A POLYMORPHIC STUDY OF MITOCHONDRIAL DNA IN THE CODING REGION AND ITS FORENSIC APPLICATION

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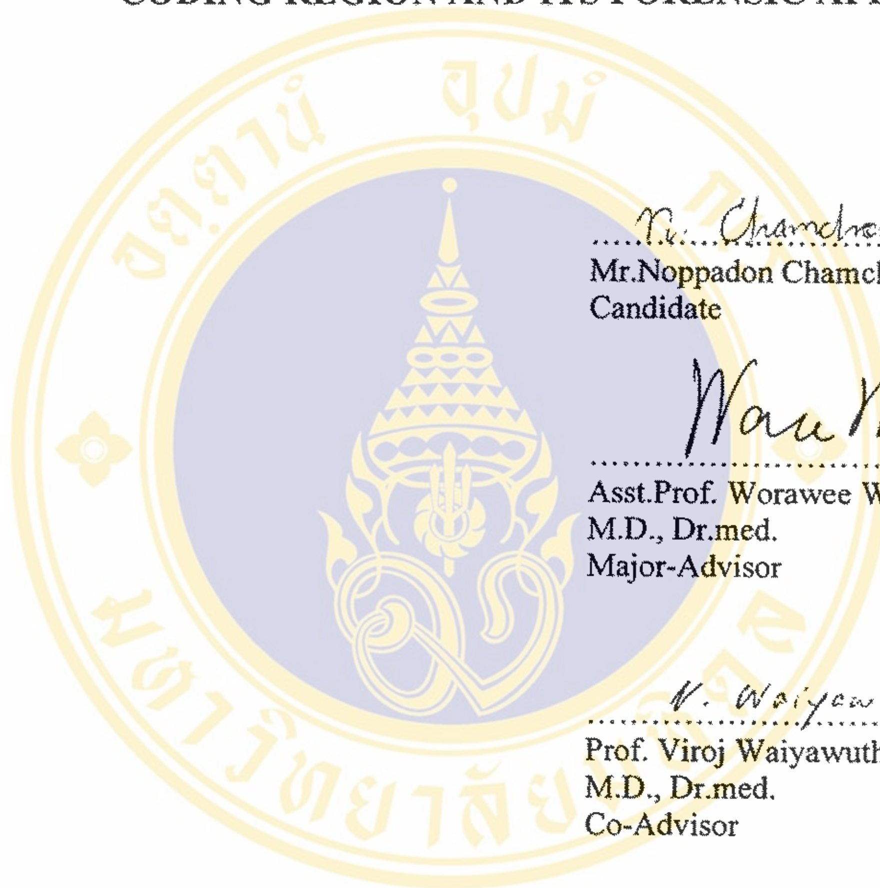

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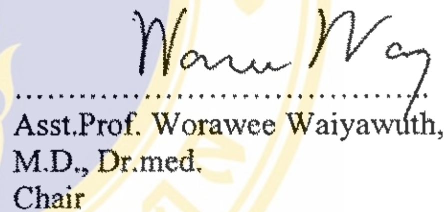

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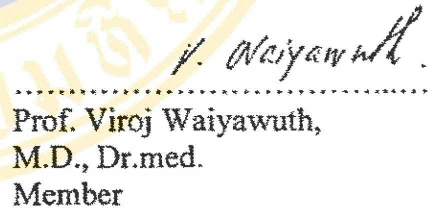

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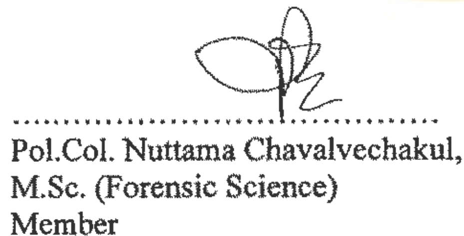

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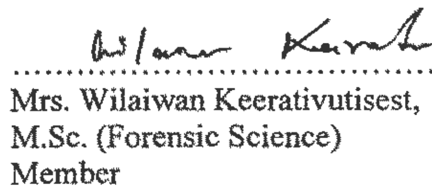

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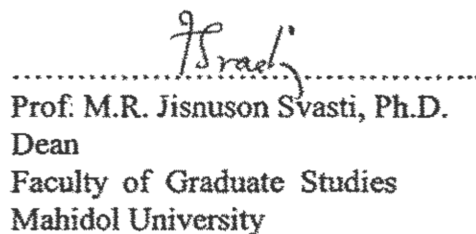

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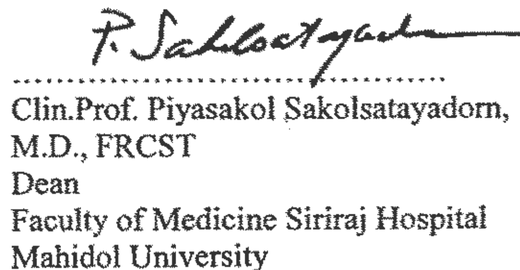

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A POLYMORPHIC STUDY OF MITOCHONDRIAL DNA IN THE CODING REGION AND ITS FORENSIC APPLICATION

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ABSTRACT

Mitochondrial DNA typing has been widely applied in forensic casework. It can be used even when DNA samples are minute or severely degraded and analysis by short tandem repeat may not be successful. The hypervariable regions I and II of the control region are currently used for mtDNA testing in forensics. However, these regions are limited due to their low power of discrimination. Therefore, the coding region of mitochondrial DNA is an alternative test region.

In this study, the purpose was to analyze mitochondrial DNA polymorphisms in the coding region and provide a basis for application in forensic science. We investigated a coding region encompassing position 8320 to 9160 in 80 unrelated individuals from a Thai population. The DNA sequences were determined by polymerase chain reaction and direct sequencing. The results showed that the sequence polymorphisms of this region differed from Cambridge reference sequence in 33 sites, and there were 23 haplotypes. The genetic diversity was estimated at 0.8598 and power of discrimination was 0.8491. The combination of this region with two hypervariable regions causes the discrimination power to rise to 0.9875.

These results suggest that polymorphic sites within nucleotide sequence 8320 to 9160 of mitochondrial DNA can be used as a marker for forensic casework.

KEY WORDS: MITOCHONDRIAL DNA / CODING REGION / POLYMORPHISM / FORENSIC APPLICATION / THAI POPULATION

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การศึกษา POLYMORPHISM ของไมโทคอนเดรียลดีเอ็นเอบริเวณ CODING REGION และการประยุกต์ใช้ทางนิติวิทยาศาสตร์ (A POLYMORPHIC STUDY OF MITOCHONDRIAL DNA IN THE CODING REGION AND ITS FORENSIC APPLICATION)

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บทคัดย่อ

การตรวจวิเคราะห์สารพันธุกรรมจากไมโทคอนเดรียเพื่อใช้ในงานด้านนิติวิทยาศาสตร์ได้รับความสนใจอย่างมาก โดยเฉพาะในกรณีที่ตัวอย่างมีปริมาณน้อยมากหรือเกิดการสลาย ซึ่งการตรวจจาก short tandem repeat ไม่สามารถให้ผลได้ การตรวจพิสูจน์จากไมโทคอนเดรียลดีเอ็นเอจะทำการวิเคราะห์บริเวณ hypervariable region I และ II ของ control region แต่บริเวณนี้มีข้อจำกัด เนื่องจากให้ค่ากำลังการแยกแยะบุคคลต่ำ ดังนั้นการใช้ coding region จึงเป็นทางเลือกสำหรับการแก้ปัญหานี้ การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษา polymorphism ในประชากรไทยและนำไปประยุกต์ใช้ในงานด้านนิติวิทยาศาสตร์

ในการศึกษา coding region ของไมโทคอนเดรียลดีเอ็นเอบริเวณลำดับเบสตำแหน่ง 8320-9160 ของประชากรไทยจำนวน 80 คนที่ไม่มีความสัมพันธ์กันทางสายเลือดฝ่ายมารดา โดยทำการวิเคราะห์ด้วยวิธี polymerase chain reaction และ direct sequencing ผลการศึกษาพบลำดับเบสที่แตกต่างจาก Cambridge reference sequence ซึ่งเป็นลำดับเบสอ้างอิงจำนวน 33 ตำแหน่งและประกอบด้วย 23 haplotypes อีกทั้งให้ค่าความหลากหลายทางพันธุกรรมเท่ากับ 0.8589 และค่ากำลังการแยกแยะบุคคลเท่ากับ 0.8491 โดยเมื่อรวม coding region กับ hypervariable I และ II พบว่าค่ากำลังการแยกแยะเพิ่มขึ้นเป็น 0.9875

การศึกษาครั้งนี้พบว่า coding region ในตำแหน่ง 8320-9160 ของไมโทคอนเดรียลดีเอ็นเอสามารถนำไปประยุกต์ใช้ในงานด้านนิติวิทยาศาสตร์ได้

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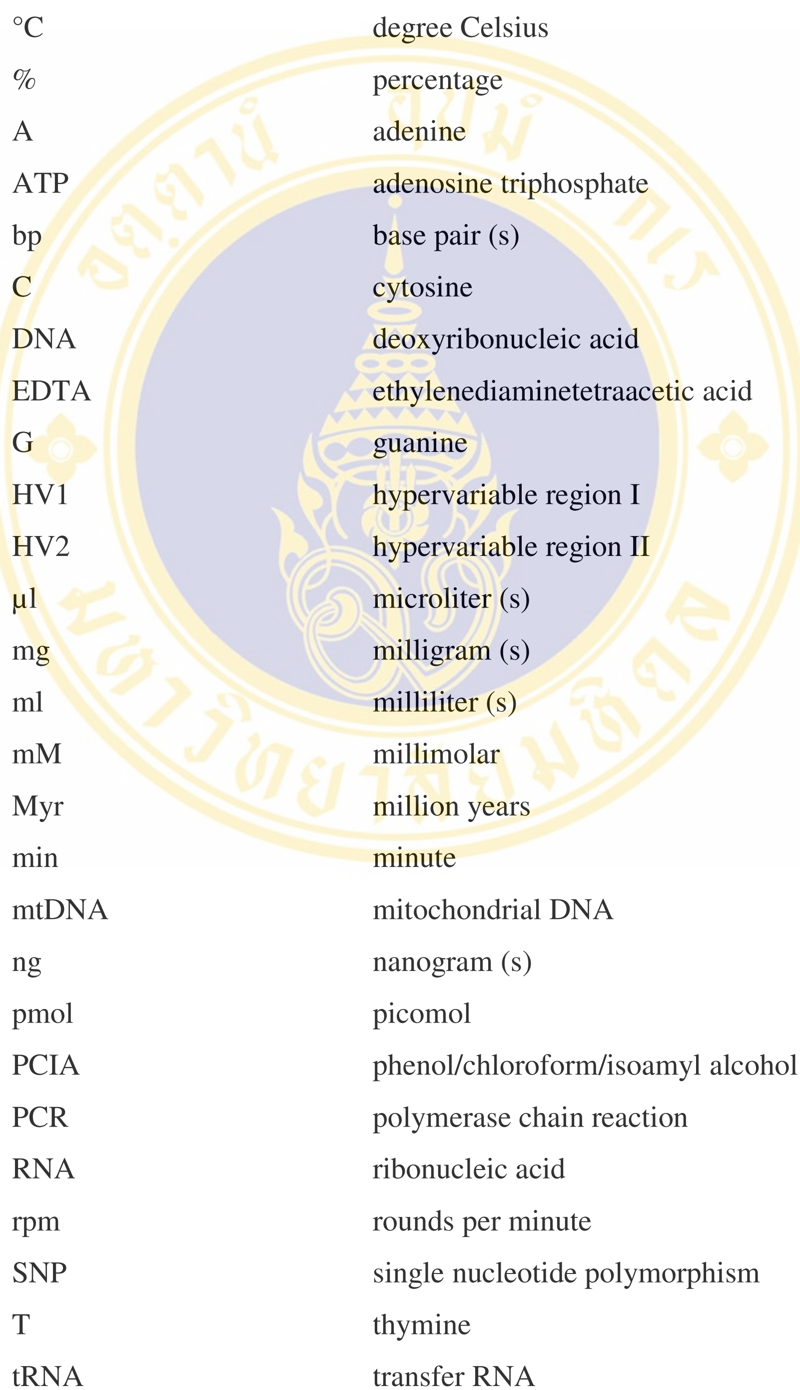
LIST OF ABBREVIATIONS



°C	degree Celsius
%	percentage
A	adenine
ATP	adenosine triphosphate
bp	base pair (s)
C	cytosine
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
G	guanine
HV1	hypervariable region I
HV2	hypervariable region II
µl	microliter (s)
mg	milligram (s)
ml	milliliter (s)
mM	millimolar
Myr	million years
min	minute
mtDNA	mitochondrial DNA
ng	nanogram (s)
pmol	picomol
PCIA	phenol/chloroform/isoamyl alcohol
PCR	polymerase chain reaction
RNA	ribonucleic acid
rpm	rounds per minute
SNP	single nucleotide polymorphism
T	thymine
tRNA	transfer RNA

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CHAPTER I
INTRODUCTION

Currently, DNA typing is significant implement for the forensic science and criminal justice system that DNA sequence was reported and used for individual identification since 1985 by Alec J. Jeffreys and his colleagues at the University of Leicester, UK. They studied that DNA probes based on a core sequence of the tandem repeats found in the hypervariable region of the human myoglobin gene could detect many highly polymorphic minisatellite simultaneously that this method is known as the DNA fingerprinting. The DNA fingerprints produced by minisatellite probes 33.15 and 33.6 are sufficiently stable and individual-specificity therefore they could use in human identification and paternity testing for the forensic application (2,3). This procedure has undergone modification and evolution led to using short tandem repeat (STR) loci or microsatellite in forensic DNA analysis, nowadays. It is DNA region with repeating units that are 2-6 base pairs in length (4). STR typing was DNA testing which has remarkable proficiency and sensitivity. However, it may fail because of the degradation of the nuclear DNA in sample, therefore mitochondrial DNA was good choice for analysis.

Human mitochondrial DNA (mtDNA) is a double-stranded circular molecule consisting of 16,569 base pairs in organelles known as mitochondria (5). The mitochondrial DNA could use even when samples were old or badly degraded and encompass hair shaft without root. Because mitochondrial DNA has hundreds to thousands of copies per cell while nuclear DNA has only two copies in cell. As a result, some mitochondrial DNA molecules are more likely survivor than nuclear DNA. The specific features of the mitochondrial genome such as high copy number per cell, high substitution rate and uniparental inheritance made mitochondrial DNA suitable for forensic analysis. The mtDNA testing consists primarily of sequence analysis of hypervariable region I (HV1) and hypervariable region II (HV2) within the control region. The analysis was performed by comparison of sample sequences with

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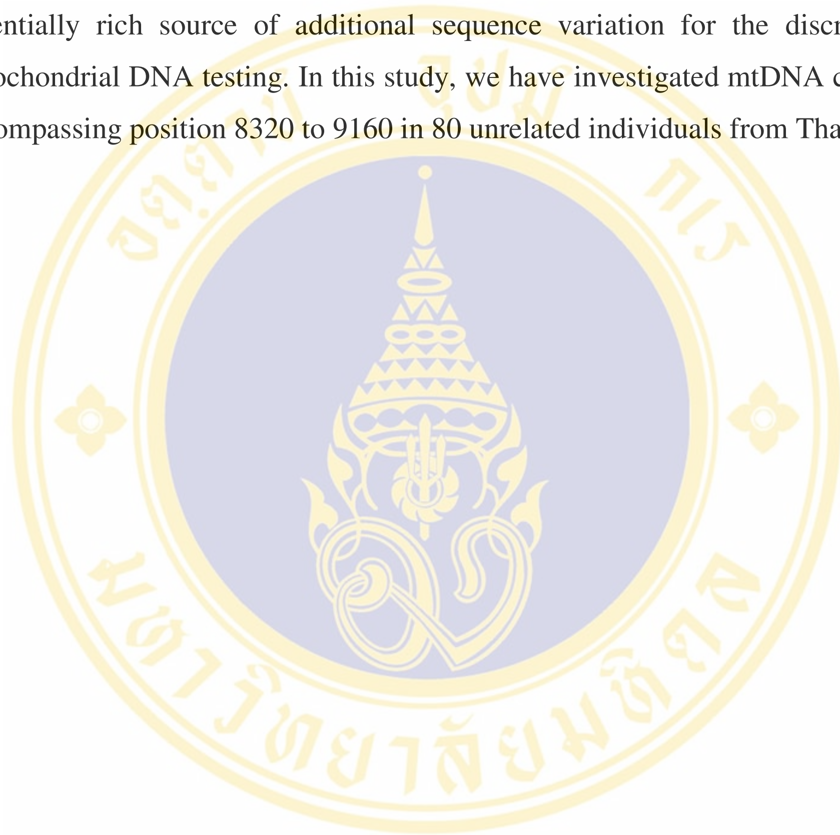
the reference sequence. However, the utility of hypervariable regions of mtDNA are limited since its low power of discrimination. Looking for other mtDNA regions may increase the meaningfulness of mtDNA result that the analysis of additional information in the coding region seems to be a good strategy to pass this drawback. The coding region is about fifteen times larger than the control region that making a potentially rich source of additional sequence variation for the discrimination of mitochondrial DNA testing. In this study, we have investigated mtDNA coding region encompassing position 8320 to 9160 in 80 unrelated individuals from Thai population.



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CHAPTER II
LITERATURE REVIEW

1. Structure of DNA

Deoxyribonucleic acid or DNA is genetic material of organism. The DNA is a polynucleotide consisting of a pentose sugar, a nitrogenous base, and a phosphate group. The sugar in DNA is deoxyribose, and the nitrogenous base divide purine and pyrimidine. The purines are adenine (A) and guanine (G) while pyrimidines are thymine (T) and cytosine (C). The nitrogenous base attached to the 1'carbon atom of the sugar by an N-glycosylic bond. While, the phosphate attached to the 5'carbon of the sugar by phosphoester linkage. The structure of DNA was exposed by Watson and Crick (6) that DNA is a double helix with sugar-phosphate backbone on the outside of the molecule. The bases are on the inside of the helix and liked together by hydrogen bonds. The base pairing is very specific: adenine is always paired with thymine by two hydrogen bonds, and guanine is always paired with cytosine by three hydrogen bonds. The two DNA strands are complementary to each other, so each DNA strand contains the template information for synthesis of a new copy of the other strand.

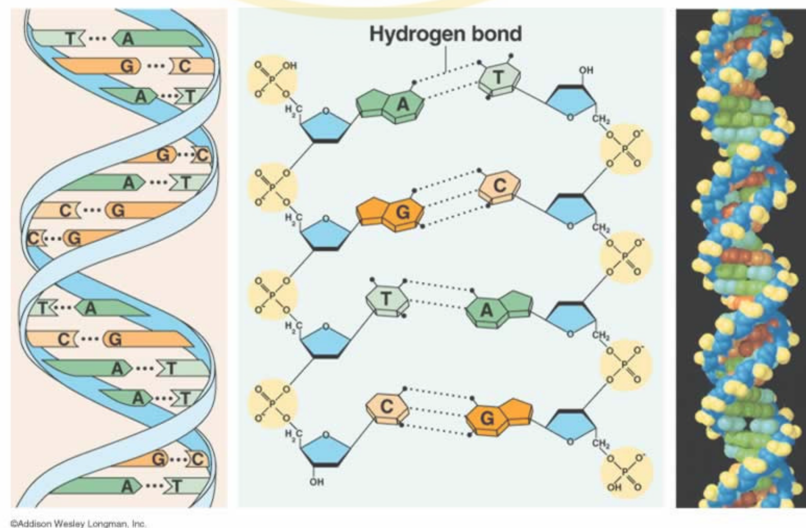

Figure 1 Structure of DNA

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2. Mitochondrial DNA

Mitochondrial DNA (mtDNA) is located in the energy-producing organelles known as mitochondria. Which mitochondria are found in almost all eukaryotic cells. They are the sites of oxidative phosphorylation process and are responsible for generating most of the adenosine triphosphate (ATP) derived from the breakdown of organic molecules. Mitochondria are bounded by a double-membrane system that consists of inner and outer mitochondrial membranes separated by an intermembrane space. The inner membrane forms numerous folds (cristae), which extend into the interior (or matrix) of the organelle (7). Mitochondria are thought to have evolved from aerobic bacteria that developed a symbiotic relationship in which they lived within larger cell. This hypothesis was affirmed from genome sequence analysis, indicating that *Rickettsia prowazekii* is closely related to mitochondria (8). It is an obligate intracellular parasite and the causative agent of epidemic typhus. This reinforces the idea which mitochondria originated from ancestor of the *Rickettsia*.

Mitochodria possess own genetic system which is separate from the nuclear genome of the cell. The human mitochondrial genome is about 16,569 base pair and is circular molecule. The two DNA strands have significantly different base compositions. The heavy (H) strand contains most of the guanine (G) nucleotides, while the light (L) strand is rich in cytosine (C) nucleotides (9). Each human cell is present hundred to thousands of mtDNA. The mitochondrial DNA encodes 13 proteins involved in electron transport and oxidative phosphorylation. Moreover, it encodes 12S and 16S ribosomal RNAs and 22 tranfer RNAs that are required for translation of the proteins. The 13 protein-coding are components of respiratory complexes that consist of seven subunits of complex I (NADH dehydrogenase), three subunits of complex IV (cytochrome c oxidase), two subunits of complex V (ATP synthase), and cytochrome b (a subunit of complex III) (10). All other mitochondrial proteins are encoded by the nuclear genome before imported into the mitochondria. In addition, the some genetic code of human mitochondrial DNA differs from the universal code. Which the terminator codon UGA is read as tryptophan, and AUA that normally codes for isoleucine is read as methionine. AGA and AGG are termination rather than arginine codons, and AUA and AUU can serve as initiator codon as well as AUG (5).

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Besides mitochondrial DNA compose of the non-coding region or the control region, also known as the displacement loop (D-loop). There is an approximately 1,100 bp region that situated between the mitochondrial $tRNA^{Pro}$ and $tRNA^{Phe}$ gene. The D-loop is a triplex structure formed by the synthesis of a short daughter H strand which remains stably associated with the parent L strand. Therefore, the D-loop is apparent template (L strand) primed for H strand synthesis. Using the L strand as a template, replication of the H strand begins at the D-loop and continues in a clockwise direction. When the synthesis of the daughter H strand is two-thirds complete, the L strand origin of synthesis is exposed on the displaced H strand. The L strand synthesis begins and proceeds in the opposite direction (11). The D-loop contains the origin of replication and hypervariable regions, HV1 and HV2. The HV1 is sequence between nucleotides 16,024 and 16,365 while HV2 encompasses positions 73 to 340. These two hypervariable regions approximately 610 bases are used for forensic propose.

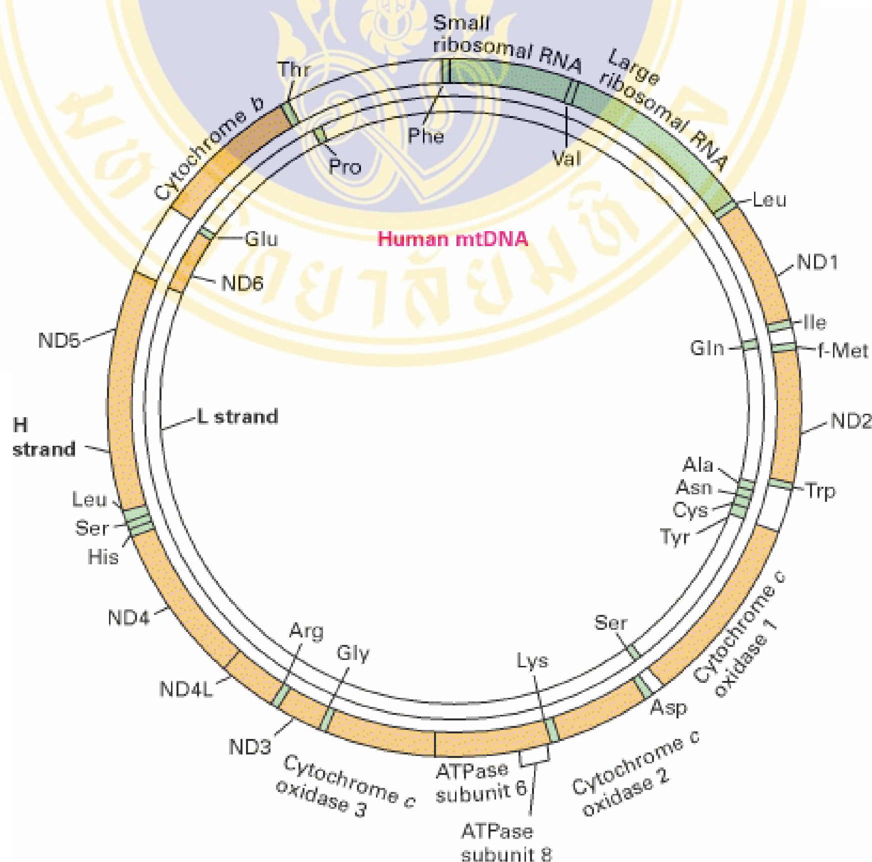

Figure 2 The human mitochondrial genome

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2.1 Mutation rate

The mitochondrial genome has a very high mutation rate that was estimated to be $1.70x10^{-8}$ substitutions per site per year for the whole genome excluding the D-loop (12). While the mutation rate of control region on the basis of pedigree studies was estimated to be 0.0043 per generation or 0.32/site/Myr (13). Heyer et al. studied mutation using deep-rooting pedigrees that can be estimated at 0.0079 per generation (14). Moreover, Howell et al. reported from analysis in control region that found the pedigree divergence rate was 0.95 mutations/bp/Myr (15).While the mutation rate of HV1 and HV2 was 0.2415 mutations/site/Myr (16). The most common type of mutation is the single base substitution. The rate of substitution in HV1 was approximately twice as high as in HV2 and that this difference was mainly due to a higher frequency of pyrimidine transition in HV1 (17). The high mutation rate in the mitochondrial genome is probably caused by the low fidelity of mtDNA polymerase, the lack of mtDNA repair mechanisms and to the move frequent exposure of mtDNA to reactive oxygen metabolites (18). The significance of each mtDNA mutation to human variability and disease depends on where in the genome and when in the human life cycle the mutation occurs. The mtDNA mutation can be either neutral or deleterious. Neutral and mildly deleterious mutations have accumulated along human female lineages that are enhancing human diversity while the moderate to severely deleterious mutation cause degenerative disease (19).

2.2 High copy number

Mitochondrial DNA is present in high copy number, with hundreds to thousands mtDNA molecules in each cell (20). The human oocyte contains ~100,000 mitochondrial genomes (21). Which each somatic cell has just two copies of nuclear DNA. In cases samples where there are minute amounts of DNA or highly degraded, the mtDNA typing is a greater likelihood of success than the nuclear DNA typing since the higher copy number of mtDNA. This property makes mtDNA suitable for analyzing ancient DNA and forensic DNA application.

2.3 Maternal inheritance

Human mitochondrial DNA is maternally inherited without recombination (22). At fertilization, only the nucleus of sperm enters the egg cell and joins directly with egg's nucleus. Although the sperm mitochondria may enter a fertilized egg but

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they are destroyed by selective elimination that the occurrence continues throughout embryogenesis (23). Therefore, the mitochondrial DNA of mother are transmitted to her children and from her daughters to the next generation cause the mtDNA sequence of sibling and all maternal relatives are identical. This characteristic can be useful in analyzing the remains of a missing person or mass disaster investigation by comparison the mtDNA sequence with known maternal relatives. For example, identification of remains from Vietnam War by a comparison of mitochondrial DNA sequence from bone with the maternal reference from oral swabs of family members (24) and to confirm the remains of Tsar Nicholas II (25).

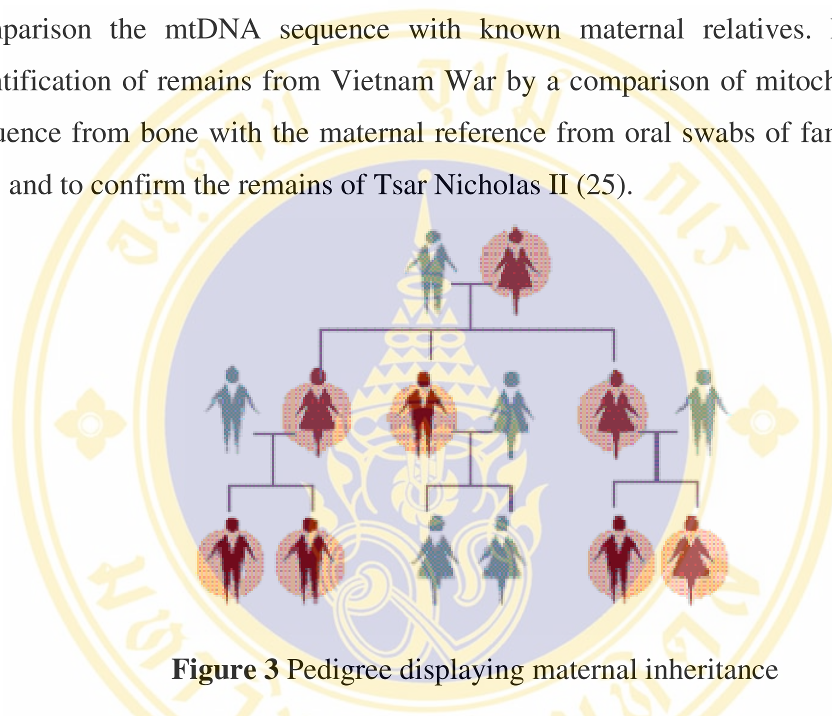

Figure 3 Pedigree displaying maternal inheritance

2.4 Lack of recombination

The mitochondrial genome is uniparental inheritance and does not undergo recombination. However, there is report for recombination in mitochondrial DNA (26). Hagelberg et al. detected an extremely rare point mutation at high frequency in the small island of Nguna in the Melanesian archipelago of Vanuatu. The multiple occurrence of a rare mutation event in one isolated locality is highly improbable that could be explained by the occurrence of recombination. Besides Awadalla et al. studies the relation between linkage disequilibrium (LD) and distance between sites (27) that provided evidence for recombination. In contrast, the complete sequence study by measure of linkage disequilibrium found no evidence for mtDNA recombination (12,28). Therefore, there is no evidence sufficiently reason to overturn the standard paradigm which the human mitochondrial genome is maternally inherited and lack of recombination.

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2.5 Heteroplasmy

Heteroplasmy defines the state where more than one mtDNA type within an individual. Heteroplasmy usually differ at only one base. It manifests in different ways: a) an individual may show more than one mtDNA type in a single tissue, b) an individual may be heteroplasmic in one tissue sample and homoplasmic in another tissue sample, and c) an individual may exhibit one mtDNA type in one tissue and a different type in another tissue (29). Besides heteroplasmy divide point and length heteroplasmy. Calloway et al. found that the frequency of heteroplasmy in human mtDNA differed across tissue types and appeared to be more frequent in muscle (30). The occurrence of heteroplasmy associated with mitochondrial disease also (31).

3. Mitochondrial DNA Application

3.1 Human evolution

The mitochondrial DNA has been used in study of the human evolution that was evidence shown the modern humans originated in Africa about 200,000 years ago and subsequently spread to the rest of the world (32). The Africa origin of human was also supported from sequence analysis in two hypervariable segments of mtDNA. Analysis of sequences from 189 individuals confirmed that the ancestor existed about 166,000 to 249,000 years ago in Africa (33). Besides analysis Neanderthal remains in fragment of HV1 shown phylogenetically distinct from modern humans, suggesting that their mtDNA types have not contributed to the modern human mtDNA pool. The divergence of modern human and Neanderthal mtDNA was estimated to be between 365,000 and 853,000 years. The age estimated of the earliest modern human divergences to be between 106,000 and 246,000 years before the present (34). Haak et al. analyze mtDNA from early European farmers that found Europeans today have a 150-times lower frequency of this mtDNA type. The finding lends weight to a proposed Paleolithic ancestry for modern Europeans (35).

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3.2 Population history

The mitochondrial DNA was used in study of human populations for understanding of population correlations, of population migrations, or population origins. For examples, the mtDNA analysis indicates that diversity among the negroids was much larger than that among the caucasoids or the mongoloids (36). Analysis of Southeast Asian mtDNA variation found that all extant populations were derived from a common ancestral population (37). Polynesian genetic affinities to populations of Asia were studied using mtDNA markers that mtDNA types related to the Polynesian motif are highest in frequency in the corridor from Taiwan south through the Philippines and east Indonesia, and the highest diversity for these types was in Taiwan (38). Besides the mtDNA analyzing shown that although East Asian haplotype are regionally specific, they all derive ultimately from one or two ancestral lineages of African origin (39).

3.3 Forensic science

The identification in forensic application, mtDNA testing consists primarily of sequence analysis of hypervariable region I (HV1) and hypervariable region II (HV2) that is routine method. The mtDNA typing can successfully applied even when samples were limited amounts or severely degraded such as teeth, old bones and hair shafts (40,41). Mitochondrial DNA polymorphism has been widely used for individual identification and maternity testing. Sequences from control region are highly variable within human populations. There have been various reports on hypervariable regions, including Koreans, Japanese, Indian, and German (42-45). Besides the mtDNA typing has been used for species identification of the conservation animals such as tigers, leopard cats, clouded leopards, and lion. The partial sequence of cytochrome *b* gene was adopted as a marker for animal identification (46). Matsuda et al. developed a PCR-based method using newly designed primers to amplify portion of the mitochondrial cytochrome *b* region for identify DNA as being of human origin (47). The main limitation of mtDNA analysis is relatively low power of discrimination associated with HV1 and HV2. The investigation of nucleotide sequences within the other portion obtained interesting that especially coding region.

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3. Extraction of DNA

The DNA extraction methods have been developed to separate proteins and other cellular materials from the DNA molecules. The success of DNA typing relies on the isolation of DNA. The quantity and quality of DNA depends on the typing of biological samples and the extraction technique.

Ideally, extraction protocols should be simple and inexpensive to perform. The DNA extraction procedure varies depending on the type of biological evidence being examined. The standard DNA extraction procedures may entail (i) organic solvents, (ii) salting out methods, or (iii) cation exchange rasins such as Chelex 100.

The organic extraction procedure or phenol-chloroform extraction yields highly purified DNA. The process involves (i) lysis of the red cell with SSC buffer (NaCl, sodium citrate), (ii) centrifugation, (iii) lysis of the pelleted white cells, and (iv) digestion of proteinaceous materials by incubation in a sodium acetate, sodium dodecylsulfate (SDS), and proteinase K solution. DNA released into the solution is extracted with phenol to remove proteinaceous material, and chloroform is present to remove residual phenol. The DNA is precipitated from the aqueous layer by the addition of cold ethanol and salt and by subjecting the solution to centrifugation. Considerable care must be exercised not to disturb the DNA pellet when removing the ethanol. Residual ethanol may be removed under the vacuum in a specially designed centrifuge. The resultant should be slightly moist to ensure resuspension in Tris-EDTA (TE) buffer (48).

Nonorganic extraction methods or salting out method is the procedure which avoids using phenol and chloroform. This method is using high salt concentrations to remove proteins which it is rapid, safe and inexpensive. The DNA obtained from this simple technique yielded quantities comparable to those obtained from the phenol-chloroform extraction (49).

The Chelex method is the use of a chelating resin suspension such as Chelex-100. The biological samples are added to Chelex suspension and boiled for several minutes bring about lysis of cell membrane. This procedure is an advantage for PCR-based typing methods because it removes inhibitors of PCR and uses only a single tube for the DNA extraction that reduces the potential for contamination from laboratory.

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4. Analytical Method

4.1 Polymerase Chain Reaction

The polymerase chain reaction or PCR is a technique for the *in vitro* amplification on of specific DNA sequences which was devised and named by Kary Mullis and colleagues. The process is similar to the mechanism by which DNA duplicates itself normally. PCR has revolutionized in the field of molecular biology with the ability to make million copies of a specific sequence of DNA in a few hours. The recently technique of PCR is developed and used in various fields. In medicine, for example, the PCR has been used in the diagnosis and screening of genetic disease such as hemophilia and thalassemia (50). In the field of forensic science, since the amount of biological materials available as evidence can be extremely small such as single shed hair or saliva traces on cigarette butt. PCR will be useful to amplify minute quantity of DNA into much larger quantity for study.

The PCR cycle is characterized by a three-step process:

1. **Denaturation** Each double-stranded segment is separated into two single strands by heating at temperature 90-95 ^{0}C
2. **Annealing** The temperature is decreased at 50-55 ^{0}C that the specific PCR primers are annealed to their complementary sequences. One primer hybridizes to 3' end of the segment on one strand, and the other hybridizes to the 3' end of the segment on the opposite strand.
3. **Primer extension** The temperature is raised to 70-75 ^{0}C. The thermostable DNA polymerase extends the primer and synthesizes a new strand of DNA along each of the original strands.

The process of denaturation, annealing, and primer extension are repeated many times until the desired quantity of DNA is generated. Theoretically the yield of polymerase chain reaction process is 2^n when n is the number of times the reaction is cycled.

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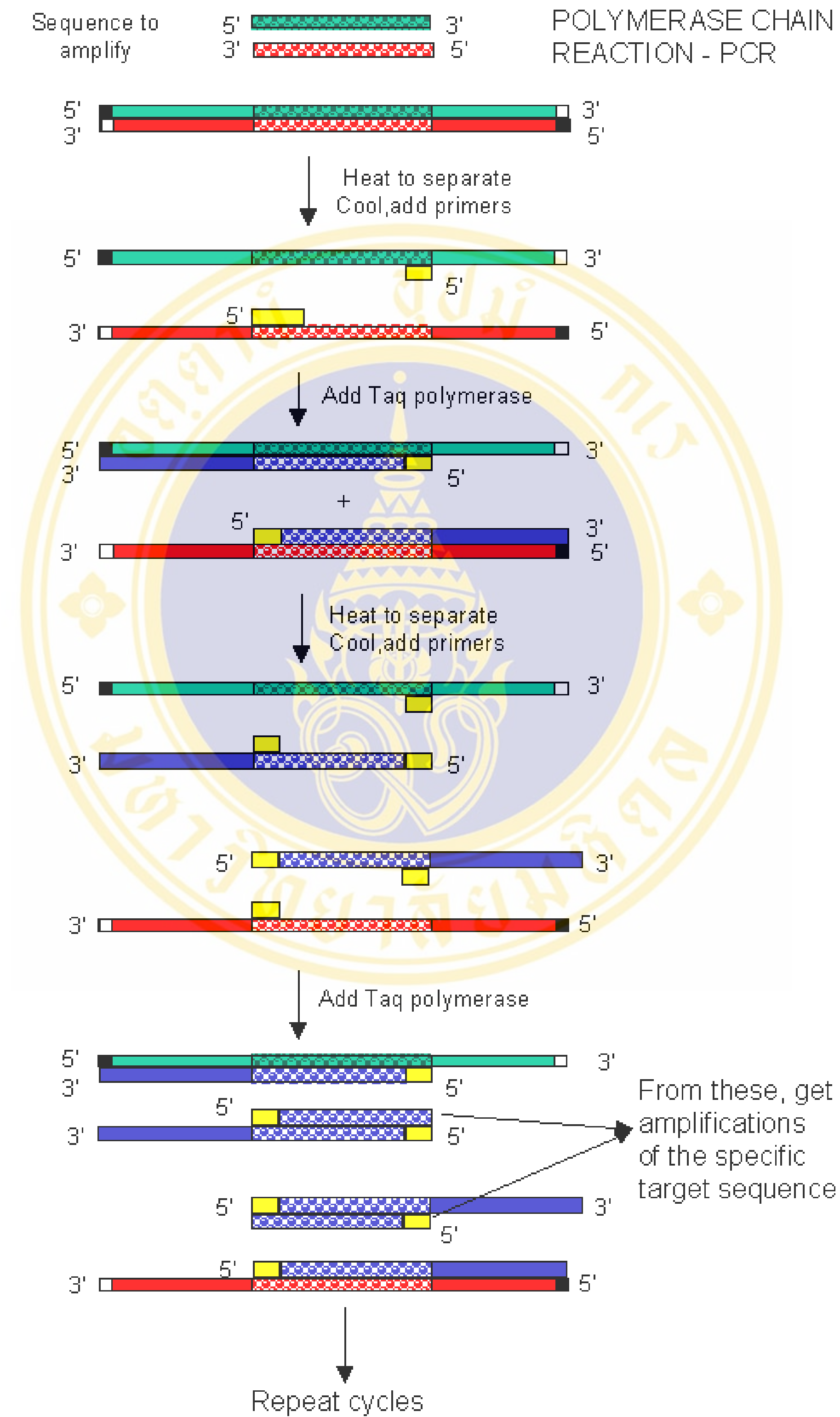

Figure 4 Polymerase Chain Reaction

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PCR Components

1. Thermostable DNA polymerase
2. Deoxynucleotide triphosphate (dNTPs)
3. Primers
4. Reaction buffer
5. Magnesium chloride ($MgCl_2$)
6. DNA template
7. Deionized water

4.2 DNA Sequencing

After the PCR cycles have been completed, the amplified DNA can be analyzed by several methods to reveal genetic variation at defined sites. The most popular DNA sequencing method is Sanger method or dideoxy sequencing (51). This technique use single-stranded DNA template for making a new complementary DNA strand *in vitro* using a suitable DNA polymerase. The components required to perform cycle sequencing are thermostable DNA polymerase, sequencing primer, buffer deoxynucleotide triphosphates (dATP, dTTP, dCTP, and dGTP), DNA template, and dideoxynucleotide triphosphates (ddNTPs) of all four bases. The denaturation step which many copies of double-stranded DNA template is split into single strands. The sequencing primer is allowed to bind with single-stranded DNA molecules. The primer is extended by action of the thermostable DNA polymerase. The concentration of the ddNTPs to be very much lower than that of its normal dNTP analog, these will be competition between a specific ddNTP molecule and an excess of analogous dNTP molecules for inclusion in the growing DNA chain. The ddNTP lack a 3' hydroxyl group that is necessary for phosphodiester bond formation. Thus, chain elongation is terminated when a dideoxynucleotide is incorporated into the growing chain by complementary base pairing to the template. The fragments of different lengths are generated corresponding to the specific sites where a particular dideoxynucleotide is inserted. In former times DNA sequencing reactions used radioisotope labeling such as ^{35}S or ^{33}P and after expositing the dried sequencing gel to an X-ray film, the sequence was read manually by following successive bands on the autoradiograph. This manual sequencing is time-consuming and labor-intensive, particularly for to interpret data.

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This cumbersome approach has been overtaken by the automated DNA sequencing methods.

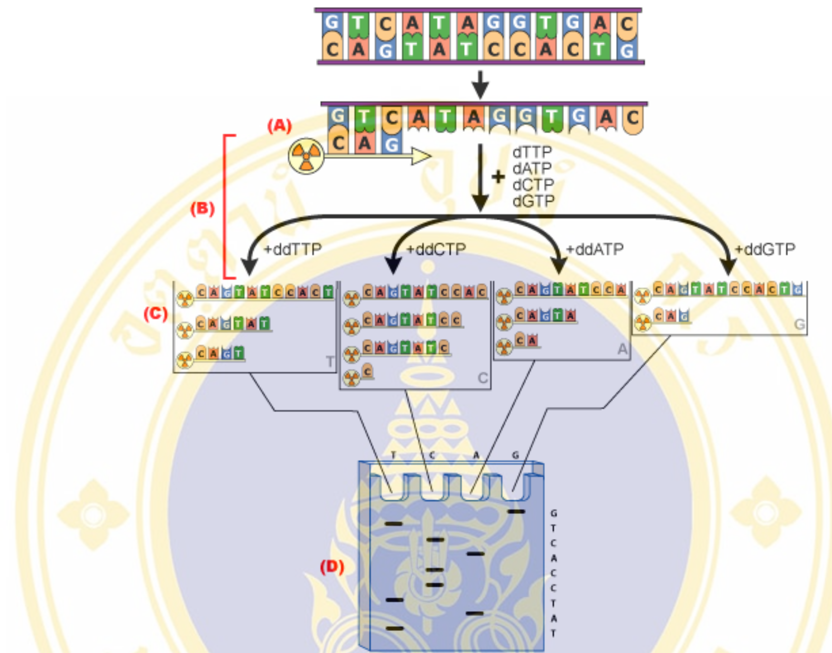

Figure 5 DNA Sequencing

Automated DNA sequencing

The automated DNA sequencer is fluorescent-based system that the extended fragments are labeled by incorporation of a primer or ddNTPs which carries a fluorophore. The use of different fluorophores in the four base-specific reactions means that, unlike conventional DNA sequencing, all four reactions can be loaded into a single lane. During electrophoresis, a monitor detects and records the fluorescence signal as the DNA passes through a fixed point in the gel. This allows an output in the form of intensity profiles for each of the differently colored fluorophores while simultaneously storing the information electronically. Recently, the high throughout DNA sequencing often uses capillary sequences where DNA samples migrate through very thin long glass capillary tubes.

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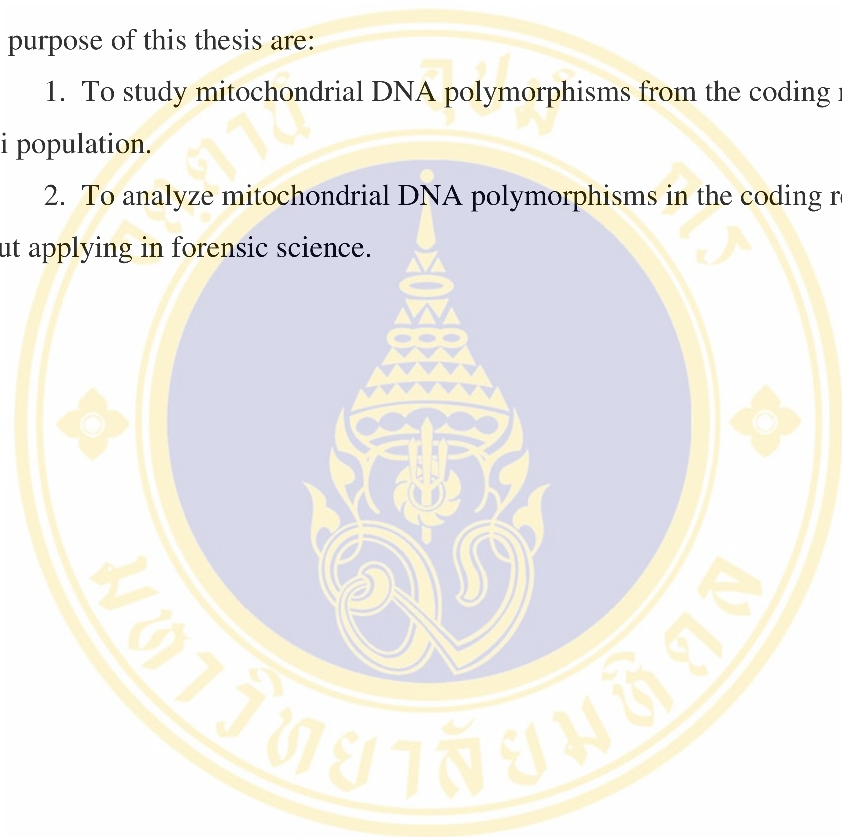
CHAPTER III
OBJECTIVES

The purpose of this thesis are:

1. To study mitochondrial DNA polymorphisms from the coding region in Thai population.
2. To analyze mitochondrial DNA polymorphisms in the coding region bring about applying in forensic science.



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CHAPTER IV
MATERIALS AND METHODS

1. Materials

1.1 Samples

The eighty blood samples of healthy unrelated Thai population from testing in serology unit, Department of Forensic Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University.

1.2 Equipment

1. Automated DNA sequencer (ABI 3130)
2. GeneAmp PCR System 9600, Applied Biosystems, USA
3. Heated stirring plate, IKAMAG® REC-G
4. High performance ultraviolet transilluminator, USA
5. Horizontal gel apparatus, Life Technologies, USA
6. Microcentrifuge, UniCen 15DR, Germany
7. Micropipettor, Gilson, France
8. QIAquick PCR purification kit, Qiagen, Germany
9. Sterilizer-autoclave, Sanyo, Japan
10. Vortex mixer, Scientific Industries, USA
11. Water bath, Life Science, USA

1.3 Miscellaneous

1. Beakers
2. Cylinders
3. Disposable gloves
4. EDTA vacutainer tubes
5. MicroAmp reaction tubes
6. Microcentrifuge tubes
7. Microcentrifuge tube rack

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8. Pipet tips
9. Stirring rod, Magnetic stirrer

1.4 Reagents

1.4.1 Reagents for DNA extraction

1. Absolute alcohol
2. 70% EtOH
3. Phenol /Chloroform /Isoamyl Alcohol, 25:24:1 (PCIA)
4. Proteinase K (20 mg/ml)
5. 0.2 M sodium acetate
6. 2.0 M sodium acetate
7. 10% SDS (Sodium Dodecyl Sulfate)
8. 1X SSC
9. TE (Tris-EDTA) buffer

1.4.2 Reagents for agarose gel electrophoresis

1. Agarose powder
2. 0.5X TBE buffer
3. 1X TBE buffer
4. Ethidium bromide (EtBr)
5. Gel loading dye

1.4.3 Reagents for polymerase chain reaction (PCR)

1. Deionized water
2. Deoxynucleotide triphosphates mixture (dNTPs)
3. 25 mM $MgCl_2$ solution
4. 10X PCR buffer (containing 15 mM $MgCl_2$)
5. Primers (25 pmol)
6. *Taq* DNA polymerase (5 Units/µl) from Qiagen, Germany

1.4.4 Reagents for DNA sequencing

1. BigDye® Terminator 5X Sequencing Buffer
2. Deionized water
3. 125 mM EDTA
4. 70% Ethanol
5. 100% Ethanol

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6. Single sequencing primer (3.2 ρmol)
7. Terminator Ready Reaction Mix:
   - A-Dye Terminator labeled with dichloro[R6G]
   - C-Dye Terminator labeled with dichloro[ROX]
   - G-Dye Terminator labeled with dichloro[R110]
   - T-Dye Terminator labeled with dichloro[TAMRA]
   - AmpliTaq Polymerase
   - Deoxynucleoside triphosphate
   - $MgCl_2$
   - Tris-HCl buffer, pH 9.0

2. Methods

2.1 Sample Collection

A liquid blood will be collected by venipuncture in EDTA vacutainer tube. The blood samples are stored at temperature 4 °C.

2.2 DNA Extraction

The DNA was extracted from the blood samples using the PCIA method (48).

1. Add 800 µl 1X SSC to 700 µl of liquid blood, mix and centrifuge at 14,000 rpm for 1 minute in a microcentrifuge.
2. Remove 1.0 ml of supernatant and discard the supernatant into a disinfectant solution.
3. Add 1.0 ml 1X SSC to the tube, vortex-mix, and centrifuge for 1 minute. Remove and discard as much of the supernatant as possible. Do not disturb the pellet.
4. To the pellet, add 375 µl of 0.2 M sodium acetate, 25 µl 10% SDS, and 5 µl proteinase K (20 mg/ml). Vortex-mix the contents briefly and incubate at 56 °C for 1 hour.
5. Add 120 µl PCIA to the tube and vortex-mix for 30 second. Perform this step in the fume hood.
6. Centrifuge for 2 minute.

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7. The aqueous layer (i.e., top layer), which contains DNA, is carefully removed and placed in a new 1.5-ml Eppendorf tube. Try not to remove the layer of denatured protein that collects at the interface of the aqueous and organic layers. Discard the original tube and PCIA in an appropriate waste container housed in the fume hood.
8. Add 1.0 ml cold, absolute EtOH to the aqueous layer. Mix by inverting the tube a couple of times. Place the tube at -20 °C for 15 minute. And centrifuge for 2 minute.
9. Decant and discard the supernatant. Remove any additional alcohol using a micropipettor. Be careful to avoid drawing any of the pellets up into the pipet tip.
10. Add 180 µl TE buffer to the pellet and vortex-mix briefly.
11. Incubate the contents at 56°C for 10 minute.
12. Add 20 µl 2.0 M sodium acetate and mix by hand for 5 second.
13. Add 500 µl cold, absolute EtOH and gently mix by hand until the solution is homogeneous.
14. Centrifuge for 1 minute.
15. Decant and discard the supernatant.
16. Wash the pellet with 1.0 ml ambient temperature 70% EtOH.
17. Centrifuge for 1 minute, decant and discard the supernatant. Remove as much EtOH as possible using a micropipette.
18. Resuspend the DNA by adding 200 µl TE buffer. Mix and incubate the contents at 56 °C overnight.
19. The next day, vortex-mix the tube for 10 to 30 second.
20. The DNA is ready for quantification.

2.3 DNA Quantification

The DNA was quantified by using ethidium bromide-stained agarose gel electrophoresis that compared with GeneRuler™ 100 bp DNA Ladder (Fermentas, Life Science)

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Agarose gel electrophoresis:

1. Prepare 1% agarose (obtained from 0.3 g of agarose powder and 30 ml of 1X TBE buffer with ethidium bromide.
2. Bring the solution to a boil for to dissolve agarose.
3. The agarose solution to equilibrate at 56 °C.
4. Pour agarose into horizontal gel form and insert the well-forming comb.
5. When the gel is ready setting (approximately 20 min), and pour 0.5X TBE buffer into electrophoresis tank.
6. Remove the comb and prepare the samples.
7. Pipet the sample 5 µl mixed with loading solution 1 µl, after that load into the well of agarose gel.
8. Set the voltage of power-source.
9. Run the gel and stop when obtained suitable length.
10. Romove the agarose gel from the tank.
11. Examine the gel on a UV light transilluminator and take the photograph of the agarose gel.
12. Evaluate the quantity of DNA by comparison with the DNA standard.

2.4 PCR Amplification

The mitochondrial DNA was amplified using the primer pairs:

HV1	L15997	5'-CAC CAT TAG CAC CCA AAG CT-3'
	H16401	5'-TGA TTT CAC GGA GGA TGG TG-3'
HV2	L 29	5'-GGT CTA TCA CCC TAT TAA CCA C-3'
	H 408	5'-CTG TTA AAA GTG CAT ACC GCC A-3'

PCR conditions

Initial denaturation at 94 °C	4.0 minutes
For 32 cycles	
Denaturation at 94 °C	45 seconds
Annealing at 55°C	30 seconds
Extension at 72 °C	3.0 minutes
Final extension 72 °C	7.0 minutes

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Coding Region: (position 8320-9160)

Forward Primer: 5'-CCCCTCTAGAGCCCACTGTAAAGC-3'

Reverse Primer**:** 5'-TTATGTGTTGTCGTGCAGGT-3'

PCR amplification was performed in a 50 µl of reaction mixture containing 0.5 µl of DNA template, 25 pmol of forward and reverse primers, 5 µl of 10× PCR buffer, 1.5 µl of 25mM $MgCl_2$, 4 µl of dNTP, and 1 U *Taq* DNA polymerase.

The PCR reaction was carried out in a DNA thermal cycler that the conditions consist of initial denaturation step at 95 ˚C for 10 min, followed by 30 cycles each of denaturation at 94 ˚C for 1 min, annealing at 56 ˚C for 1 min, and extension at 72 ˚C for 1 min. The final extension condition was 72 ˚C for 10 min.

2.5 Post-PCR Quantification

The PCR products were determined by 1% agarose gel stained with ethidium bromide. The GeneRuler^{TM} 100 bp DNA Ladder (Fermentas, Life Science) was also for PCR product size estimation and quantification. These yield gel were imaged and analyzed with GeneSnap/GeneTools from SynGene.

2.6 Purification

PCR products of HV1, HV2, and coding region were purified by using the QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions.

Procedure

1. Add 5 volumes of Buffer PB to 1 volume of the PCR reaction and mix.
2. Place a QIAquick spin column in a provided 2-ml collection tube.
3. To bind DNA, apply the sample to the QIAquick column and centrifuge at 14,000 rpm for 1 minute.
4. Discard flow-through. Place the QIAquick column back into the same tube.
5. To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge at 14,000 rpm for 1 minute.
6. Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 minute at maximum speed.
7. Place QIAquick column in a clean 1.5-ml microcentrifuge tube.
8. To elute DNA, add 20 µl Buffer EB (10 mM Tris-Cl, pH 8.5) to the center of the QIAquick membrane. Let the column stand for 5 min and centrifuge the column for 1 minute.

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2.7 DNA Sequencing

The purified PCR products were sequenced by using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The sequenced products were ethanol/EDTA precipitated.

Reagents

Terminator Ready Reaction Mix	4 µl
5X Sequencing Buffer	2 µl
Primer (3.2 pmol)	1 µl
Template	1 µl
Deionized water	12 µl
Total Volume	20 µl

The sequencing condition

Initial denaturation at 96 °C	1 minute
Repeat the following for 25 cycles:	
Denaturation at 96 °C	10 seconds
Annealing at 50 °C	5 seconds
Extension at 60 °C	4 minutes
Final extension 72 °C	7 minutes

Ethanol/EDTA Precipitation

1. Take product from PCR tubes to 1.5 ml Eppendorf tubes, and add 5 µl of 125 mM EDTA to each tube.
2. Add 60 µl of 100% ethanol
3. Close the lid and mix by inverting 4 times
4. Incubate at room temperature for 15 minute.
5. Centrifuge at 4°C, 3000 x g for 30 minute.
6. Take the reaction off from the tubes as well as you can.
7. Add 60 µl of 70 % ethanol to each tube.
8. Centrifuge set to 4°C, spin at 1650 x g for 15 minute.

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9. Then remove the content from the tubes.
10. Dry the content off by thermal plate or another.

2.8 Sequencing Analysis

The nucleotide sequences were analyzed by an ABI 3130 Genetic Analyzer using POP-4 polymer. The sequences were aligned and compared with the Cambridge reference sequence (or Anderson sequence) by SeqScape software version 2.5.

2.9 Statistical Analysis

Random match probability:

$$P = \sum (Xi^2)$$

Discrimination power:

$$Dp = 1-\sum (Xi^2)$$

The genetic diversity is calculated according to the formula (52):

$$h = \frac{\left(1-\sum x^2\right)n}{(n-1)}$$

where n is the sample size

x is the frequency of each mtDNA type

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CHAPTER V
RESULTS

1. The Quantity of DNA Extraction

Figure 6 The quantity of DNA extraction

The band of DNA was extracted from blood samples with using PCIA method that indicated in figure 6. The quantity of DNA was showed:

	Base pairs	Quantity (ng)
Track 1	3905	176
Track 2	3905	163
Track 3	3940	271

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Track 4	**(Ladder)**	
1	1031	169
2	900	147
3	800	131
4	700	115
5	600	98
6	500	164
7	400	65
8	300	49
9	200	33
10	100	16
Track 5	3905	321
Track 6	3940	322
Track 7	4049	426

2. The Result of PCR Amplification

Figure 7 Amplification products of coding region

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The amplified products 5 µl were electrophored on 1% agarose gel and stained with ethidium bromide. The size and quantity of PCR products were indicated:

	Base pairs	Quantity (ng)
Track 1	830	82
Track 2	820	153
Track 3	835	116
Track 4	**(Ladder)**	
1	1031	169
2	900	147
3	800	131
4	700	115
5	600	98
6	500	164
7	400	65
8	300	49
9	200	33
10	100	16
Track 5	862	132
Track 6	872	76
Track 7	867	89
Track 8	Negative Control	

3. Analysis of mtDNA Sequences

The results that the hypervariable region I (positions 16024-16365), hypervariable region II (position 73-340), and the coding region (position 8320-9160) of 80 unrelated Thais were compared the different of the sequence with the Cambridge reference sequence (or Anderson sequence).

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3.1 The sequences of the hypervariable region I

Table 1 Sequence polymorphism in the hypervariable region I of 80 unrelated Thais

SAMPLE	Hypervariable region I
1	16214A 16223T 16256T 16278T
2	16183C 16193.1+C 16212G 16232A 16249C 16304C 16362C
3	16108T 16129A 16162G 16172C 16189C 16304C
4	16124C 16148T 16223T
5	16172C 16223T 16260T 16264T 16271C
6	16140C 16182C 16183C 16189C 16261T 16266A
7	16111T 16145A 16192T 16223T 16291T 16304C
8	16108T 16129A 16162G 16172C 16241T 16256T 16304C
9	16304C 16362C
10	16129A 16192T 16223T 16297C
11	16124C 16148T 16223T
12	16223T 16331C 16355T 16362C
13	16145A 16185T 16239T 16311C 16325C
14	16031-A 16059-A 16129A 16172C 16304C
15	16093C 16189C 16260T 16298C
16	16108T 16129A 16161G 16172C 16304C 16334G
17	16223T 16259T 16278T 16291T 16362C
18	16183C 16188.1+C 16189C 16300G 16304C
19	16059-A 16184T 16223T 16274A 16278T
20	16042A 16058-A 16129A 16172C 16304C
21	16108T 16129A 16162G 16172C 16309C
22	16042A 16223T 16298C 16327T
23	16129A 16223T 16270T 16362C
24	16051G 16206C 16271C 16328T
25	16183C 16189C 16223T 16357C 16362C
26	16094C 16111T 16223T 16290T 16319A 16320T 16362C
27	16223T 16257A 16261T 16292T
28	16140C 16182C 16183C 16189C 16261T 16266A 16304C
29	16183C 16189C 16223T 16298C 16327T
30	16129A 16172C 16304C
31	16223T 16257A 16261T 16292T
32	16140C 16183C 16189C
33	16093C 16147T 16249C 16288C 16325C

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Table 1 Sequence polymorphism in the hypervariable region I of 80 unrelated Thais (Continued)

SAMPLE	Hypervariable region I
34	16140C 16183C 16189C 16266A
35	16108T 16129A 16172C 16223T 16234T 16290T
36	16181G
37	16217C 16223T 16362C
38	16129A 16169T 16172C 16304C
39	16086C 16172C 16223T 16257A 16261T 16295T
40	16145A 16192T 16223T 16291T 16294T 16304C
41	16223T 16224C 16266T 16290T 16311C
42	16124C 18183C 16189C 16278T 16362C
43	16298C 16327T
44	16129A 16223T 16297C 16298-T
45	16185T 16223T 16260T 16298C 16302G
46	16185T 16189C 16223T 16232A 16319A 16362C
47	16223T 16311C 16362C
48	16223T 16297C
49	16051C 16070C 16113C 16189C 16270T 16304C
50	16223T 16295T 16311C
51	16147T 16183C 16184A 16189C 16217C 16234T
52	16129A 16182C 16183C 16189C
53	16108T 16129A 16162G 16172C 16304C
54	16189C 16223T 16256T
55	16129A 16223T 16248T 16297C
56	16193T 16223T
57	16136C 16223T 16312G
58	16172C 16223T 16257A 16261T 16316G
59	16223T 16286T
60	16093C 16129A 16209C 16235C
61	16181G
62	16157C 16278T 19304C
63	16140C 16183C 16189C 16266A 16267T 16293G
64	16140C 16183C 16189C 16218T 16266A
65	16223T 16298C 16327T 16357C
66	16108T 16129A 16172C 16223T 16234T 16287T 16290T
67	16136C 16182C 16183C 16189C 16217C
68	16244C 16257T 16259T 16288C 16304C
69	16108T 16129A 16162G 16172C 16256T 16304C
70	16129A 16172C 16304C

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Table 1 Sequence polymorphism in the hypervariable region I of 80 unrelated Thais (Continued)

SAMPLE	Hypervariable region I
71	16181C 16182C 16183C 16189C 16213A 16217C 16261T 16292T 16311C 16320T
72	16129A 16162G 16168T 16172C 16304C 16399G
73	16129A 16187T 16189C 16223T 16297C
74	16223T 16266T 16311C 16325C 16362C
75	16183C 16189C 16193.1+C 16223T 16266T 16291T 16311C
76	16086C 16129A 16209C 16223T 16263C 16272G
77	16209C 16223T 16233G 16274A 16304C 16311C
78	16223T 16235G 16290T 16311C 16319A 16362C
79	16223T 16278T
80	16129A 16162G 16172C 16304C

3.2 The sequences of the hypervariable region II

Table 2 Sequence polymorphism in the hypervariable region II of 80 unrelated Thais

SAMPLE	Hypervariable region II
1	73G 152C 229G 263G 309.1+C 315.1+C
2	73G 152C 229G 249-A 263G 309.1+C 315.1+C
3	73G 150T 195C 229G 249-A 263G 315.1+C
4	73G 195C 229G 263G 309.1+C 315.1+C
5	73G 151T 229G 263G 315.1+C
6	73G 152C 210G 229G 263G 309.2+2C 315.1+C
7	73G 210G 229G 263G 315.1+C
8	73G 229G 249-A 263G 293C 309.1+C 315.1+C
9	73G 229G 263G 315.1+C
10	73G 150T 199C 204C 229G 263G 309.1+C 315.1+C
11	73G 195C 229G 263G 309.2+2C 315.1+C
12	73G 152C 195C 229G 263G 315.1+C
13	73G 152C 229G 163G 309.2+2C 315.1+C
14	73G 229G 249-A 263G 315.1+C
15	73G 207A 229G 259-A 263G 309.1+C 315.1+C
16	73G 229G 249-A 263G 309.1+C 315.1+C
17	73G 228A 229G 263G 271272273274-ATTT 309.1+C 315.1+C
18	73G 146C 150T 195C 198T 229G 263G 309.1+C 315.1+C
19	73G 146C 152C 229G 263G 309.1+C 315.1+C
20	73G 204C 229G 249-A 263G 315.1+C
21	73G 229G 249-A 263G 309.1+C 315.1+C
22	73G 146C 229G 249-A 263G 315.1+C
23	73G 146C 152C 229G 263G 309.1+C 315.1+C
24	73G 194T 229G 263G 315.1+C
25	73G 150T 229G 263G 309.1+C 315.1+C
26	73G 143A 152C 204C 207A 229G 235G 309.1+C 315.1+C
27	73G 149.1+T 229G 263G 309.1+C 315.1+C
28	73G 152C 210G 229G 263G 309.2+2C 315.1+C 324G

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Table 2 Sequence polymorphism in the hypervariable region II of 80 unrelated Thais (Continued)

SAMPLE	Hypervariable region II
29	73G 229G 249-A 263G 309.1+C 315.1+C
30	73G 229G 249-A 263G 315.1+C
31	73G 150T 229G 263G 309.1+C 315.1+C
32	73G 152C 210G 229G 263G 309.1+C 315.1+C
33	73G 150T 229G 263G 315.1+C 329A
34	73G 210G 229G 263G 309.1+C 315.1+C
35	73G 185A 189G 229G 263G 309.1+C 315.1+C
36	73G 146A 199C 229G 309.2+2C 315.1+C
37	73G 229G 263G 309.1+C 315.1+C
38	73G 229G 249-A 263G 315.1+C
39	73G 150T 229G 263G 309.1+C 315.1+C
40	73G 189G 210G 229G 263G 315.1+C
41	73G 125C 127C 128T 146C 152C 229G 263G 309.1+C 315.1+C 318C 326G
42	73G 229G 263G 315.1+C
43	73G 229G 249-A 263G 309.1+C 315.1+C
44	73G 150T 199C 204C 229G 263G 309.1+C 315.1+C 332T
45	73G 151T 152C 229G 249-A 263G 309.1+C 315.1+C
46	73G 229G 263G 315.1+C
47	73G 299G 309.1+C 315.1+C
48	73G 150T 189G 199C 204C 229G 263G 315.1+C
49	73G 229G 263G 315.1+C
50	73G 146C 152C 199C 229G 263G 315.1+C
51	73G 229G 263G 309.1+C 315.1+C
52	73G 229G 263G 273G 309.1+C 315.1+C
53	73G 229G 249-A 263G 309.1+C 315.1+C
54	73G 229G 239C 263G 309.1+C 315.1+C
55	73G 150T 199C 204C 207A 229G 263G 309.1+C 315.1+C
56	73G 150T 195C 229G 263G 315.1+C
57	73G 229G 263G 315.1+C
58	73G 150T 195C 204C 229G 263G 315.1+C
59	73G 229G 263G 315.1+C
60	73G 146C 153G 229G 263G 309.1+C 315.1+C
61	73G 146A 199C 229G 263G 309.1+C 315.1+C
62	73G 146C 151T 229G 263G 309.2+2C 315.1+C
63	73G 152C 210G 229G 263G 315.1+C
64	73G 210G 229G 263G 315.1+C
65	73G 189G 214G 229G 249-A 263G 309.1+C 315.1+C
66	73G 185A 189G 229G 263G 309.1+C 315.1+C
67	73G 199C 229G 263G 315.1+C
68	73G 152C 229G 263G 315.1+C 329A
69	73G 229G 249-A 263G 293C 309.1+C 315.1+C
70	73G 229G 249-A 263G 309.2+2C 315.1+C

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Table 2 Sequence polymorphism in the hypervariable region II of 80 unrelated Thais (Continued)

SAMPLE	Hypervariable region II
71	73G 229G 263G 309.2+2C 315.1+C
72	73G 229G 249-A 263G 309.1+C 315.1+C
73	73G 150T 199C 204C 229G 263G 309.1+C 315.1+C
74	73G 173C 229G 263G 309.1+C 315.1+C
75	73G 146C 152C 195C 229G 263G 309.1+C 315.1+C
76	73G 152C 229G 249-A 263G 315.1+C 316A
77	73G 143A 182T 229G 263G 309.1+C 315.1+C
78	73G 152C 199C 229G 234G 235G 259G 263G 309.1+C 315.1+C
79	73G 150T 151T 195C 229G 263G 309.1+C 315.1+C
80	73G 229G 249-A 263G 309.1+C 315.1+C

3.3 The sequences of coding region

Table 3 Sequence polymorphism in coding region of 80 unrelated Thais (position 8320-9160)

SAMPLE	CODING REGION
1	8701G 8860G
2	8860G
3	8860G 9053A
4	8701G 8860G 9078C
5	8521G 8701G 8718G 8860G
6	8584A 8860G
7	8509T 8701G 8860G 8865A
8	8860G 9053A
9	8614C 8724G 8860G
10	8701G 8860G
11	8701G 8860G 9078C
12	8701G 8860G
13	8464T 8743A 8784G 8860G 8887G 9116C
14	8860G 9053A
15	8860G
16	8701G 8860G 9103C
17	8701G 8860G
18	8730G 8860G
19	8701G 8860G
20	8860G 9053A
21	8860G 9053A
22	8584A 8701G 8860G
23	8414T 8473C 8701G 8860G

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Table 3 Sequence polymorphism in coding region of 80 unrelated Thais (Continued)

SAMPLE	CODING REGION
24	8860G 9053A
25	8701G 8860G
26	8459G 8794T 8860G 9115G
27	8860G
28	8584A 8860G
29	8584A 8701G 8860G
30	8860G 9053A
31	8860G
32	8584A 8860G
33	8860G
34	8584A 8860G
35	8584A 8829T 8860G
36	8701G 8860G
37	8701G 8860G
38	8860G 9053A
39	8860G
40	8509T 8701G 8860G 8865A
41	8701G 8860G
42	8701G 8860G
43	8584A 8701G 8860G 9040T
44	8701G 8860G
45	8584A 8701G 8860G 9090C
46	8414T 8701G 8860G
47	8701G 8860G
48	8701G 8860G
49	8724G 8860G
50	8701G 8860G
51	8701G 8860G 9078C
52	8860G
53	8860G 9053A
54	8701G 8860G
55	8701G 8860G
56	8701G 8860G
57	8562T 8701G 8860G
58	8860G
59	8414T 8701G 8860G
60	8701G 8860G

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Table 3 Sequence polymorphism in coding region of 80 unrelated Thais (Continued)

SAMPLE	CODING REGION
61	8701G 8860G
62	8860G
63	8584A 8860G
64	8584A 8860G
65	8584A 8701G 8860G
66	8701G 8860G
67	8860G
68	8860G
69	8860G 9053A
70	8860G 9053A
71	8860G
72	8860G 9053A
73	8701G 8860G
74	8519A 8555C 8701G 8715C 8860G
75	8701G 8860G
76	8701G 8853G 8860G 9127G
77	8701G 8860G
78	8794T 8860G 9126C
79	8701G 8860G
80	8860G 9053A

The sequence polymorphisms have been found 33 sites. The most common variable sites besides A8860G were at the position 8701, where 42 out of 80 individuals (52.5%) had nucleotide transition from A to G. The second most common variable site showed G to A change at position 9053 in 13 individuals (16.25%). The third was at position 8584 in 12 individuals (15%) where had G to A transition. The nucleotide sequences were compared with Cambridge Reference Sequence (CRS) that indicated in table 4.

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Table 4 List of variable sites of coding region between positions 8320-9160

	8414	8459	8464	8473	8509	8519	8521	8555	8562	8584	8614	8701	8715	8718	8724	8730	8743	8784	8794	8829	8853	8860	8865	8887	9040	9053	9078	9090	9103	9115	9116	9126	9127
CRS	C	A	C	T	C	G	A	T	C	G	T	A	T	A	A	A	G	A	C	C	A	A	G	A	C	G	T	T	T	A	T	T	A
1												G										G											
2																						G											
3																						G				A							
4												G										G					C						
5							G					G		G								G											
6										A												G											
7					T							G										G	A										
8																						G				A							
9											C				G							G											
10												G										G											
11												G										G					C						
12												G										G											
13			T														A	G				G		G							C		
14																						G				A							
15																						G											
16												G										G							C				
17												G										G											
18																G						G											
19												G										G											
20																						G				A							
21																						G				A							
22										A		G										G											
23	T			C								G										G											
24																						G				A							
25												G										G											

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Table 4 List of variable sites of coding region between positions 8320-9160 (Continued)

	8414	8459	8464	8473	8509	8519	8521	8555	8562	8584	8614	8701	8715	8718	8724	8730	8743	8784	8794	8829	8853	8860	8865	8887	9040	9053	9078	9090	9103	9115	9116	9126	9127
CRS	C	A	C	T	C	G	A	T	C	G	T	A	T	A	A	A	G	A	C	C	A	A	G	A	C	G	T	T	T	A	T	T	A
26		G																	T			G								G			
27																						G											
28										A												G											
29										A		G										G											
30																						G				A							
31																						G											
32										A												G											
33																						G											
34										A												G											
35										A										T		G											
36												G										G											
37												G										G											
38																						G				A							
39																						G											
40					T							G										G	A										
41												G										G											
42												G										G											
43										A		G										G			T								
44												G										G											
45										A		G										G						C					
46	T											G										G											
47												G										G											
48												G										G											
49															G							G											
50												G										G											

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Table 4 List of variable sites of coding region between positions 8320-9160 (Continued)

	8414	8459	8464	8473	8509	8519	8521	8555	8562	8584	8614	8701	8715	8718	8724	8730	8743	8784	8794	8829	8853	8860	8865	8887	9040	9053	9078	9090	9103	9115	9116	9126	9127
CRS	C	A	C	T	C	G	A	T	C	G	T	A	T	A	A	A	G	A	C	C	A	A	G	A	C	G	T	T	T	A	T	T	A
51												G										G					C						
52																						G											
53																						G				A							
54												G										G											
55												G										G											
56												G										G											
57									T			G										G											
58																						G											
59	T											G										G											
60												G										G											
61												G										G											
62																						G											
63										A												G											
64										A												G											
65										A		G										G											
66												G										G											
67																						G											
68																						G											
69																						G				A							
70																						G				A							
71																						G											
72																						G				A							
73												G										G											
74						A		C				G	C									G											
75												G										G											

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Table 4 List of variable sites of coding region between positions 8320-9160 (Continued)

	8414	8459	8464	8473	8509	8519	8521	8555	8562	8584	8614	8701	8715	8718	8724	8730	8743	8784	8794	8829	8853	8860	8865	8887	9040	9053	9078	9090	9103	9115	9116	9126	9127
CRS	C	A	C	T	C	G	A	T	C	G	T	A	T	A	A	A	G	A	C	C	A	A	G	A	C	G	T	T	T	A	T	T	A
76												G									G	G											G
77												G										G											
78																			T			G										C	
79												G										G											
80																						G				A							
	3	1	1	1	2	1	1	1	1	12	1	42	1	1	2	1	1	1	2	1	1	80	2	1	1	13	3	1	1	1	1	1	1

In comparison to the Cambridge Reference Sequence (CRS)

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3.4 The haplotype in the mitochondrial DNA

Table 5 The haplotypes from coding region

Haplotype	Coding Region	Frequency	Xi	Xi²
1	8414T 8473C 8701G 8860G	1	0.0125	0.000156
2	8414T 8701G 8860G	2	0.025	0.000625
3	8459G 8794T 8860G 9115G	1	0.0125	0.000156
4	8464T 8743A 8784G 8860G 8887G 9116C	1	0.0125	0.000156
5	8509T 8701G 8860G 8865A	2	0.025	0.000625
6	8519A 8555C 8701G 8715C 8860G	1	0.0125	0.000156
7	8521G 8701G 8718G 8860G	1	0.0125	0.000156
8	8562T 8701G 8860G	1	0.0125	0.000156
9	8584A 8701G 8860G	3	0.0375	0.001406
10	8584A 8701G 8860G 9040T	1	0.0125	0.000156
11	8584A 8701G 8860G 9090C	1	0.0125	0.000156
12	8584A 8829T 8860G	1	0.0125	0.000156
13	8584A 8860G	6	0.075	0.005625
14	8614C 8724G 8860G	1	0.0125	0.000156
15	8701G 8853G 8860G 9127G	1	0.0125	0.000156
16	8701G 8860G	24	0.3	0.09
17	8701G 8860G 9078C	3	0.0375	0.001406
18	8701G 8860G 9103C	1	0.0125	0.000156
19	8724G 8860G	1	0.0125	0.000156
20	8730G 8860G	1	0.0125	0.000156
21	8794T 8860G 9126C	1	0.0125	0.000156
22	8860G	12	0.15	0.0225
23	8860G 9053A	13	0.1625	0.026406
	SUM	80	1	0.150933
	1-SUM [1-∑(Xi²)]			0.849067

The most common haplotypes are: 8701G 8860G (n=24); 8860G 9053A (n=13); 8860G (n=12).

Random match probability

$$P = \sum (Xi^2)$$

$$= 0.15093$$

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Discrimination power

$$Dp = 1-\sum (Xi^2)$$

$$= 0.84906$$

The genetic diversity

$$h = \frac{\left(1-\sum x^2\right)n}{(n-1)}$$

$$= 0.85981$$

Table 6 The haplotypes from HV1 and coding region

HV1 and Coding Region	Frequency	Xi	Xi²
16031-A 16059-A 16129A 16172C 16304C 8860G 9053A	1	0.0125	0.000156
16042A 16058-A 16129A 16172C 16304C 8860G 9053A	1	0.0125	0.000156
16042A 16223T 16298C 16327T 8584A 8701G 8860G	1	0.0125	0.000156
16051C 16070C 16113C 16189C 16270T 16304C 8724G 8860G	1	0.0125	0.000156
16051G 16206C 16271C 16328T 8860G 9053A	1	0.0125	0.000156
16059-A 16184T 16223T 16274A 16278T 8701G 8860G	1	0.0125	0.000156
16086C 16129A 16209C 16223T 16263C 16272G 8701G 8853G 8860G 9127G	1	0.0125	0.000156
16086C 16172C 16223T 16257A 16261T 16295T 8860G	1	0.0125	0.000156
16093C 16129A 16209C 16235C 8701G 8860G	1	0.0125	0.000156
16093C 16147T 16249C 16288C 16325C 8860G	1	0.0125	0.000156
16093C 16189C 16260T 16298C 8860G	1	0.0125	0.000156
16094C 16111T 16223T 16290T 16319A 16320T 16362C 8459G 8794T 8860G 9115G	1	0.0125	0.000156
16108T 16129A 16161G 16172C 16304C 16334G 8701G 8860G 9103C	1	0.0125	0.000156

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Table 6 The haplotypes from HV1 and coding region (Continued)

HV1 and Coding Region	Frequency	Xi	Xi²
16108T 16129A 16162G 16172C 16189C 16304C 8860G 9053A	1	0.0125	0.000156
16108T 16129A 16162G 16172C 16241T 16256T 16304C 8860G 9053A	1	0.0125	0.000156
16108T 16129A 16162G 16172C 16256T 16304C 8860G 9053A	1	0.0125	0.000156
16108T 16129A 16162G 16172C 16304C 8860G 9053A	1	0.0125	0.000156
16108T 16129A 16162G 16172C 16309C 8860G 9053A	1	0.0125	0.000156
16108T 16129A 16172C 16223T 16234T 16287T 16290T 8701G 8860G	1	0.0125	0.000156
16108T 16129A 16172C 16223T 16234T 16290T 8584A 8829T 8860G	1	0.0125	0.000156
16111T 16145A 16192T 16223T 16291T 16304C 8509T 8701G 8860G 8865A	1	0.0125	0.000156
16124C 16148T 16223T 8701G 8860G 9078C	2	0.025	0.000625
16124C 18183C 16189C 16278T 16362C 8701G 8860G	1	0.0125	0.000156
16129A 16162G 16168T 16172C 16304C 16399G 8860G 9053A	1	0.0125	0.000156
16129A 16162G 16172C 16304C 8860G 9053A	1	0.0125	0.000156
16129A 16169T 16172C 16304C 8860G 9053A	1	0.0125	0.000156
16129A 16172C 16304C 8860G 9053A	2	0.025	0.000625
16129A 16182C 16183C 16189C 8860G	1	0.0125	0.000156
16129A 16187T 16189C 16223T 16297C 8701G 8860G	1	0.0125	0.000156
16129A 16192T 16223T 16297C 8701G 8860G	1	0.0125	0.000156
16129A 16223T 16248T 16297C 8701G 8860G	1	0.0125	0.000156
16129A 16223T 16270T 16362C 8414T 8473C 8701G 8860G	1	0.0125	0.000156
16129A 16223T 16297C 16298-T 8701G 8860G	1	0.0125	0.000156
16136C 16182C 16183C 16189C 16217C 8860G	1	0.0125	0.000156
16136C 16223T 16312G 8562T 8701G 8860G	1	0.0125	0.000156
16140C 16182C 16183C 16189C 16261T 16266A 16304C 8584A 8860G	1	0.0125	0.000156
16140C 16182C 16183C 16189C 16261T 16266A 8584A 8860G	1	0.0125	0.000156
16140C 16183C 16189C 16218T 16266A 8584A 8860G	1	0.0125	0.000156

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Table 6 The haplotypes from HV1 and coding region (Continued)

HV1 and Coding Region	Frequency	Xi	Xi²
16140C 16183C 16189C 16266A 16267T 16293G 8584A 8860G	1	0.0125	0.000156
16140C 16183C 16189C 16266A 8584A 8860G	1	0.0125	0.000156
16140C 16183C 16189C 8584A 8860G	1	0.0125	0.000156
16145A 16185T 16239T 16311C 16325C 8464T 8743A 8784G 8860G 8887G 9116C	1	0.0125	0.000156
16145A 16192T 16223T 16291T 16294T 16304C 8509T 8701G 8860G 8865A	1	0.0125	0.000156
16147T 16183C 16184A 16189C 16217C 16234T 8701G 8860G 9078C	1	0.0125	0.000156
16157C 16278T 19304C 8860G	1	0.0125	0.000156
16172C 16223T 16257A 16261T 16316G 8860G	1	0.0125	0.000156
16172C 16223T 16260T 16264T 16271C 8521G 8701G 8718G 8860G	1	0.0125	0.000156
16181C 16182C 16183C 16189C 16213A 16217C 16261T 16292T 16311C 16320T 8860G	1	0.0125	0.000156
16181G 8701G 8860G	2	0.025	0.000625
16183C 16188.1+C 16189C 16300G 16304C 8730G 8860G	1	0.0125	0.000156
16183C 16189C 16193.1+C 16223T 16266T 16291T 16311C 8701G 8860G	1	0.0125	0.000156
16183C 16189C 16223T 16298C 16327T 8584A 8701G 8860G	1	0.0125	0.000156
16183C 16189C 16223T 16357C 16362C 8701G 8860G	1	0.0125	0.00015
16183C 16193.1+C 16212G 16232A 16249C 16304C 16362C 8860G	1	0.0125	0.000156
16185T 16189C 16223T 16232A 16319A 16362C 8414T 8701G 8860G	1	0.0125	0.000156
16185T 16223T 16260T 16298C 16302G 8584A 8701G 8860G 9090C	1	0.0125	0.000156
16189C 16223T 16256T 8701G 8860G	1	0.0125	0.000156
16193T 16223T 8701G 8860G	1	0.0125	0.000156
16209C 16223T 16233G 16274A 16304C 16311C 8701G 8860G	1	0.0125	0.000156
16214A 16223T 16256T 16278T 8701G 8860G	1	0.0125	0.000156

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Table 6 The haplotypes from HV1 and coding region (Continued)

HV1 and Coding Region	Frequency	Xi	Xi²
16217C 16223T 16362C 8701G 8860G	1	0.0125	0.000156
16223T 16224C 16266T 16290T 16311C 8701G 8860G	1	0.0125	0.000156
16223T 16235G 16290T 16311C 16319A 16362C 8794T 8860G 9126C	1	0.0125	0.000156
16223T 16257A 16261T 16292T 8860G	2	0.025	0.000625
16223T 16259T 16278T 16291T 16362C 8701G 8860G	1	0.0125	0.000156
16223T 16266T 16311C 16325C 16362C 8519A 8555C 8701G 8715C 8860G	1	0.0125	0.000156
16223T 16278T 8701G 8860G	1	0.0125	0.000156
16223T 16286T 8414T 8701G 8860G	1	0.0125	0.000156
16223T 16295T 16311C 8701G 8860G	1	0.0125	0.000156
16223T 16297C 8701G 8860G	1	0.0125	0.000156
16223T 16298C 16327T 16357C 8584A 8701G 8860G	1	0.0125	0.000156
16223T 16311C 16362C 8701G 8860G	1	0.0125	0.000156
16223T 16331C 16355T 16362C 8701G 8860G	1	0.0125	0.000156
16244C 16257T 16259T 16288C 16304C 8860G	1	0.0125	0.000156
16298C 16327T 8584A 8701G 8860G 9040T	1	0.0125	0.000156
16304C 16362C 8614C 8724G 8860G	1	0.0125	0.000156
SUM	80	1	0.013726
1-SUM [1-∑(Xi²)]			0.986274

Random match probability

$$P = \sum (Xi^2)$$
$$= 0.01373$$

Discrimination power

$$Dp = 1-\sum (Xi^2)$$
$$= 0.98627$$

The genetic diversity

$$h = \frac{\left(1-\sum x^2\right)n}{(n-1)}$$
$$= 0.99875$$

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Table 7 The haplotypes from HV2 and coding region

HV2 and Coding Region	Frequency	Xi	Xi²
73G 125C 127C 128T 146C 152C 229G 263G 309.1+C 315.1+C 318C 326G 8701G 8860G	1	0.0125	0.000156
73G 143A 152C 204C 207A 229G 235G 309.1+C 315.1+C 8459G 8794T 8860G 9115G	1	0.0125	0.000156
73G 143A 182T 229G 263G 309.1+C 315.1+C 8701G 8860G	1	0.0125	0.000156
73G 146A 199C 229G 263G 309.1+C 315.1+C 8701G 8860G	1	0.0125	0.000156
73G 146A 199C 229G 309.2+2C 315.1+C 8701G 8860G	1	0.0125	0.000156
73G 146C 150T 195C 198T 229G 263G 309.1+C 315.1+C 8730G 8860G	1	0.0125	0.000156
73G 146C 151T 229G 263G 309.2+2C 315.1+C 8860G	1	0.0125	0.000156
73G 146C 152C 195C 229G 263G 309.1+C 315.1+C 8701G 8860G	1	0.0125	0.000156
73G 146C 152C 199C 229G 263G 315.1+C 8701G 8860G	1	0.0125	0.000156
73G 146C 152C 229G 263G 309.1+C 315.1+C 8414T 8473C 8701G 8860G	1	0.0125	0.000156
73G 146C 152C 229G 263G 309.1+C 315.1+C 8701G 8860G	1	0.0125	0.000156
73G 146C 153G 229G 263G 309.1+C 315.1+C 8701G 8860G	1	0.0125	0.00015
73G 146C 229G 249-A 263G 315.1+C 8584A 8701G 8860G	1	0.0125	0.000156
73G 149.1+T 229G 263G 309.1+C 315.1+C 8860G	1	0.0125	0.000156
73G 150T 151T 195C 229G 263G 309.1+C 315.1+C 8701G 8860G	1	0.0125	0.000156
73G 150T 189G 199C 204C 229G 263G 315.1+C 8701G 8860G	1	0.0125	0.000156
73G 150T 195C 204C 229G 263G 315.1+C 8860G	1	0.0125	0.000156
73G 150T 195C 229G 249-A 263G 315.1+C 8860G 9053A	1	0.0125	0.000156
73G 150T 195C 229G 263G 315.1+C 8701G 8860G	1	0.0125	0.000156
73G 150T 199C 204C 207A 229G 263G 309.1+C 315.1+C 8701G 8860G	1	0.0125	0.000156

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Table 7 The haplotypes from HV2 and coding region (Continued)

HV2 and Coding Region	Frequency	Xi	Xi²
73G 150T 199C 204C 229G 263G 309.1+C 315.1+C 332T 8701G 8860G	1	0.0125	0.000156
73G 150T 199C 204C 229G 263G 309.1+C 315.1+C 8701G 8860G	2	0.025	0.000625
73G 150T 229G 263G 309.1+C 315.1+C 8701G 8860G	1	0.0125	0.000156
73G 150T 229G 263G 309.1+C 315.1+C 8860G	2	0.025	0.000625
73G 150T 229G 263G 315.1+C 329A 8860G	1	0.0125	0.000156
73G 151T 152C 229G 249-A 263G 309.1+C 315.1+C 8584A 8701G 8860G 9090C	1	0.0125	0.000156
73G 151T 229G 263G 315.1+C 8521G 8701G 8718G 8860G	1	0.0125	0.000156
73G 152C 195C 229G 263G 315.1+C 8701G 8860G	1	0.0125	0.000156
73G 152C 199C 229G 234G 235G 259G 263G 309.1+C 315.1+C 8794T 8860G 9126C	1	0.0125	0.000156
73G 152C 210G 229G 263G 309.1+C 315.1+C 8584A 8860G	1	0.0125	0.000156
73G 152C 210G 229G 263G 309.2+2C 315.1+C 324G 8584A 8860G	1	0.0125	0.000156
73G 152C 210G 229G 263G 309.2+2C 315.1+C 8584A 8860G	1	0.0125	0.000156
73G 152C 210G 229G 263G 315.1+C 8584A 8860G	1	0.0125	0.000156
73G 152C 229G 163G 309.2+2C 315.1+C 8464T 8743A 8784G 8860G 8887G 9116C	1	0.0125	0.000156
73G 152C 229G 249-A 263G 309.1+C 315.1+C 8860G	1	0.0125	0.000156
73G 152C 229G 249-A 263G 315.1+C 316A 8701G 8853G 8860G 9127G	1	0.0125	0.000156
73G 152C 229G 263G 309.1+C 315.1+C 8701G 8860G	1	0.0125	0.000156
73G 152C 229G 263G 315.1+C 329A 8860G	1	0.0125	0.000156
73G 173C 229G 263G 309.1+C 315.1+C 8519A 8555C 8701G 8715C 8860G	1	0.0125	0.000156
73G 185A 189G 229G 263G 309.1+C 315.1+C 8584A 8829T 8860G	1	0.0125	0.000156
73G 185A 189G 229G 263G 309.1+C 315.1+C 8701G 8860G	1	0.0125	0.000156

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Table 7 The haplotypes from HV2 and coding region (Continued)

HV2 and Coding Region	Frequency	Xi	Xi²
73G 189G 210G 229G 263G 315.1+C 8509T 8701G 8860G 8865A	1	0.0125	0.000156
73G 189G 214G 229G 249-A 263G 309.1+C 315.1+C 8584A 8701G 8860G	1	0.0125	0.000156
73G 194T 229G 263G 315.1+C 8860G 9053A	1	0.0125	0.000156
73G 195C 229G 263G 309.1+C 315.1+C 8701G 8860G 9078C	1	0.0125	0.000156
73G 195C 229G 263G 309.2+2C 315.1+C 8701G 8860G 9078C	1	0.0125	0.000156
73G 199C 229G 263G 315.1+C 8860G	1	0.0125	0.000156
73G 204C 229G 249-A 263G 315.1+C 8860G 9053A	1	0.0125	0.000156
73G 207A 229G 259-A 263G 309.1+C 315.1+C 8860G	1	0.0125	0.000156
73G 210G 229G 263G 309.1+C 315.1+C 8584A 8860G	1	0.0125	0.000156
73G 210G 229G 263G 315.1+C 8509T 8701G 8860G 8865A	1	0.0125	0.000156
73G 210G 229G 263G 315.1+C 8584A 8860G	1	0.0125	0.000156
73G 228A 229G 263G 271272273274-ATTT 309.1+C 315.1+C 8701G 8860G	1	0.0125	0.000156
73G 229G 239C 263G 309.1+C 315.1+C 8701G 8860G	1	0.0125	0.000156
73G 229G 249-A 263G 293C 309.1+C 315.1+C 8860G 9053A	2	0.025	0.000625
73G 229G 249-A 263G 309.1+C 315.1+C 8584A 8701G 8860G	1	0.0125	0.000156
73G 229G 249-A 263G 309.1+C 315.1+C 8584A 8701G 8860G 9040T	1	0.0125	0.000156
73G 229G 249-A 263G 309.1+C 315.1+C 8701G 8860G 9103C	1	0.0125	0.000156
73G 229G 249-A 263G 309.1+C 315.1+C 8860G 9053A	4	0.05	0.0025
73G 229G 249-A 263G 309.2+2C 315.1+C 8860G 9053A	1	0.0125	0.000156
73G 229G 249-A 263G 315.1+C 8860G 9053A	3	0.0375	0.001406
73G 229G 263G 273G 309.1+C 315.1+C 8860G	1	0.0125	0.000156
73G 229G 263G 309.1+C 315.1+C 8701G 8860G	1	0.0125	0.000156
73G 229G 263G 309.1+C 315.1+C 8701G 8860G 9078C	1	0.0125	0.000156
73G 229G 263G 309.2+2C 315.1+C 8860G	1	0.0125	0.000156

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Table 7 The haplotypes from HV2 and coding region (Continued)

HV2 and Coding Region	Frequency	Xi	Xi²
73G 229G 263G 315.1+C 8414T 8701G 8860G	2	0.025	0.000625
73G 229G 263G 315.1+C 8562T 8701G 8860G	1	0.0125	0.000156
73G 229G 263G 315.1+C 8614C 8724G 8860G	1	0.0125	0.000156
73G 229G 263G 315.1+C 8701G 8860G	1	0.0125	0.000156
73G 229G 263G 315.1+C 8724G 8860G	1	0.0125	0.000156
73G 299G 309.1+C 315.1+C 8701G 8860G	1	0.0125	0.000156
SUM	80	1	0.01654
1-SUM [1-∑(Xi²)]			0.98346

Random match probability

$$P = \sum (Xi^2)$$
$$= 0.01654$$

Discrimination power

$$Dp = 1-\sum (Xi^2)$$
$$= 0.98346$$

The genetic diversity

$$h = \frac{\left(1-\sum x^2\right)n}{(n-1)}$$
$$= 0.9959$$

Table 8 The haplotypes from HV1, HV2 and coding region

HV1, HV2 and Coding Region	Frequency	Xi	Xi²
16031-A 16059-A 16129A 16172C 16304C 73G 229G 249-A 263G 315.1+C 8860G 9053A	1	0.0125	0.000156
16042A 16058-A 16129A 16172C 16304C 73G 204C 229G 249-A 263G 315.1+C 8860G 9053A	1	0.0125	0.000156
16042A 16223T 16298C 16327T 73G 146C 229G 249-A 263G 315.1+C 8584A 8701G 8860G	1	0.0125	0.000156
16051C 16070C 16113C 16189C 16270T 16304C 73G 229G 263G 315.1+C 8724G 8860G	1	0.0125	0.000156
16051G 16206C 16271C 16328T 73G 194T 229G 23-63G 315.1+C 8860G 9053A	1	0.0125	0.000156

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Table 8 The haplotypes from HV1, HV2 and coding region (Continued)

HV1, HV2 and Coding Region	Frequency	Xi	Xi²
16059-A 16184T 16223T 16274A 16278T 73G 146C 152C 229G 263G 309.1+C 315.1+C 8701G 8860G	1	0.0125	0.000156
16086C 16129A 16209C 16223T 16263C 16272G 73G 152C 229G 249-A 263G 315.1+C 316A 8701G 8853G 8860G 9127G	1	0.0125	0.000156
16086C 16172C 16223T 16257A 16261T 16295T 73G 150T 229G 263G 309.1+C 315.1+C 8860G	1	0.0125	0.000156
16093C 16129A 16209C 16235C 73G 146C 153G 229G 263G 309.1+C 315.1+C 8701G 8860G	1	0.0125	0.000156
16093C 16147T 16249C 16288C 16325C 73G 150T 229G 263G 315.1+C 329A 8860G	1	0.0125	0.000156
16093C 16189C 16260T 16298C 73G 207A 229G 259-A 263G 309.1+C 315.1+C 8860G	1	0.0125	0.000156
16094C 16111T 16223T 16290T 16319A 16320T 16362C 73G 143A 152C 204C 207A 229G 235G 309.1+C 315.1+C 8459G 8794T 8860G 9115G	1	0.0125	0.000156
16108T 16129A 16161G 16172C 16304C 16334G 73G 229G 249-A 263G 309.1+C 315.1+C 8701G 8860G 9103C	1	0.0125	0.000156
16108T 16129A 16162G 16172C 16189C 16304C 73G 150T 195C 229G 249-A 263G 315.1+C 8860G 9053A	1	0.0125	0.000156
16108T 16129A 16162G 16172C 16241T 16256T 16304C 73G 229G 249-A 263G 293C 309.1+C 315.1+C 8860G 9053A	1	0.0125	0.000156
16108T 16129A 16162G 16172C 16256T 16304C 73G 229G 249-A 263G 293C 309.1+C 315.1+C 8860G 9053A	1	0.0125	0.000156
16108T 16129A 16162G 16172C 16304C 73G 229G 249-A 263G 309.1+C 315.1+C 8860G 9053A	1	0.0125	0.000156
16108T 16129A 16162G 16172C 16309C 73G 229G 249-A 263G 309.1+C 315.1+C 8860G 9053A	1	0.0125	0.000156
16108T 16129A 16172C 16223T 16234T 16287T 16290T 73G 185A 189G 229G 263G 309.1+C 315.1+C 8701G 8860G	1	0.0125	0.000156

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Table 8 The haplotypes from HV1, HV2 and coding region (Continued)

HV1, HV2 and Coding Region	Frequency	Xi	Xi²
16108T 16129A 16172C 16223T 16234T 16290T 73G 185A 189G 229G 263G 309.1+C 315.1+C 8584A 8829T 8860G	1	0.0125	0.000156
16111T 16145A 16192T 16223T 16291T 16304C 73G 210G 229G 263G 315.1+C 8509T 8701G 8860G 8865A	1	0.0125	0.000156
16124C 16148T 16223T 73G 195C 229G 263G 309.1+C 315.1+C 8701G 8860G 9078C	1	0.0125	0.000156
16124C 16148T 16223T 73G 195C 229G 263G 309.2+2C 315.1+C 8701G 8860G 9078C	1	0.0125	0.000156
16124C 18183C 16189C 16278T 16362C 73G 229G 263G 315.1+C 8701G 8860G	1	0.0125	0.000156
16129A 16162G 16168T 16172C 16304C 16399G 73G 229G 249-A 263G 309.1+C 315.1+C 8860G 9053A	1	0.0125	0.000156
16129A 16162G 16172C 16304C 73G 229G 249-A 263G 309.1+C 315.1+C 8860G 9053A	1	0.0125	0.000156
16129A 16169T 16172C 16304C 73G 229G 249-A 263G 315.1+C 8860G 9053A	1	0.0125	0.000156
16129A 16172C 16304C 73G 229G 249-A 263G 309.2+2C 315.1+C 8860G 9053A	1	0.0125	0.000156
16129A 16172C 16304C 73G 229G 249-A 263G 315.1+C 8860G 9053A	1	0.0125	0.000156
16129A 16182C 16183C 16189C 73G 229G 263G 273G 309.1+C 315.1+C 8860G	1	0.0125	0.000156
16129A 16187T 16189C 16223T 16297C 73G 150T 199C 204C 229G 263G 309.1+C 315.1+C 8701G 8860G.	1	0.0125	0.000156
16129A 16192T 16223T 16297C 73G 150T 199C 204C 229G 263G 309.1+C 315.1+C 8701G 8860G	1	0.0125	0.000156
16129A 16223T 16248T 16297C 73G 150T 199C 204C 207A 229G 263G 309.1+C 315.1+C 8701G 8860G	1	0.0125	0.000156
16129A 16223T 16270T 16362C 73G 146C 152C 229G 263G 309.1+C 315.1+C 8414T 8473C 8701G 8860G	1	0.0125	0.000156
16129A 16223T 16297C 16298-T 73G 150T 199C 204C 229G 263G 309.1+C 315.1+C 332T 8701G 8860G	1	0.0125	0.000156
16136C 16182C 16183C 16189C 16217C 73G 199C 229G 263G 315.1+C 8860G	1	0.0125	0.000156

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Table 8 The haplotypes from HV1, HV2 and coding region (Continued)

HV1, HV2 and Coding Region	Frequency	Xi	Xi²
16136C 16223T 16312G 73G 229G 263G 315.1+C 8562T 8701G 8860G	1	0.0125	0.000156
16140C 16182C 16183C 16189C 16261T 16266A 16304C 73G 152C 210G 229G 263G 309.2+2C 315.1+C 324G 8584A 8860G	1	0.0125	0.000156
16140C 16182C 16183C 16189C 16261T 16266A 73G 152C 210G 229G 263G 309.2+2C 315.1+C 8584A 8860G	1	0.0125	0.000156
16140C 16183C 16189C 16218T 16266A 73G 210G 229G 263G 315.1+C 8584A 8860G	1	0.0125	0.000156
16140C 16183C 16189C 16266A 16267T 16293G 73G 152C 210G 229G 263G 315.1+C 8584A 8860G	1	0.0125	0.000156
16140C 16183C 16189C 16266A 73G 210G 229G 263G 309.1+C 315.1+C 8584A 8860G	1	0.0125	0.000156
16140C 16183C 16189C 73G 152C 210G 229G 263G 309.1+C 315.1+C 8584A 8860G	1	0.0125	0.000156
16145A 16185T 16239T 16311C 16325C 73G 152C 229G 263G 309.2+2C 315.1+C 8464T 8743A 8784G 8860G 8887G 9116C	1	0.0125	0.000156
16145A 16192T 16223T 16291T 16294T 16304C 73G 189G 210G 229G 263G 315.1+C 8509T 8701G 8860G 8865A	1	0.0125	0.000156
16147T 16183C 16184A 16189C 16217C 16234T 73G 229G 263G 309.1+C 315.1+C 489C 553T 8701G 8860G 9078C	1	0.0125	0.000156
16157C 16278T 19304C 73G 146C 151T 229G 263G 309.2+2C 315.1+C 8860G	1	0.0125	0.000156
16172C 16223T 16257A 16261T 16316G 73G 150T 195C 204C 229G 263G 315.1+C 8860G	1	0.0125	0.000156
16172C 16223T 16260T 16264T 16271C 73G 151T 229G 263G 315.1+C 8521G 8701G 8718G 8860G	1	0.0125	0.000156
16181C 16182C 16183C 16189C 16213A 16217C 16261T 16292T 16311C 16320T 73G 229G 263G 309.2+2C 315.1+C 8860G	1	0.0125	0.000156
16181G 73G 146A 199C 229G 263G 309.1+C 315.1+C 8701G 8860G	1	0.0125	0.000156

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Table 8 The haplotypes from HV1, HV2 and coding region (Continued)

HV1, HV2 and Coding Region	Frequency	Xi	Xi²
16181G 73G 146A 199C 229G 309.2+2C 315.1+C 8701G 8860G	1	0.0125	0.000156
16183C 16188.1+C 16189C 16300G 16304C 73G 146C 150T 195C 198T 229G 263G 309.1+C 315.1+C 8730G 8860G	1	0.0125	0.000156
16183C 16189C 16193.1+C 16223T 16266T 16291T 16311C 73G 146C 152C 195C 229G 263G 309.1+C 315.1+C 8701G 8860G	1	0.0125	0.000156
16183C 16189C 16223T 16298C 16327T 73G 229G 249-A 263G 309.1+C 315.1+C 8584A 8701G 8860G	1	0.0125	0.000156
16183C 16189C 16223T 16357C 16362C 73G 150T 229G 263G 309.1+C 315.1+C 8701G 8860G	1	0.0125	0.000156
16183C 16193.1+C 16212G 16232A 16249C 16304C 16362C 73G 152C 229G 249-A 263G 309.1+C 315.1+C 8860G	1	0.0125	0.000156
16185T 16189C 16223T 16232A 16319A 16362C 73G 229G 263G 315.1+C 8414T 8701G 8860G	1	0.0125	0.000156
16185T 16223T 16260T 16298C 16302G 73G 151T 152C 229G 249-A 263G 309.1+C 315.1+C 8584A 8701G 8860G 9090C	1	0.0125	0.000156
16189C 16223T 16256T 73G 229G 239C 263G 309.1+C 315.1+C 8701G 8860G	1	0.0125	0.000156
16193T 16223T 73G 150T 195C 229G 263G 315.1+C 8701G 8860G	1	0.0125	0.000156
16209C 16223T 16233G 16274A 16304C 16311C 73G 143A 182T 229G 263G 309.1+C 315.1+C 8701G 8860G	1	0.0125	0.000156
16214A 16223T 16256T 16278T 73G 152C 229G 263G 309.1+C 315.1+C 8701G 8860G	1	0.0125	0.000156
16217C 16223T 16362C 73G 229G 263G 309.1+C 315.1+C 8701G 8860G	1	0.0125	0.000156
16223T 16224C 16266T 16290T 16311C 73G 125C 127C 128T 146C 152C 229G 263G 309.1+C 315.1+C 318C 326G 8701G 8860G	1	0.0125	0.000156

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Table 8 The haplotypes from HV1, HV2 and coding region (Continued)

HV1, HV2 and Coding Region	Frequency	Xi	Xi²
16223T 16235G 16290T 16311C 16319A 16362C 73G 152C 199C 229G 234G 235G 259G 263G 309.1+C 315.1+C 8794T 8860G 9126C	1	0.0125	0.000156
16223T 16257A 16261T 16292T 73G 149.1+T 229G 263G 309.1+C 315.1+C 8860G	1	0.0125	0.000156
16223T 16257A 16261T 16292T 73G 150T 229G 263G 309.1+C 315.1+C 8860G	1	0.0125	0.000156
16223T 16259T 16278T 16291T 16362C 73G 228A 229G 263G 271272273274-ATTT 309.1+C 315.1+C 8701G 8860G	1	0.0125	0.000156
16223T 16266T 16311C 16325C 16362C 73G 173C 229G 263G 309.1+C 315.1+C 8519A 8555C 8701G 8715C 8860G	1	0.0125	0.000156
16223T 16278T 73G 150T 151T 195C 229G 263G 309.1+C 315.1+C 8701G 8860G	1	0.0125	0.000156
16223T 16286T 73G 229G 263G 315.1+C 8414T 8701G 8860G	1	0.0125	0.000156
16223T 16295T 16311C 73G 146C 152C 199C 229G 263G 315.1+C 8701G 8860G	1	0.0125	0.000156
16223T 16297C 73G 150T 189G 199C 204C 229G 263G 315.1+C 8701G 8860G	1	0.0125	0.000156
16223T 16298C 16327T 16357C 73G 189G 214G 229G 249-A 263G 309.1+C 315.1+C 8584A 8701G 8860G	1	0.0125	0.000156
16223T 16311C 16362C 73G 229G 309.1+C 315.1+C 8701G 8860G	1	0.0125	0.000156
16223T 16331C 16355T 16362C 73G 152C 195C 229G 263G 315.1+C 8701G 8860G	1	0.0125	0.000156
16244C 16257T 16259T 16288C 16304C 73G 152C 229G 263G 315.1+C 329A 8860G	1	0.0125	0.000156
16298C 16327T 73G 229G 249-A 263G 309.1+C 315.1+C 8584A 8701G 8860G 9040T	1	0.0125	0.000156
16304C 16362C 73G 229G 263G 315.1+C 8614C 8724G 8860G	1	0.0125	0.000156
SUM	80	1	0.01248
1-SUM [1-∑(Xi²)]			0.98752

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Random match probability

$$P = \sum (Xi^2)$$

$$= 0.01248$$

Discrimination power

$$Dp = 1-\sum (Xi^2)$$

$$= 0.98752$$

The genetic diversity

$$h = \frac{\left(1-\sum x^2\right)n}{(n-1)}$$

$$= 1.00$$

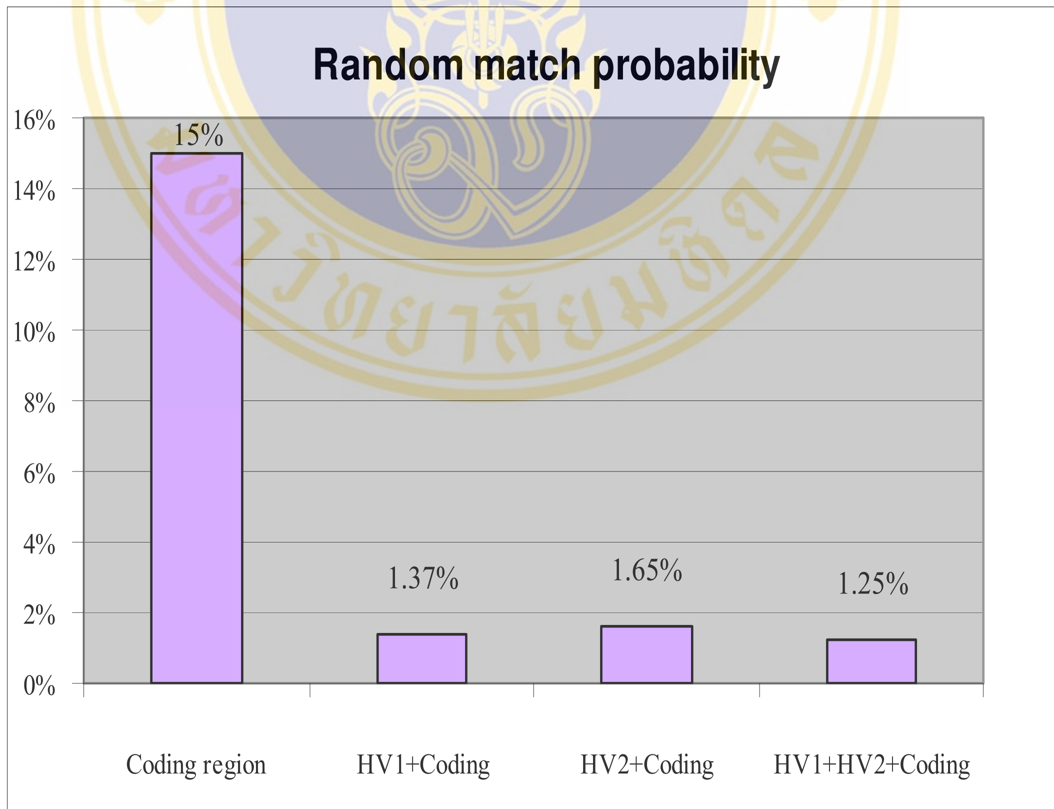

Figure 8 Random match probability in Thai population

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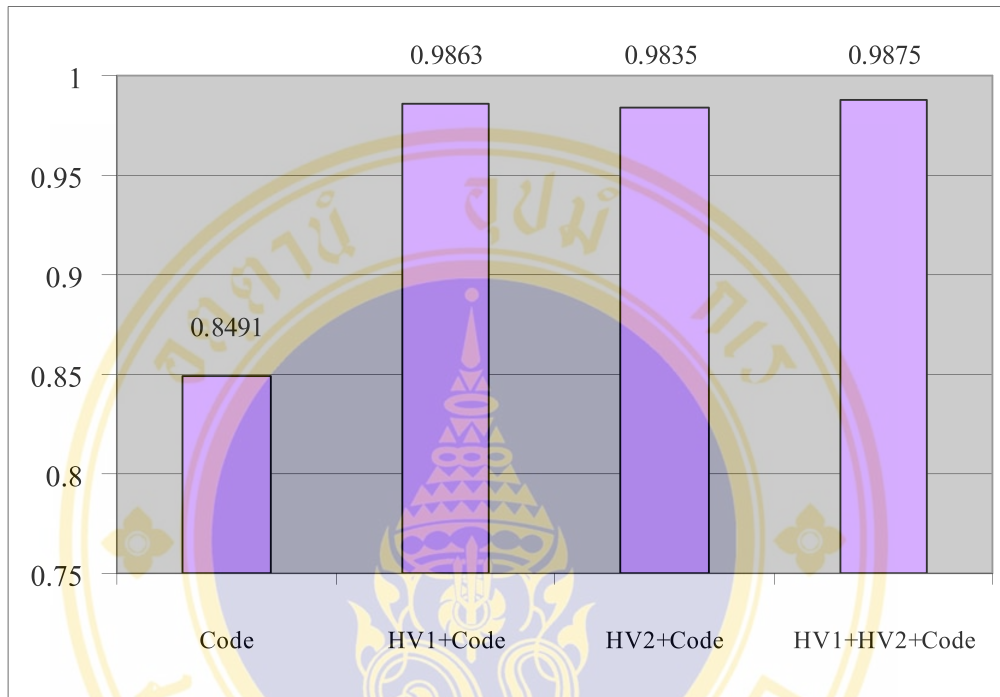

Figure 9 Power of Discrimination in Thai population

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3.5 The nucleotide change pattern

The pattern of sequence polymorphisms showed that the most change is transition types.

Table 9 The nucleotide changes of mtDNA in 80 unrelated Thais

Type of nucleotide change	HV1	HV2	CODING REGION	TOTAL	
Substitution					
Transition					
T → C	133	61	11	205	
C → T	127	20	11	158	761
G → A	34	8	29	71	
A → G	19	175	133	327	
Transversion					
A → C	27	-	-	27	
C → A	13	-	-	13	
A → T	1	-	-	1	131
T → A	-	4	-	4	
G → T	-	2	-	2	
T → G	-	1	-	1	
G → C	2	-	-	2	
C → G	-	81	-	81	
Insertion	3	170	-	173	
Deletion	5	21	-	26	
% Transition[a]	87.92	75	100		85.31
% Transversion[a]	12.08	25	0		14.69

[a] % transition and % transversion are calculated as number of observations divided by total substitutions times 100.

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CHAPTER VI
DISCUSSION

This mitochondrial DNA analysis which the blood samples from 80 unrelated Thais were obtained from routine paternity cases. This study not classify the gender but in all cases must were maternally unrelated individuals. The DNA was extracted from whole blood using the organic extraction procedure. The phenol-chloroform-isoamyl alcohol method was used that yields highly purified DNA and inexpensive (48). However it is an irritant and toxic which should be use carefully. Besides the DNA extraction was successful since using from fresh blood samples that have abundantly DNA amount. The polymerase chain reaction method of hypervariable region I (HV1) and hypervariable region II (HV2) were used according to the protocol of routinely forensic laboratory. The primers used L15997 and H16401 in HV1, and L29 and H408 in HV2 (43). While PCR condition of the coding region encompassing position 8320 to 9160 applied from Tzen et al (53). The PCR amplification of all regions attained satisfactory that this study has strictly control of contamination such as always wear a laboratory coat, change gloves frequently and run negative control. The PCR yields were estimated by comparison with GeneRuler DNA Ladder that electrophoresed in a 1% agarose gel stained with ethidium bromide. The amount of product can be estimated based on the intensity of the fluorescent bands. This study was imaged and analyzed with GeneSnap/GeneTools software. The PCR products were purified prior to cycle sequencing for to remove excess primers, dNTP, enzyme, and buffer components. The DNA sequencing process consists of cycle sequencing and purifying extension products. The ethanol/EDTA precipitation was used that it is particularly good at getting rid of unincorporated dye-labeled terminators prior to electrophoresis. Excess dye terminators in sequencing reactions obscure data in the early part of the sequence and can interfere with basecalling. This method produces the cleanest signal but it may cause loss of small molecular weight fragments (54).

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The nucleotide sequences were analyzed by an ABI 3130 Genetic Analyzer that it has four capillary. It performs convenient and rapid which the runtime approximately 40 min/run. The ABI 3130 Genetic Analyzer that use capillary length 36 cm. and POP-4 polymer. Length of read is useable rang of high-quality or high-accuracy that is 400 bases. While the coding region of this study has approximately 840 base pairs therefore the sequencing primers was used in both forward and reverse direction cause in some parts of nucleotide sequences was confirmed. Really, the nucleotide sequences can read to 600-800 base pairs.

The nucleotide sequences of 80 individuals were compared with the Cambridge reference sequence (5) and the sequences was confirmed by SeqScape software version 2.5 (Applied Biosystems). The majority pattern of nucleotide changes in hypervariable regions of this study were nucleotide substitution the same as Korean, Japanese and German populations (42,43,45). The hypervariable region I found transition type more than in hypervariable region II. The other hand, transversion, insertion, and deletion found in HV2 more than HV1. The transition types are more common than the transversion types. The hypervariable region I was 87.92% and hypervariable region II was 75%. The most of the transversion are C to G which occurred predominately in the HV2 region. The most insertions occur in the HV2 region at site 309 and 315. The insertions of an additional cytosine (C) are common in all population groups (55,56).

In this study focused in coding region between position 8320 and 8160 of mitochondrial DNA. This region is the most of ATP synthase 6 and 8. This region was selected since it has no large size. Besides the mutation has been reported only little associated with disease. The position was confirmed that was 8344, 8356, 8363, and 8993 (57). The myoclonic epilepsy and ragged-red fiber (MERRF) and neurogenic muscle weakness, ataxia, and retinitis pigmentosa (NARP) disease have been associated with mutation of those positions. (58,59). This study investigated sequence polymorphism of coding region which no found nucleotide position of MERRF and NARP disease. The region approximately 840 base pairs was studied that the eighty samples analysis showed the sequence polymorphism 33 sites at differed from the Cambridge reference sequence (CRS). However all individual sequence showed a guanine (G) in position 8860. The confirmation by Andrews et al. reanalyzed the

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original CRS which demonstrated an adenine (A) at position 8860 was a rare polymorphism (60). The study observed the nucleotide change of coding region that found transition type at 100%. The deletion and insertion was not found. The four types of nucleotide transition were identified: A to G (72%), G to A (16%), T to C (6%), and C to T (6%). The most nucleotide transition besides A8860G was at the position 8701 the same as in Taiwan population (53). While German population found mostly position 8697A (61,62).

The genetic diversity of coding region in positions 8320-8160 is 0.8598. However combination of coding region and hypervariable regions found to increase of genetic diversity. The genetic diversity of coding region with HV1 was 0.9987, and it combined with HV2 was 0.9959. Moreover, the discrimination power within 8320-8160 is 0.8491 which the probability of two random individuals having the same genotype is 15%. Considering all three regions combined that they can provide the random match probability equals 1.25%, and the discrimination power was 0.9875. This study performed only a small portion of the coding region that approximately 840 base pair from 16,569 base pairs of human mitochondrial genome. The remaining has numerous that the potential for increase the power of forensic testing. The study of Parsons et al.(63) through analysis of the entire mitochondrial DNA genome demonstrated the potential for variation in the mtDNA coding region to provide discrimination within the common HV1/HV2 types, and to greatly decrease the limitation of forensic mtDNA testing. Coble et al. have identified single nucleotide polymorphisms (SNPs) from the entire mtDNA genome that discrimination provided by the multiplex panels of SNP sites can reduce the frequency of the common type in Caucasian population (64). The methods for SNP analysis have been developed numerous such as Pyrosequencing, SNaPshot and DNA microarray (65,66). The optimal mtDNA typing systems of the future will likely be based on whole mtGenome sequence information and the single nucleotide polymorphisms are being developed for forensic application.

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CHAPTER VII
CONCLUSION

Sequence polymorphisms of coding region encompassing position 8320-9160 from 80 unrelated Thais are 23 different haplotypes defined by 33 variable positions for comparison with the Cambridge reference sequence. The most common haplotypes are: 8701G 8860G. The position 8701 is the most common variable site, which 43 out of 80 individuals (53.45%) has nucleotide transition from A to G. The nucleotide change patterns are transition types in all cases.

The genetic diversity is 0.8598, and the discrimination power is 0.8491. The probability of two randomly selected individual having identical mtDNA type is 15%. However when is combined with HV1 region, the discrimination power is 0.9862 or combined with HV2 region is 0.9834 ,and random match probability are 1.4% and 1.6% respectively. The discrimination power of all three region rise to 0.9875 and random match probability descend to 1.25%.

The polymorphic sites within coding region can be useful in combination with control region in order to increase discrimination power for forensic application.

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APPENDIX

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APPENDIX

Cambridge Reference Sequence (Anderson sequence)

1. Mitochondrial DNA Sequences of HV1

15970 15980 15990 16000 16010 16020

GAAAAAGTCT TTAACTC**CAC CATTAGCACC CAAAGCT**AAG ATTCTAATTT AAACTATTCT

CTTTTTCAGA AATTGAGGTG GTAATCGTGG GTTTCGATTC TAAGATTAAA TTTGATAAGA

16030 16040 16050 16060 16070 16080

CTGTTCTTTC ATGGGGAAGC AGATTTGGGT ACCACCCAAG TATTGACTCA CCCATCAACA

GACAAGAAAG TACCCCTTCG TCTAAACCCA TGGTGGGTTC ATAACTGAGT GGGTAGTTGT

16090 16100 16110 16120 16130 16140

ACCGCTATGT ATTTCGTACA TTACTGCCAG CCACCATGAA TATTGTACGG TACCATAAAT

TGGCGATACA TAAAGCATGT AATGACGGTC GGTGGTACTT ATAACATGCC ATGGTATTTA

16150 16160 16170 16180 16190 16200

ACTTGACCAC CTGTAGTACA TAAAAACCCA ATCCACATCA AAACCCCCTC CCCATGCTTA

TGAACTGGTG GACATCATGT ATTTTTGGGT TAGGTGTAGT TTTGGGGGAG GGGTACGAAT

16210 16220 16230 16240 16250 16260

CAAGCAAGTA CAGCAATCAA CCCTCAACTA TCACACATCA ACTGCAACTC CAAAGCCACC

GTTCGTTCAT GTCGTTAGTT GGGAGTTGAT AGTGTGTAGT TGACGTTGAG GTTTCGGTGG

16270 16280 16290 16300 16310 16320

CCTCACCCAC TAGGATACCA ACAAACCTAC CCACCCTTAA CAGTACATAG TACATAAAGC

GGAGTGGGTG ATCCTATGGT TGTTTGGATG GGTGGGAATT GTCATGTATC ATGTATTTCG

16330 16340 16350 16360 16370 16380

CATTTACCGT ACATAGCACA TTACAGTCAA ATCCCTTCTC GTCCCCATGG ATGACCCCCC

GTAAATGGCA TGTATCGTGT AATGTCAGTT TAGGGAAGAG CAGGGGTACC TACTGGGGGG

16390 16400 16410 16420 16430 16440

TCAGATAGGG GTCCCTTGAC CACCATCCTC CGTGAAATCA ATATCCCGCA CAAGAGTGCT

AGTCTATCCC CAGGGAACTG **GTGGTAGGAG GCACTTTAGT** TATAGGGCGT GTTCTCACGA

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2. Mitochondrial DNA Sequences of HV2

10 20 30 40 50 60

GATCACA**GGT CTATCACCCT ATTAACCA**CT CACGGGAGCT CTCCATGCAT TTGGTATTTT

CTAGTGTCCA GATAGTGGGA TAATTGGTGA GTGCCCTCGA GAGGTACGTA AACCATAAAA

70 80 90 100 110 120

CGTCTGGGGG GTATGCACGC GATAGCATTG CGAGACGCTG GAGCCGGAGC ACCCTATGTC

GCAGACCCCC CATACGTGCG CTATCGTAAC GCTCTGCGAC CTCGGCCTCG TGGGATACAG

130 140 150 160 170 180

GCAGTATCTG TCTTTGATTC CTGCCTCATC CTATTATTTA TCGCACCTAC GTTCAATATT

CGTCATAGAC AGAAACTAAG GACGGAGTAG GATAATAAAT AGCGTGGATG CAAGTTATAA

190 200 210 220 230 240

ACAGGCGAAC ATACTTACTA AAGTGTGTTA ATTAATTAAT GCTTGTAGGA CATAATAATA

TGTCCGCTTG TATGAATGAT TTCACACAAT TAATTAATTA CGAACATCCT GTATTATTAT

250 260 270 280 290 300

ACAATTGAAT GTCTGCACAG C**CAC**TTTCCA CACAGACATC ATAACAAAAA ATTTCCACCA

TGTTAACTTA CAGACGTGTC GGTGAAAGGT GTGTCTGTAG TATTGTTTTT TAAAGGTGGT

310 320 330 340 350 360

AACCCCCCCT **CCCCCG**CTTC TGGCCACAGC ACTTAAACAC ATCTCTGCCA AACCCCAAAA

TTGGGGGGGA GGGGGCGAAG ACCGGTGTCG TGAATTTGTG TAGAGACGGT TTGGGGTTTT

370 380 390 400 410 420

ACAAAGAACC CTAACACCAG CCTAACCAGA TTTCAAATTT TATCTTTTGG CGGTATGCAC

TGTTTCTTGG GATTGTGGTC GGATTGGTCT AAAGTTTAAA ATAGAAA**ACC GCCATACGTG**

430 440 450 460 470 480

TTTTAACAGT CACCCCCCAA CTAACACATT ATTTTCCCCT CCCACTCCCA TACTACTAAT

AAAATTGTCA GTGGGGGGTT GATTGTGTAA TAAAAGGGGA GGGTGAGGGT ATGATGATTA

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3. Mitochondrial DNA Sequences of 8320-9160

8230 8240 8250 8260 8270 8280

ATTAATTCCC CTAAAAATCT TTGAAATAGG GCCCGTATTT ACCCTATAGC ACCCCCTCTA

TAATTAAGGG GATTTTTAGA AACTTTATCC CGGGCATAAA TGGGATATCG TGGGGGAGAT

8290 8300 8310 8320 8330 8340

C**CCCCTCTAG AGCCCACTGT AAAGC**TAACT TAGCATTAAC CTTTTAAGTT AAAGATTAAG

GGGGGAGATC TCGGGTGACA TTTCGATTGA ATCGTAATTG GAAAATTCAA TTTCTAATTC

8350 8360 8370 8380 8390 8400

AGAACCAACA CCTCTTTACA GTGAAATGCC CCAACTAAAT ACTACCGTAT GGCCCACCAT

TCTTGGTTGT GGAGAAATGT CACTTTACGG GGTTGATTTA TGATGGCATA CCGGGTGGTA

8410 8420 8430 8440 8450 8460

AATTACCCCC ATACTCCTTA CACTATTCCT CATCACCCAA CTAAAAATAT TAAACACAAA

TTAATGGGGG TATGAGGAAT GTGATAAGGA GTAGTGGGTT GATTTTTATA ATTTGTGTTT

8470 8480 8490 8500 8510 8520

CTACCACCTA CCTCCCTCAC CAAAGCCCAT AAAAATAAAA AATTATAACA AACCCTGAGA

GATGGTGGAT GGAGGGAGTG GTTTCGGGTA TTTTTATTTT TTAATATTGT TTGGGACTCT

8530 8540 8550 8560 8570 8580

ACCAAAATGA ACGAAAATCT GTTCGCTTCA TTCATTGCCC CCACAATCCT AGGCCTACCC

TGGTTTTACT TGCTTTTAGA CAAGCGAAGT AAGTAACGGG GGTGTTAGGA TCCGGATGGG

8590 8600 8610 8620 8630 8640

GCCGCAGTAC TGATCATTCT ATTTCCCCCT CTATTGATCC CCACCTCCAA ATATCTCATC

CGGCGTCATG ACTAGTAAGA TAAAGGGGGA GATAACTAGG GGTGGAGGTT TATAGAGTAG

8650 8660 8670 8680 8690 8700

AACAACCGAC TAATCACCAC CCAACAATGA CTAATCAAAC TAACCTCAAA ACAAATGATA

TTGTTGGCTG ATTAGTGGTG GGTTGTTACT GATTAGTTTG ATTGGAGTTT TGTTTACTAT

8710 8720 8730 8740 8750 8760

ACCATACACA ACACTAAAGG ACGAACCTGA TCTCTTATAC TAGTATCCTT AATCATTTTT

TGGTATGTGT TGTGATTTCC TGCTTGGACT AGAGAATATG ATCATAGGAA TTAGTAAAAA

8770 8780 8790 8800 8810 8820

ATTGCCACAA CTAACCTCCT CGGACTCCTG CCTCACTCAT TTACACCAAC CACCCAACTA

TAACGGTGTT GATTGGAGGA GCCTGAGGAC GGAGTGAGTA AATGTGGTTG GTGGGTTGAT

8830 8840 8850 8860 8870 8880

TCTATAAACC TAGCCATGGC CATCCCCTTA TGAGCGGG**CA C**AGTGATTAT AGGCTTTCGC

AGATATTTGG ATCGGTACCG GTAGGGGAAT ACTCGCCCGT GTCACTAATA TCCGAAAGCG

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8890 8900 8910 8920 8930 8940

TCTAAGATTA AAAATGCCCT AGCCCACTTC TTACCACAAG GCACACCTAC ACCCCTTATC

AGATTCTAAT TTTTACGGGA TCGGGTGAAG AATGGTGTTC CGTGTGGATG TGGGGAATAG

8950 8960 8970 8980 8990 9000

CCCATACTAG TTATTATCGA AACCATCAGC CTACTCATTC AACCAATAGC CCTGGCCGTA

GGGTATGATC AATAATAGCT TTGGTAGTCG GATGAGTAAG TTGGTTATCG GGACCGGCAT

9010 9020 9030 9040 9050 9060

CGCCTAACCG CTAACATTAC TGCAGGCCAC CTACTCATGC ACCTAATTGG AAGCGCCACC

GCGGATTGGC GATTGTAATG ACGTCCGGTG GATGAGTACG TGGATTAACC TTCGCGGTGG

9070 9080 9090 9100 9110 9120

CTAGCAATAT CAACCATTAA CCTTCCCTCT ACACTTATCA TCTTCACAAT TCTAATTCTA

GATCGTTATA GTTGGTAATT GGAAGGGAGA TGTGAATAGT AGAAGTGTTA AGATTAAGAT

9130 9140 9150 9160 9170 9180

CTGACTATCC TAGAAATCGC TGTCGCCTTA ATCCAAGCCT ACGTTTTCAC ACTTCTAGTA

GACTGATAGG ATCTTTAGCG ACAGCGGAAT TAGGTTCGGA TGCAAAAGTG TGAAGATCAT

9190 9200 9210 9220 9230 9240

AGCCTCTACC TGCACGACAA CACATAATGA CCCACCAATC ACATGCCTAT CATATAGTAA

TCGGAGA**TGG ACGTGCTGTT GTGTATT**ACT GGGTGGTTAG TGTACGGATA GTATATCATT

9250 9260 9270 9280 9290 9300

AACCCAGCCC ATGACCCCTA ACAGGGGCCC TCTCAGCCCT CCTAATGACC TCCGGCCTAG

TTGGGTCGGG TACTGGGGAT TGTCCCCGGG AGAGTCGGGA GGATTACTGG AGGCCGGATC

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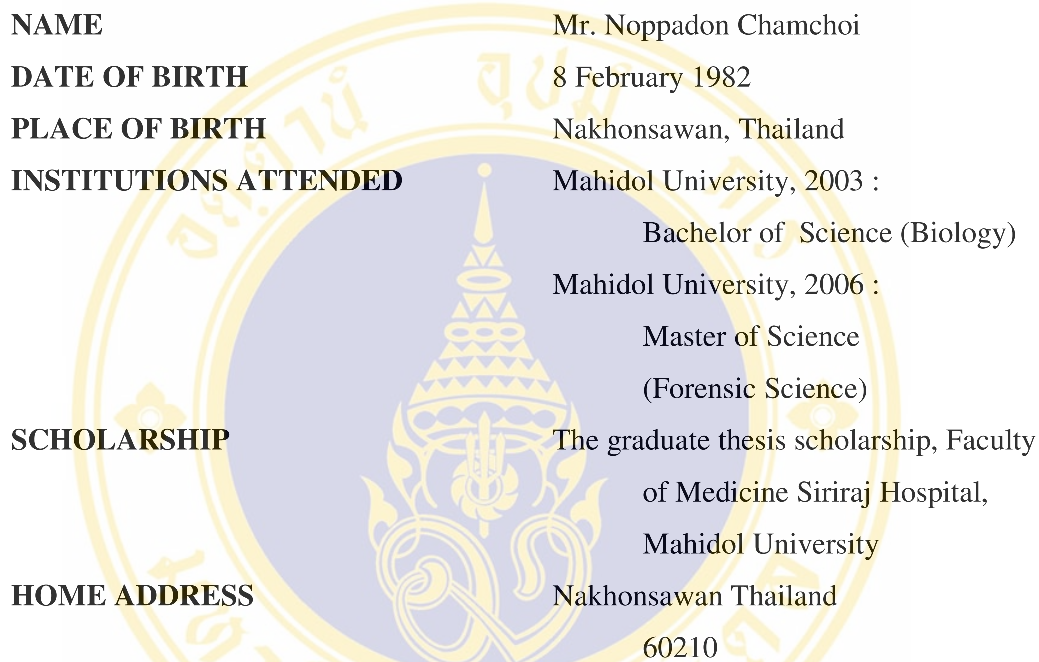

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