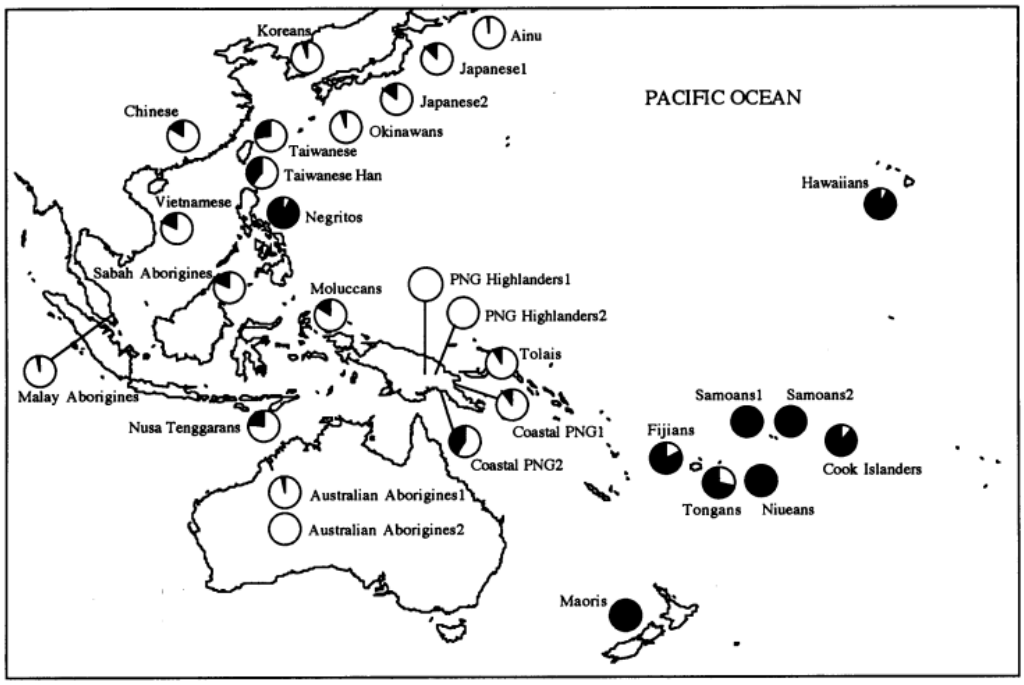

Figure 2.4 Distribution of 9-bp deletion in the COII/tRNALys intergenic region of the mtDNA in the Asian and Pacific populations (Black portion of the circle indicates the presence of 9-bp deletion) (Redd et al., 1995).

The loss of one of the two copies of the 9 bp tandem repeat sequence (CCCCCTCTA) in this region has been widely applied as a genetic marker in population group studies (Giuliano et al., 2024) and has been used as a genetic marker to trace population movements throughout Asia. The 9-bp deletion in the COII/tRNALys intergenic region of the mtDNA acts as a defining marker for haplogroup B population (Derenko et al., 2012) (Giuliano et al., 2024), which is particularly important in population group studies. In addition to that, the 9-bp deletion has also been researched in clinical studies where there are associations between the 9-bp deletion and diseases such as Hepatocellular Carcinoma (HCC) (Jin et al., 2012), Polycystic Ovary Syndrome (PCOS) (Moosa et al., 2022), dilated cardiomyopathy (DCM) and certain neurological disorders hypothesised linked to the ATP generation dysfunction by the mitochondria (Komandur et al., 2011).

2.2.3 Application of mtDNA in population genetic studies

The mtDNA has been applied in many populations' genetic studies due to the features of the mtDNA that make it suitable for the identification of historical remains or degraded DNA that has been exposed to harsh weather like extremely hot or cold (Connell et al., 2023).

The mtDNA could be used to study and understand evolution and history. Understanding the population history of such groups is important to understand previous migration patterns, population bottlenecks, founder effects, and other demographic events that may have shaped the current genetic diversity observed (Connell et al., 2023). In addition to that, historical factors like colonisation migration, isolation, and certain cultural practices specific to the population may also impact

genetic diversity (Arias et al., 2018). The ancient mtDNA could be used to understand past human diet, track historical civilizations and trade routes, and identify geographical domestication origins and lineage relationships (Merheb et al., 2019).

For example, the population history of the indigenous people in Southeast Asia have been hypothesised to be shaped by two migrations, i.e. the “Out of Africa” migration and the “Out of Taiwan” migration (Jinam et al., 2012). Most Austronesian-speaking population groups were hypothesised to come “Out of Taiwan” (Jinam et al., 2012). By analysing the mtDNA of the population group, we could understand the migration pattern and ancestry of those of the Murut ethnic group. The distribution and patterns of these haplogroups offer insights into the historical processes that shaped the region's population (Schurr & Wallace, 2002).

2.3 Haplogroup B

A haplotype is a specific pattern of sequential single nucleotide polymorphism (SNP); a single base sequence polymorphism on a single chromosome. A haplogroup is a collection of similar haplotypes in the mtDNA inherited from a common ancestor, reflecting an accumulation of sequential mutation through the maternal lineage (Mitchell et al., 2014). This inheritance that differentiates the various haplotypes is especially useful for determining geographic populations (Giuliano et al., 2024).

The major mtDNA haplogroups found in Asians are haplogroups A, B, C, D, E, F, G, P, Q, Y, Z (Chen et al., 2015). In Southeast Asia, the haplogroups B, F and M constitute the majority of the mtDNAs in this region (Schurr & Wallace, 2002). In addition to that, Derenko et al. (2012) observed that the haplogroup B is also found at a commonly in Southeast Asia with a frequency of; in mainland Southeast Asia (20.6%) and Island Southeast Asia (15.5%). The distribution and pattern of these haplogroups provide ideas on the historical process of people placement in the region (Schurr & Wallace, 2002).

The haplogroup B is characterised by the 9-bp deletion in the COII/tRNALys intergenic region of the mtDNA; located at SNP positions 8280–8290 = A [delCCCCCTCTA] G (Derenko et al., 2012) (Chen et al., 2015). Thus, the 9-bp deletion acts as an important marker to examine and understand the genetic relationships between populations within haplogroup B.

2.3.1 Haplogroup B dataset among Southeast Asians

Referring to research done by Schurr & Wallace (2002) and (Trejaut et al., 2005), the dataset for the haplogroup B in Southeast Asian populations was compiled as stated in Table 2.1.

Table 2.1 Haplogroup B Percentage (Schurr & Wallace, 2002) (Trejaut et al., 2005).

Population	**n**	**Haplogroup B**
Taiwanese Han	20	7.1
Malaysian Han	14	32.0
Vietnamese	28	17.9
Orang Asli	31	3.1
Malays	14	14.3
Sabah Aborigines	32	18.6
Papua New Guinea	119	41.8
Koreans	13	15.4
Tibetans	54	11.1
Nepalese	50	4.0
Philippines	59	40.7
Indonesia	54	20.4

2.4 Polymerase Chain Reaction (PCR)

The polymerase chain reaction was first introduced by Kary B. Mullins in 1985 (Mullis, 1990) which resulted in him winning The Nobel Prize in Chemistry in 1993. The PCR functions to amplify specific segments of DNA into larger quantities (Tytgat, 2022). The PCR is a rapid, straightforward and efficient technique for amplifying particular segments of the DNA (Li et al., 2008).

There are three stages of the PCR, denaturation, annealing and elongation. In the denaturation stage, the double-stranded DNA (dsDNA) is heated to a temperature, usually 90 °C and above, causing the hydrogen bonds between the double-stranded helix to break into single-stranded DNA (ssDNA) (Tytgat, 2022). Next, is annealing where the primers in the PCR reaction mix will anneal to the specific sequences on the ssDNA. This is usually done at a lower temperature around 60 °C. The final stage is the elongation step, where the deoxynucleotide triphosphates (dNTPs) which are the building blocks of the DNA will be hybridised complementary to the template strand. This is done with the help of a DNA polymerase at 72 °C which is considered the optimum temperature for the enzyme (Tytgat, 2022). A common DNA polymerase used is the *Taq* polymerase due to its thermostability. The repetition of these steps multiple times causes the target region to be amplified as each new amplified DNA strand will act as a template for the following cycle (Tytgat, 2022). The PCR process is usually performed in 20 – 30 cycles (Li et al., 2008). The PCR stages can be referred to in Figure 2.3.

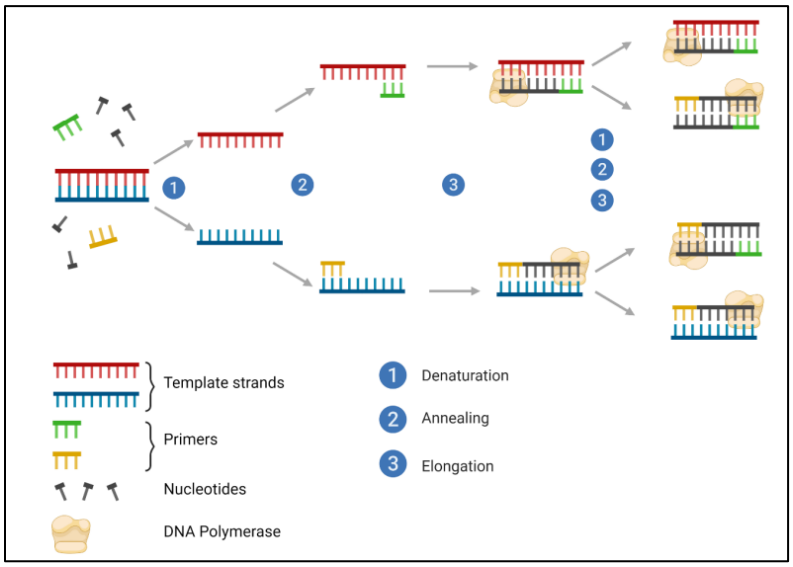

Figure 2.5 Polymerase Chain Reaction consisting of three stages: (1) Denaturation, (2) Annealing and (3) Elongation (Tytgat, 2022).

CHAPTER 3

METHODOLOGY

3.1 Materials

3.1.1 Chemicals and Reagents

The list of chemicals, reagents and consumables used in this are listed in Table 3.1.

Table 3.1 List of chemicals, reagents and consumables used in this research.

Chemical	**Production Company**
Tris base	Sigma-Aldrich
Boric acid	Promega Corporation
Disodium ethylenediaminetetraacetate dehydrate (Na_2EDTA)	Sigma-Aldrich
PrimeWay Genomic DNA Extraction Kit	1st Base
Ethidium Bromide	Sigma-Aldrich
Agarose powder	1st Base
Primer	Sigma-Aldrich
1kbp DNA ladder	Gene DireX
100bp ladder	ThermoFisher Scientific
Orange G Dye	Sigma-Aldrich
Consumables	**Production Company**
Pipette tips	Eppendorf
0.5 mL PCR tube	Biologix
1.5 mL centrifuge tube	Biologix
Kim wipes	Kimberly-Clarck Worldwide Inc.

3.1.2 Instruments and Apparatus

The list of instruments and apparatus used in this research, their brand and model are shown in Table 3.2.

Table 3.2 List of instruments and apparatus used in this research.

Instrument	**Brand**	**Model**
PCR Thermal Cycler	Applied Biosystem	Veriti 96 Well
Analytical Balance	Shimadzu	ATX224
DNA Electrophoresis System	Owl	BI
Hot plate and Magnetic Stirrer	ERLA	EMS-HP-700
Laminar Air Flow Cabinet	ERLA	CFM-4
pH meter	Hanna Instruments	PH211
Microwave Oven	Elba	EMO-1706
Pipette 10 µL, 20 µL, 100 µL	Eppendorf	Research plus
Power Supply	Bio-Rad	Power-Pac 3000V
Spectrophotometer	DeNovix	DS-11
Vortex Mixer	ERLA	EVM 6000

3.1.3 Chemical and Reagent Preparation

3.1.3.1 Preparation of 10X Tris-Borate-EDTA TBE Buffer (pH 8.3)

A total of 108 g Tris base, 55 g boric acid and 9.3 g Na_2EDTA was mixed into 827.7 mL of distilled water into a 1 L Schott bottle. The solution was then autoclaved and then stored at room temperature.

3.1.3.2 Preparation of 1X Tris-Borate-EDTA TBE Buffer (pH 8.3)

A total of 100 mL 10X TBE buffer was mixed into 900 mL of distilled water in 1 L Schott bottle.

3.2 Methodology

3.2.1 Sample Collection

The ethical clearance (USM/JEPeM/15100366) was approved by the Ethical Committee for Research involving Human Subjects, USM. A total of 100 unrelated Murut individuals were interviewed, and their blood samples were collected. For each participant, 10 mL of blood sample was collected and kept inside a vacutainer blood collection tube with their consent. Blood samples were stored under -80 °C for long-term preservation.

3.2.2 Sampling Method

The Murut individuals were selected through verbal interviews before being accepted as subjects for this research. All consensual participants were required to fill out consent forms.

The inclusion and exclusion criteria of the biological sample are:

Inclusion criteria:

i. Subjects are unrelated males or females of Murut ethnicity without mixed marriage for at least three (3) generations.

ii. Participants should be normal or healthy individuals without chronic or genetic conditions.

iii. Adults aged above 18 years old.

Exclusion criteria:

i. Subjects with a medical history.

ii. Subjects with a family history of mixed marriage (non-Murut ancestry).

3.2.3 Genomic DNA Extraction

The Genomic DNA (gDNA) extraction was carried out using the PrimeWay Genomic DNA Extraction Kit (KIT-9020-50). There are 5 main steps: lysis, binding, washing, drying and elution. Before lysis, the blood sample was equilibrated from the EDTA tube at room temperature. Then, 200 μL of the sample was transferred into a new 1.5 mL microcentrifuge tube.

Next, lysis of the blood samples was done by adding 25 µL of Proteinase K to the samples followed by 200 µL of TB2 Buffer and vortexed vigorously for 20 seconds to mix. The sample was then incubated at room temperature for 5 minutes, and then vortexed again. The samples were then incubated at 70 °C for 15 minutes, producing a brownish solution during the incubation.

This is followed by the binding process, 210 µL of ethanol was added to the sample and vortexed. Next, the One PrimeWay Genomic Column was placed into a Collection Tube and the lysate was transferred onto the PrimeWay Genomic Column. Then, the Collection Tube was centrifuged at 11000 rpm for 1 minute. The Primeway Genomic Column was placed into a new Collection Tube.

The next process is washing. At this step, 500 µL of Wash Buffer T1 was added into the column and centrifuged at 11000 rpm for 1 minute. The follow-through was discarded and the column was placed back into the Collection Tube. A 600 µL of Wash Buffer T2 was added into the column and then centrifuged at 11000 rpm for 1 minute. The follow-through was discarded and the column was placed back into the Collection Tube.

For the drying process, the Collection Tube with the PrimeWay Genomic Column was centrifuged at 11000 rpm for 1 minute to ensure the ethanol residue was fully removed.

Lastly, for the elution process, the PrimeWay Genomic Column was placed into a new 1.5 mL centrifuge tube and 100 µL of elution buffer was added to the centre of the membrane. The column was left to stand at room temperature for 1 minute. Finally, centrifuged at 11000 rpm for 1 minute to allow the DNA to elute.

3.2.4 Agarose Gel Electrophoresis of Genomic DNA

An agarose gel electrophoresis was run to confirm and visualise the presence of the extracted genomic DNA. For this purpose, a 1% agarose gel was prepared by dissolving 0.5 g of agarose powder with 50 mL 1 XTBE buffer and was heated for 2 minutes. The solution was then left to cool and 1.5 µL of ethidium bromide (EtBr) (10 mg/mL) was added into the solution using a micropipette and swirled until dissolved.

The agarose gel solution was poured onto a horizontal electrophoresis tray and left to solidify for 20 minutes. A 3 µL of 1K bp DNA ladder was loaded into the first well as a size reference and1 µL of Orange G dye was mixed with 2 µL of extracted gDNA and then loaded into the wells. The electrophoresis was conducted at 90 V for 45 minutes. Finally, the gel was observed under UV light to observe the presence of gDNA.

3.2.5 Genomic DNA Quantification

The quantity and the quality of the extracted gDNA were analysed using a UV-Vis Spectrophotometer using the DeNovix DS-11 Spectrophotometer. Before the instrument is used, the lens should be cleaned using 1 µL of ddH_2O by loaded on the lower pedestal of the instrument. The sampling arm was brought down to wash both the upper and lower pedestals. Next, the upper and lower pedestal surfaces were cleaned with Kim wipes to remove any residue. A blank measurement was done by loading 1.0 µL of TE Buffer (the same from the extraction of the DNA) onto the lower pedestal and lowering the sampling arm to set a baseline reading. Both the pedestal surfaces were then wiped with Kim wipes. Then, 1.0 µL of the DNA sample was loaded onto the lower pedestal and measurement of concentration and purity was taken after the sampling arm

was lowered. After each DNA sample, the pedestal surfaces were wiped with Kim wipes to prevent any carryover between samples. The results were extracted and analysed, where a desirable 260/280 ratio of 1.8 – 2.0 and 260/230 absorbance ratio of 2.0 – 2.2 were suggested for the extracted gDNA of high purity.

3.2.6 Polymerase Chain Reaction (PCR) Amplification of COII/tRNALys intergenic region

The PCR amplification of the mtDNA COII/tRNALys intergenic region was done using established primers 8215F and 8385R (Wrischnik et al., 1987). A 15 µL was prepared as displayed in Table 3.3. Details regarding the primer used for the PCR was shown in Table 3.4 and the PCR parameters were displayed in Table 3.5. To monitor the cross-contamination during PCR amplification, the negative control was included and ran simultaneously with the samples. The amplified PCR products were then verified using an agarose gel electrophoresis.

Table 3.3 PCR components for PCR reaction mixture.

Component		**Volume (µL)**
PCR ready mix		13.2
Primer (10 pmol)	**8215 F**	0.6
	8385 R	0.6
ddH_2O		0.3
DNA		0.3
Total		15

Table 3.4 Primer used for amplification of the COII/tRNALys intergenic region.

Primer	Primer sequence (5' – 3')	Length (bases)	Tm (°C)	PCR product size (bp)
8215F	ACAGTTTCATGCCCATCGTC	20	60	211 bp (without 9-bp deletion)
8385R	GTAATTATGGTGGGCCATACGG	22	66	202 bp (with 9-bp deletion)

Table 3.5 PCR cycling parameters for Veriti 96 Well Thermal Cycler.

Stage	Temperature (°C)	Duration
Denaturation	95	4 minutes
Annealing (30 cycles)	95	30 seconds
	60	30 seconds
	72	30 seconds
Elongation	60	30 seconds
	72	7 minutes

3.2.7 Agarose Gel Electrophoresis for amplified PCR products

After PCR amplification, an agarose gel electrophoresis was run to confirm and visualise the success of the amplification. A 3% agarose gel was prepared by adding 1.5 g of agarose powder into 50 mL of 1X TBE buffer and was heated using a microwave oven for 2 minutes. The solution was then left to cool and 1.5 µL of ethidium bromide (EtBr) (10 mg/mL) was added into the solution using a micropipette and swirled until dissolved.