

**MITOCHONDRIAL DNA ANALYSIS OF PREHISTORIC
HUMAN REMAINS FROM NOEN U-LOKE,
NAKHON RATCHASIMA**

THITIMA SANPACHUDAYAN

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Thesis
Entitled

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NAKHON RATCHASIMA**

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**MITOCHONDRIAL DNA ANALYSIS OF PREHISTORIC HUMAN REMAINS
FROM NOEN U-LOKE, NAKHON RATCHASIMA**

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THESIS ADVISORS: PATCHAREE LERTRIT, M.D. Ph.D., RACHANIE THOSARAT,
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The Iron Age sites of the Mun River Valley are among the most important and significant sites in Southeast Asia for documenting the rise of the state. Three sites around the upper Mun River Valley have been excavated which are Noen U-Loke, Ban Lum Khao and Prasat Phimai. The prehistoric cemetery at Noen U-Loke was dated in the vicinity of Iron Age about 2,400-1,500 years before present. The cultural sequence at Noen U-Loke is one of the longest continuous records. Knowledge of the prehistory was greatly increased by much residential and industrial evidence. The present study aims to generate additional information about the relationship between the prehistoric human populations in this area. An important tool for this investigation was DNA analysis. Previous studies have shown that DNA can survive in ancient remains. The twenty-six molar teeth of good quality were collected from twenty-six adult skeletons, one each, from Noen U-Loke archaeological site. The method originally used for ancient DNA extraction was very similar to a silica/guanidine thiocyanate method that described in Boom *et al*, 1990 and modified by Höss and Pääbo. The additional used of phosphate buffer was designed for DNA extraction from hydroxyapatite crystal according to Persson, 1992 and Götherström and Lidén, 1996. These modified protocols gave us a good yield of DNA and high succeed rate of DNA recovery (22 out of 26 = 80%). The ancient DNA authenticity was seriously checked, all mitochondrial DNA sequences from each of samples in this study was then trusted to be derived from the excavated remains. The modern samples from several ethnic groups in Thailand and from several part of China were included to the analyses. The investigations indicated that the Noen U-Loke area probably used by a relative population without or very few migrations from outside population through 900 years (2,400-1,500 BP) and suggested that the ancestor of the ancient population from Noen U-Loke probably moved from eastern China more than 2,400 before.

**KEY WORDS: ANCIENT DNA/ MITOCHONDRIAL DNA/ PREHISTORIC
REMAINS/ GENETIC RELATIONSHIP**

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การตรวจวิเคราะห์ดีเอ็นเอไมโทคอนเดรีย ในตัวอย่างโครงกระดูกมนุษย์ยุคก่อนประวัติศาสตร์ ที่ขุดพบที่เนินอุโลก จังหวัดนครราชสีมา (MITOCHONDRIAL DNA ANALYSIS OF PREHISTORIC HUMAN REMAINS FROM NOEN U-LOKE, NAKHON RATCHASIMA)

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บทคัดย่อ

บริเวณลุ่มแม่น้ำมูลอุดมไปด้วยแหล่งโบราณคดี ทั้งที่ยังมิได้ขุดค้นและที่ได้รับการขุดค้นอย่างเป็นระบบแล้วจำนวนมาก แต่ละแห่งมีอายุความเก่าแก่แตกต่างกันไปทั้งที่มีอายุมากสุดอยู่ในยุคหิน, ยุคสำริด จนถึงแหล่งที่มีอายุน้อยที่สุดอยู่ในยุคเหล็ก แหล่งโบราณคดีที่มีอายุอยู่ในช่วงยุคหินบนพื้นที่ลุ่มแม่น้ำมูลนี้มีความสำคัญอย่างยิ่ง เนื่องจากมีการใช้พื้นที่บริเวณนี้เป็นสุสานอย่างต่อเนื่องยาวนาน แหล่งที่ได้รับการขุดค้นจากนักโบราณคดีอย่างเป็นระบบแล้วแหล่งหนึ่งได้แก่แหล่งโบราณคดีก่อนประวัติศาสตร์เนินอุโลก จังหวัดนครราชสีมา มีอายุระหว่าง 2,400-1,500 ปีก่อน เมื่อย้อนกลับไปประมาณ 2,400 ปี มีการใช้พื้นที่บริเวณนี้เป็นสุสานอย่างต่อเนื่องยาวนานถึง 900 ปี เป็นเหตุให้พื้นที่นี้เหมาะแก่การขุดค้นเพื่อการศึกษาความเป็นไปของวัฒนธรรม ประเพณี และการดำรงชีวิตของประชากรก่อนประวัติศาสตร์ได้เป็นอย่างดี ในการศึกษาเรื่องนี้ และสนใจในอันที่จะเพิ่มพูนความรู้ความเข้าใจ ถึงความสัมพันธ์ และการสืบเชื้อสายภายในกลุ่มชนที่อาศัยอยู่ในพื้นที่บริเวณเนินอุโลกเมื่อครั้งโบราณ โดยใช้ขั้นตอนทางวิทยาศาสตร์ในการตรวจวิเคราะห์ดีเอ็นเอเพื่อหาความสัมพันธ์ทางพันธุกรรมของโครงกระดูกมนุษย์โบราณในชั้นต่างๆของบริเวณที่ขุดค้น การสกัดดีเอ็นเอจากเนื้อเยื่อภายในโพรงฟันของโครงกระดูกมนุษย์โบราณทำได้โดยใช้ silica และ guanidine thiocyanate ในขั้นตอนการสกัด เพิ่มเติมด้วยขั้นตอนการสกัดดีเอ็นเอจากผลึก hydroxyapatite ส่วนประกอบหนึ่งของตัวฟัน ผลการศึกษาพบว่าสามารถสกัดและเพิ่มจำนวนดีเอ็นเอจากตัวอย่างโครงกระดูกมนุษย์โบราณได้จำนวน 22 จากทั้งหมด 26 ตัวอย่าง คิดเป็นประมาณ 80% และจากการวิเคราะห์ดีเอ็นเอของกลุ่มชนโบราณด้วยโปรแกรมคอมพิวเตอร์ พบว่ามีการตั้งถิ่นฐานและใช้พื้นที่นี้อย่างต่อเนื่องโดยกลุ่มชนเดียวกัน โดยมีการสืบทอดเชื้อสายต่อเนื่องยาวนานตลอด 900 ปีของการใช้พื้นที่บริเวณนี้ เมื่อวิเคราะห์ดีเอ็นเอของกลุ่มชนโบราณจากเนินอุโลก เทียบกับประชากรที่อาศัยอยู่ในปัจจุบัน ในพื้นที่ประเทศไทยและกลุ่มประชากรอื่นในประเทศจีน พบว่ามีความเป็นไปได้ที่บรรพบุรุษของกลุ่มชนโบราณที่อาศัยในพื้นที่บริเวณเนินอุโลก จังหวัดนครราชสีมา มีความสัมพันธ์กันทางพันธุกรรมใกล้เคียงกับกลุ่มประชากรอื่นปัจจุบันทางตะวันออกของประเทศจีนมากกว่าประชากรที่อาศัยอยู่ในพื้นที่ประเทศไทยในปัจจุบัน จึงมีความเป็นไปได้ที่บรรพบุรุษของกลุ่มชนโบราณจากเนินอุโลกจะอพยพมาจากทางตะวันออกของประเทศจีนเมื่อกว่า 2,400 ปีมาแล้ว

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LIST OF ABBREVIATIONS

AD	Anno Domini
bp	Base pair
BC	Before century
BP	Before present
°C	Degree celcius
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphosphate
dGTP	Deoxyguanosine triphosphate
dTTP	Deoxythymidine triphosphate
dNTP	Deoxynucleotide triphosphate
DNA	Deoxyribonucleic acid
e.g.	<i>exempli gratia</i> (for example)
et al.	<i>et alii</i> (and other people)
EDTA	Ethylenediaminetetraacetic acid
g	Gravity force
i.e.	<i>id est</i> (that is)
mg	Milligram
ml	Millilitre(s)
mM	Millimolar
MgCl ₂	Magnesium chloride
ng	Nanogram
nt	Nucleotide
NaCl	Sodium chloride
No	Number

LIST OF ABBREVIATIONS (continued.)

rpm	Revolutions per minute
PCR	Polymerase chain reaction
<i>Taq</i>	<i>Thermus aquaticus</i>
Tris HCl	Tris-hydrochloride
TAE	Tris-acetate EDTA
UV	Ultraviolet
w/v	Weight Per Volume
μg	Microgram
μl	Microlitre

CHAPTER I

INTRODUCTION

Evidence of settlement by rice cultivators and rise of state has been found in river valley areas, which have highly suitable conditions for rice cultivation and agriculture. The Mun River Valley is one of the most important areas in northeast of Thailand for such evidence. It is well known that this region comprised part of the Angkorian state. Excavations in this particular area have been conducted by the University of Otago Department of Anthropology and the Fine Arts Department of Thailand from 1995 to 1998 (www.otago.ac.nz/Anthropology/Angkor.html). Three sites around the upper Mun River Valley have been excavated which are Noen U-Loke, Ban Lum Khao and Prasat Phimai. The prehistory of Thailand, on the basis of technological development, is divided into three major periods: the Stone Age, the Bronze Age and the Iron Age (1). The Iron Age sites of the Mun River Valley are among the most important and significant sites in Southeast Asia for documenting the rise of the state (2). So, the additional information about the human who live at that time was such necessary. The prehistoric cemetery at Noen U-Loke was dated in the vicinity of the Late Bronze age and the Iron Age about 3,000-1,500 years before present (2-3). The cultural sequence at Noen U-Loke is one of the longest continuous records in Southeast Asia (2). There was possible to trace back to the history of an Iron Age community. The mortuary sequence at Noen U-Loke Amphoe Non Sung, Nakhon Ratchasima was divided into five phases ranging in depth from 4.2 to 0.95 meters below the present surface of the mound. Knowledge of the prehistory was greatly increased by much residential and industrial evidence. The study of prehistoric human remain is an important aspect of archaeology and provide the biological anthropologist with a way to reconstruct the life histories, culture and behavior of the prehistoric people. In order to add more understanding, the present study aims to generate additional information about the relationship between the prehistoric human populations. An important tool for this investigation was DNA analysis. Previous

studies have shown that DNA can survive in ancient remains, and that the best subjects for genetic investigations are bone and tooth samples (4). This study addressed on tooth samples because they are often the best preserved part and sometime the only part of the skeleton to be recovered from archaeological sites. For more reason, the intact tooth could prevent the inside truly ancient DNA from outside foreign DNA that causes possible contamination. The twenty-six molar teeth of good quality were collected from twenty-six adult skeletons one each from Noen U-Loke archaeological site. All samples obtained in this study were from phase 2, 3, 4 and 5. No available grave was found in phase 1. One, six, nine and nine teeth samples were from phase 2, 3, 4, and 5 respectively.

Human DNA consists of nuclear DNA and extranuclear DNA or mitochondrial DNA (mtDNA). The study of anthropology often investigate in several part of DNA, intergenic COII/tRNA^{LYS} 9-bp deletion, β -globin gene, Y-chromosome in nuclear DNA and mitochondrial DNA etc (5-11).

Human mitochondrial DNA is a circular double stranded DNA consisting of coding regions and non-coding region (12). Because of its polymorphic nature, maternal inheritance with little or no contribution from the father (13), very well known sequence information, resistance to extreme environmental conditions and high copy number per cell, human mitochondrial DNA has proved to be more suitable for typing ancient material (14-17). The most suitable region of mitochondrial DNA for the use in research of ancient remains is in the non-coding region, or displacement loop (D-loop) called the hypervariable region (HV). This region is most suitable because of the very important feature that the nucleotide sequences in this region vary between individuals but have a high similarity within the maternal line. The high mutation rate combined with the maternal mode of transmission allows comparison of mitochondria sequence between maternally related individuals. Also, its sequence evolution is much high which results in the ability to discriminate between relatively separated population (17-20). So, the present study focused on mitochondrial hypervariable segment 1 (HVS-1) in the non-coding region.

To determined the relationship between 1,500-2,400 year-old human population from Noen U-Loke and modern population. The modern samples from several ethnic groups in Thailand and from several part of China were compared and

analyzed by phylogenetic analysis using PAUP* version 4.0 packaged program. Nucleotide diversity and evolutionary distance using Molecular Evolutionary Genetics Analysis program version 3.0 (MEGA3), and mitochondrial haplogroup were also determined.

In summary, the objectives of this study are 1) to develop procedures to successfully extract DNA from prehistoric human samples in Thailand and 2) to determine the relationship of 1,500-2,400 years-old prehistoric human population from Noen U-Loke site, Nakhon Ratchasima to modern peoples from several populations.

CHAPTER II

LITERATURE REVIEW

Archaeological study in Thailand has made a good progress from time to time in increasing the understanding of the prehistory of the people living in the area called “Thailand” at present. The basic culture, social, technological, and economic prehistoric sequence have been discovered. The excavations of many sites in Thailand (Lopburi, Kanchanaburi, Khon Kaen, Nakhon Ratchasima, and Ban Chiang) have shown evidence of rice cultivation by the prehistoric farmer, for example, implements, rice grain or even rice chaff (2). Evidence of settlement by rice cultivators and rise of state has been found in river valley areas, which have highly suitable conditions for rice cultivation and agriculture. The Mun River Valley is one of the most important areas in northeast Thailand for such evidence (Figure 1). Excavations in this particular area have been conducted. The multi-disciplinary research was conducted as part of the “Origin of Angkor Archaeological Project”, undertaken by the University of Otago Department of Anthropology and the Fine Arts Department of Thailand from 1995 to 1998 (www.otago.ac.nz/Anthropology/Angkor.html). The research program was designed to provide a basic culture and chronological sequence for the upper Mun River Valley during prehistory, with specific reference to identifying any social, technology and economic changes leading towards the development of states. It is well known that this region comprised part of the Angkorian state. Three sites around the upper Mun River Valley have been excavated which are Noen U-Loke, Ban Lum Khao and Prasat Phimai (Figure 2).

The prehistory of Thailand, on the basis of technological development, is divided into three major periods: the Stone Age dated over 2,000 BC, the Bronze Age dated between 1,500-500 BC and the Iron Age dated between 500 BC.-500 AD (1). The late Neolithic and the Bronze age was documented at Ban Lum Khao, at Noen U-Loke represented the Iron Age and the Angkorian period could be seen at late Prasat Phimai, 800 AD.-1,300 AD.



Figure 1. The map of Thailand. Mun River Valley area locates in Nakhon Ratchasima province or Korat in the northeast region.

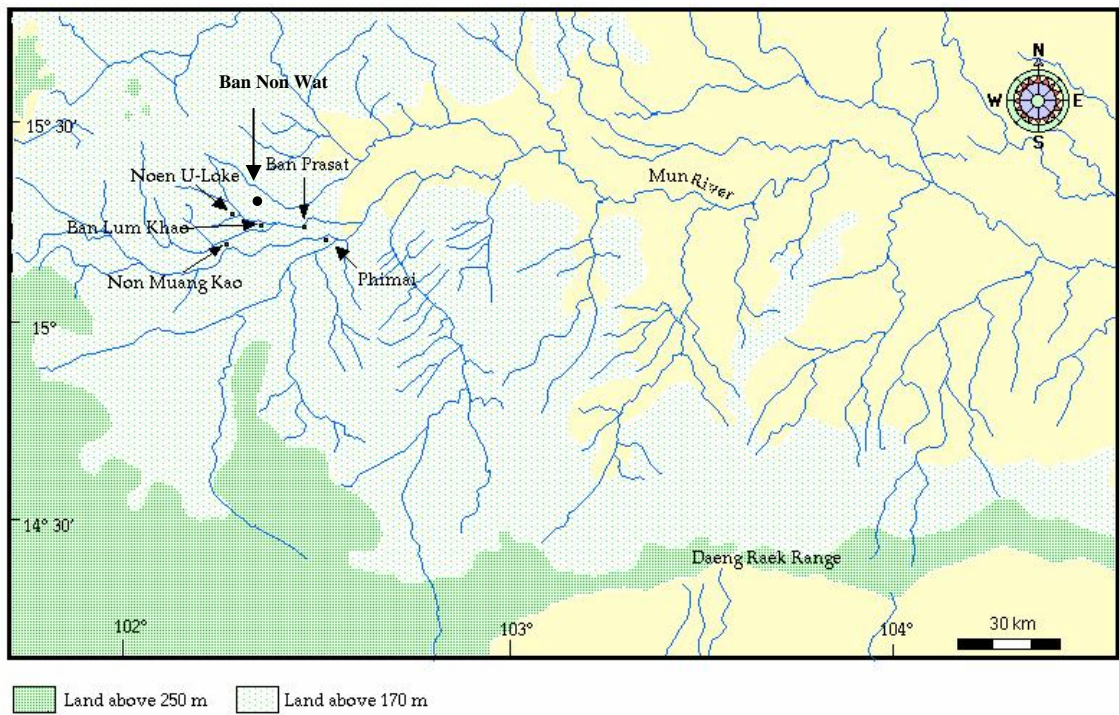


Figure 2. The Mun River Valley area

The major change in the vicinity of late Iron Age 200 AD could be seen from very rich graves of men and women associated with the quantities of the ornament made from bronze, silver and gold jewelry and the development of a very impressive ceramic industry. This was followed by a period when agricultural implements increased, suggesting intensification of rice cultivation. The Iron Age sites of the Mun River Valley are among the most important and significant sites in Southeast Asia for documenting the rise of the state (2).

The prehistoric cemetery at Noen U-Loke, from seventeen remains radiocarbon dates in four layers by Dr. Thomas Higham, Waikato University, New Zealand, was dated in the vicinity of the Late Bronze age and the Iron Age about 3,000-1,500 years before present (2-3). The cultural sequence at Noen U-Loke is one of the longest continuous records in Southeast Asia which contained evidence for industrial, occupation and mortuary activity (2). The industrial activity involved the construction of a series of clay-lined furnaces equipped with tuyeres that unsure of their precise function, but they were clearly used on several successive occasions. The occupation remains included rows of deep postholes, hearths and pits both of which have provided large samples of carbonized rice. Many of the pottery vessels and potsherds showed evidence for a white residue on the interior that has been identified as a resin of probably dipterocarp origin. The most interesting, in terms of assessing aspects of social organization at this site, derives from the 126 burials (see details in Table 1). There was possible, on the basis of uncovered 126 inhumation graves and much residential and industrial evidence, to trace back the history of an Iron Age community. The mortuary sequence at Noen U-Loke Amphoe Non Sung, Nakhon Ratchasima was divided into five phases ranging in depth from 4.2 to 0.95 meters below the present surface of the mound. Phase number 5 depth was 0.55-1.77 meters below the datum and dated between 300-500 AD. Phase number 4 was 1.54-3.65 meters from surface and was dated between 200-400 AD. Phase number 3 was 2.18-3.32 meters from surface and was dated between 200 BC.-200 AD. Phase number 2 was 3.65-4.12 meters from surface and was dated between 400 BC.-100 BC. Phase number 1 was the deepest by 3.8-4.3 meters below the datum and was the oldest, dated over 500 BC in the vicinity of the Late Bronze Age. The earliest mortuary phase, phase number 1, was represented by part of only one grave appeared in the square Mortuary Phase 2

Table 1. The details of the uncovered 66 inhumation graves. From all 126 inhumation graves, 60 graves are neonate or infant which were excluded from the investigation. This table showed the details only 66 samples of adult skeleton.

Burial number	Mortuary Phase	Skeleton dates (BP)	Depth below datum (m)	Sex of skeleton	Age of skeleton at death (year)	Status
26	2	>2,500	3.65	♂	mid-adult	available
27	2	>2,500	4.12	♂	mid-old	not available
107	2	2,400-2,100	3.99	♂	young male	not available
108	2	2,400-2,100	4.10	♀	>40	not available
114	2	2,400-2,100	4.09	♀	35-40	not available
29	3	2,200-1,800	2.51-2.61	?	3-6	not available
33	3	2,200-1,800	2.62	♂	25-30	available
35	3	2,200-1,800	2.59	♀	35-40	not available
36	3	2,200-1,800	3.14	♀	20-25	not available
37	3	2,200-1,800	3.14	♀	>40	not found
39	3	2,200-1,800	2.65	♂	20-25	not available
40	3	2,200-1,800	2.71	♀	20-25	not available
41	3	2,200-1,800	3.32	?	7-11	available
42	3	2,200-1,800	3.32	♂	>40	not available
44	3	2,200-1,800	3.21	♂	young-adult	available
45	3	2,200-1,800	2.70	♂	25-30	available
48	3	2,200-1,800	2.81	♀	25-30	available
49	3	2,200-1,800	2.92	♀	35-40	not available
50	3	2,200-1,800	2.91-3.10	♂	20-25	available
52	3	2,200-1,800	2.88	♀	>40	not found
53	3	2,200-1,800	3.03	♂	adult	not found
54	3	2,200-1,800	2.92	?	adult	not found
55	3	2,200-1,800	3.03	?	adult	not found
14	4	1,800-1,600	2.01	♂	35-40	not available
16	4	1,800-1,600	2.08	♀	20-25	available
20	4	1,800-1,600	2.45	♂?	adult	not found
21	4	1,800-1,600	2.26	♂?	adult	not found
31	4	1,800-1,600	2.18	?	~12	available
62	4	1,800-1,600	1.54	♀	35-40	available
69	4	1,800-1,600	1.82	♂	25-30	available
74	4	1,800-1,600	2.29	♂	25-30	available
75	4	1,800-1,600	2.01-2.48	?	16-20	not found
76	4	1,800-1,600	2.48	♂	adult	not found

Table 1 (continued). The details of the uncovered 66 inhumation graves. From all 126 inhumation graves, 60 graves are neonate or infant which were excluded from the investigation. This table showed the details only 66 samples of adult skeleton.

Burial number	Mortuary Phase	Skeleton dates (BP)	Depth below datum (m)	Sex of skeleton	Age of skeleton at death (year)	Status
78	4	1,800-1,600	2.39	♂?	30-35	not found
82	4	1,800-1,600	2.45	♀	35-40	not available
84	4	1,800-1,600	2.43	♂	adult	not available
86	4	1,800-1,600	2.30	♂	25-30	not available
87	4	1,800-1,600	-	?	adult	not available
94	4	1,800-1,600	-	♂	>40	not available
96	4	1,800-1,600	2.61	♂?	adult	not found
98	4	1,800-1,600	2.52	♂	adult	not available
99	4	1,800-1,600	2.43	♀	>40	available
101	4	1,800-1,600	-	?	-	not found
104	4	1,800-1,600	1.84	♀	25-30	available
105	4	1,800-1,600	1.80-2.57	♂	adult	not found
110	4	1,800-1,600	2.95	♀	37-42	not available
111	4	1,800-1,600	2.57	♂	25-30	available
113	4	1,800-1,600	3.65	♀	25-30	available
118	4	1,800-1,600	2.48	?	14-16	not available
124	4	1,800-1,600	-	?	adult	not found
1	5	1,700-1,500	1.45	♂	35-40	not available
4	5	1,700-1,500	0.76	♀	15-18	available
5	5	1,700-1,500	0.82	♂	20-25	available
8	5	1,700-1,500	1.27	?	25-60	available
10	5	1,700-1,500	1.22	♀	>40	available
12	5	1,700-1,500	0.93	♀	>40	available
57.1	5	1,700-1,500	0.55	?	12	not available
57.2	5	1,700-1,500	0.55	?	16-17	not available
59	5	1,700-1,500	-	♀?	adult	available
60	5	1,700-1,500	1.09-1.39	♂	20-25	available
61	5	1,700-1,500	1.27	♂	young	not available
66	5	1,700-1,500	1.52	♂	35-40	available
67	5	1,700-1,500	1.57	♂?	20-26	not available
68	5	1,700-1,500	1.25	♂	25-30	available
72	5	1,700-1,500	-	?	adult	not available
73	5	1,700-1,500	1.77	?	35-40	not available

incorporates six interments (Figure 3). The third mortuary phase incorporated a cluster of inhumation graves with the head pointing to the northeast (Figure 4). Apart from the tight clustering and different orientation, the first evidence for exotic glass beads and agate jewelry were found. One person had been buried prone, all others were supine. Another was found with an agate neck pendant, which was to recur in all later phases, while some of the others individuals wore bronze jewelry: finger and toe rings, bangles, earrings. The highest burial in this group was a lidded jar containing the remains of an infant. The third mortuary phase again incorporates a cluster (Figure 4). The first carnelian was recovered and bronze ornaments were common but there were few pottery vessels or iron artifacts. There are assuredly a number of changes in mortuary ritual in this phase. Although the same orientation (Figure 4), the dead were now buried on rice beds. This involved filling the base of the grave with a layer of burnt rice before interring the body. A further layer of rice then covered the human remains to a present depth of about 10 cm. Several individuals, possibly women, were buried with spindle whorls. The fourth mortuary phase was, again, interred in rice beds with the head pointing to the north or south (Figure 5). The richest, burial number 14, was associated with one of the most remarkable assemblages of bronzes. This adult male was found in association with bronze earrings. A bimetallic bronze and iron ring was found in the neck area. On each arm, there were approximately 75 bronze bangles covering the body from the elbow to the shoulder while the finger bones were covered with rings. Three belts of circular bronze were found round the waist, and the toes bore large, bronze rings. In addition to these finds, the body was associated with pottery vessels, glass beads in the area of the neck, chest and ankles, and an iron knife. An infant buried nearby was also richly endowed with bronze and glass ornaments. The final phase, the orientation remained with the head to the north but rice beds were no longer in evidence (Figure 6). Continuity was seen in the use of jar burials for infants, and the presence of agate pendants and beads in the neck area, and the bronze jewelry. A feature of these later burials was the marked increase in iron implements, which include knives, sickles and in one case, a socketed spearhead. Glass beads became extremely rare however and no carnelian was recovered. These findings support the hypothesis that the Iron Age communities recognized status differentials in the mortuary ritual. The preliminary look at the faunal remains from Noen U-Loke

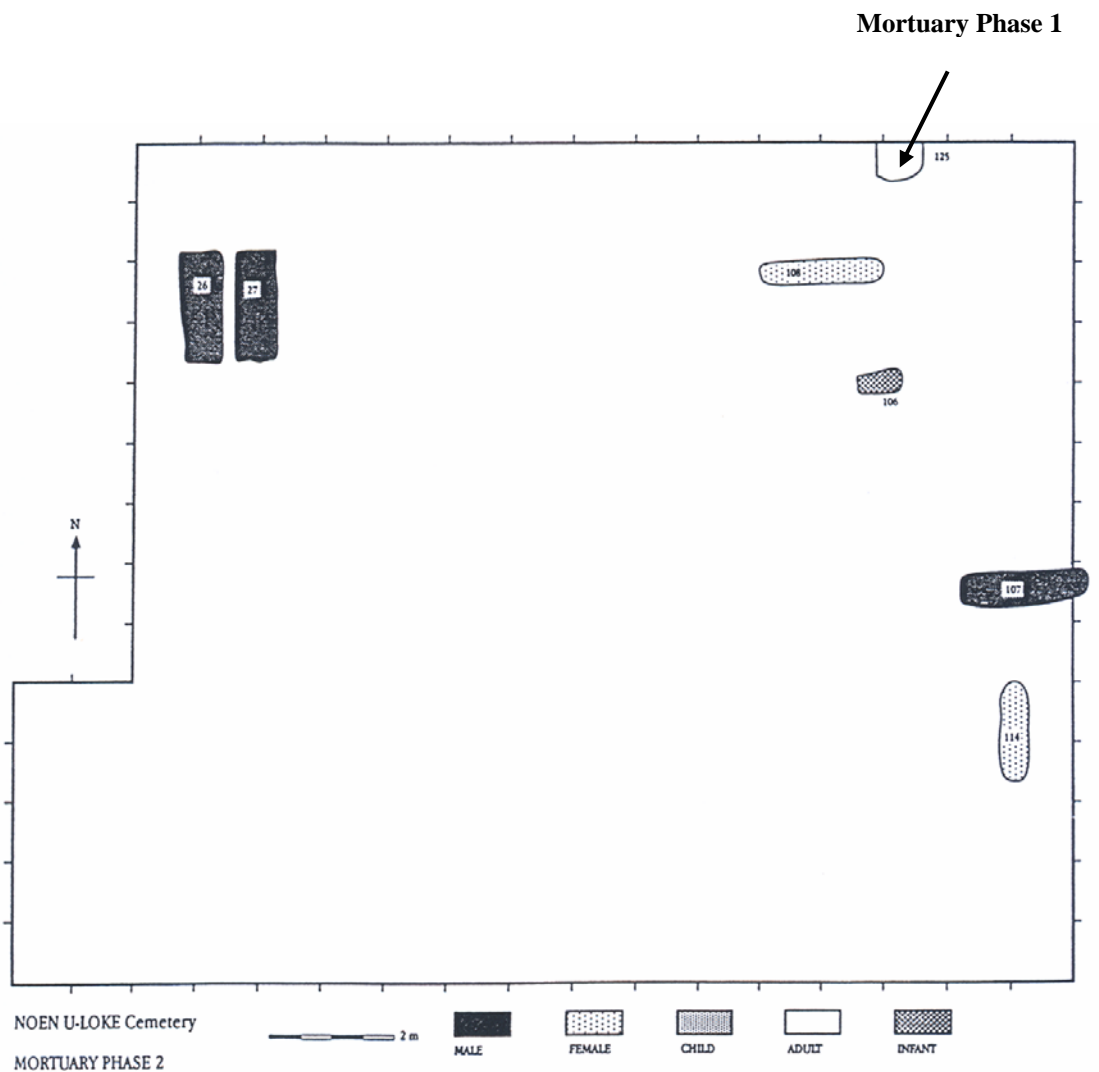


Figure 3. The diagram show position and number of each of burial in mortuary phase 1 and 2. The orientation of head of skeletons in these phase were illustrated.

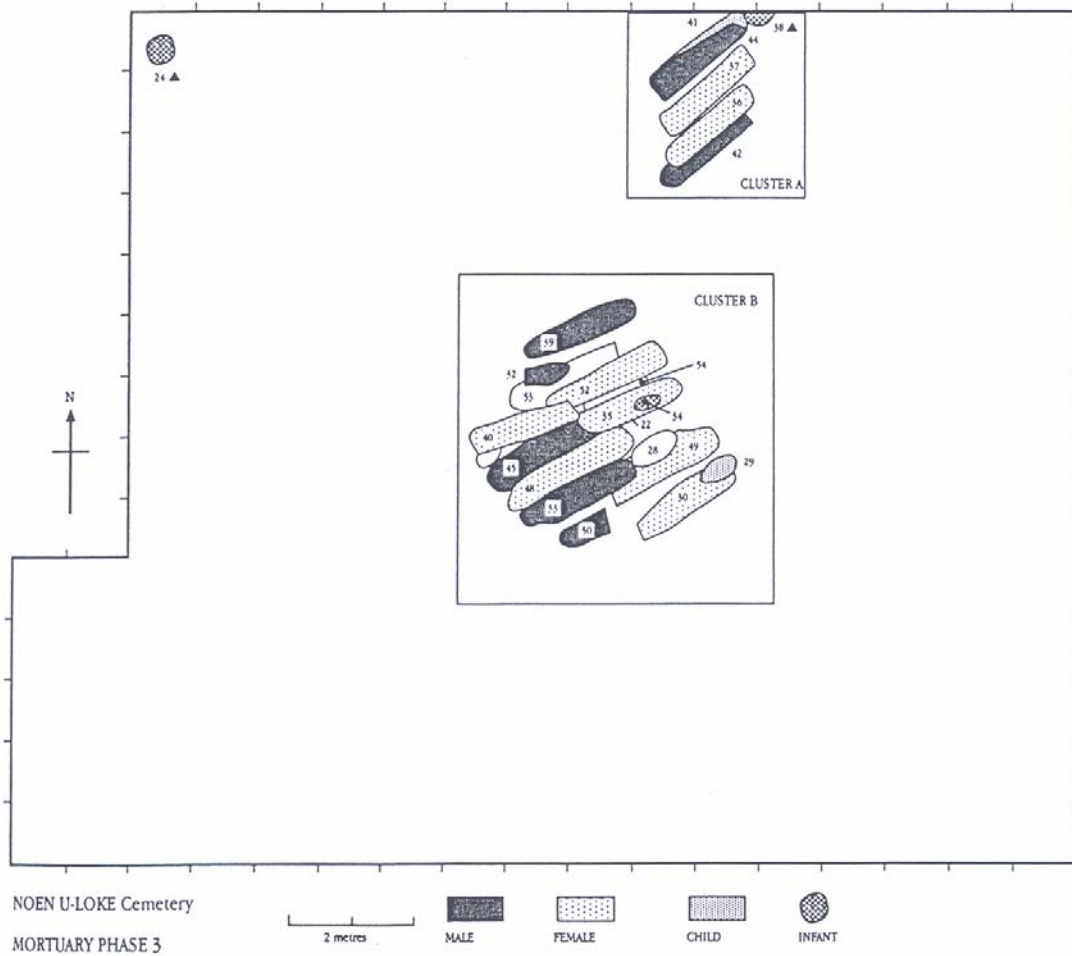


Figure 4. The diagram show position and number of each of burial in mortuary phase 3. The orientation of head of skeleton and clusters of burials in these phase were illustrated.

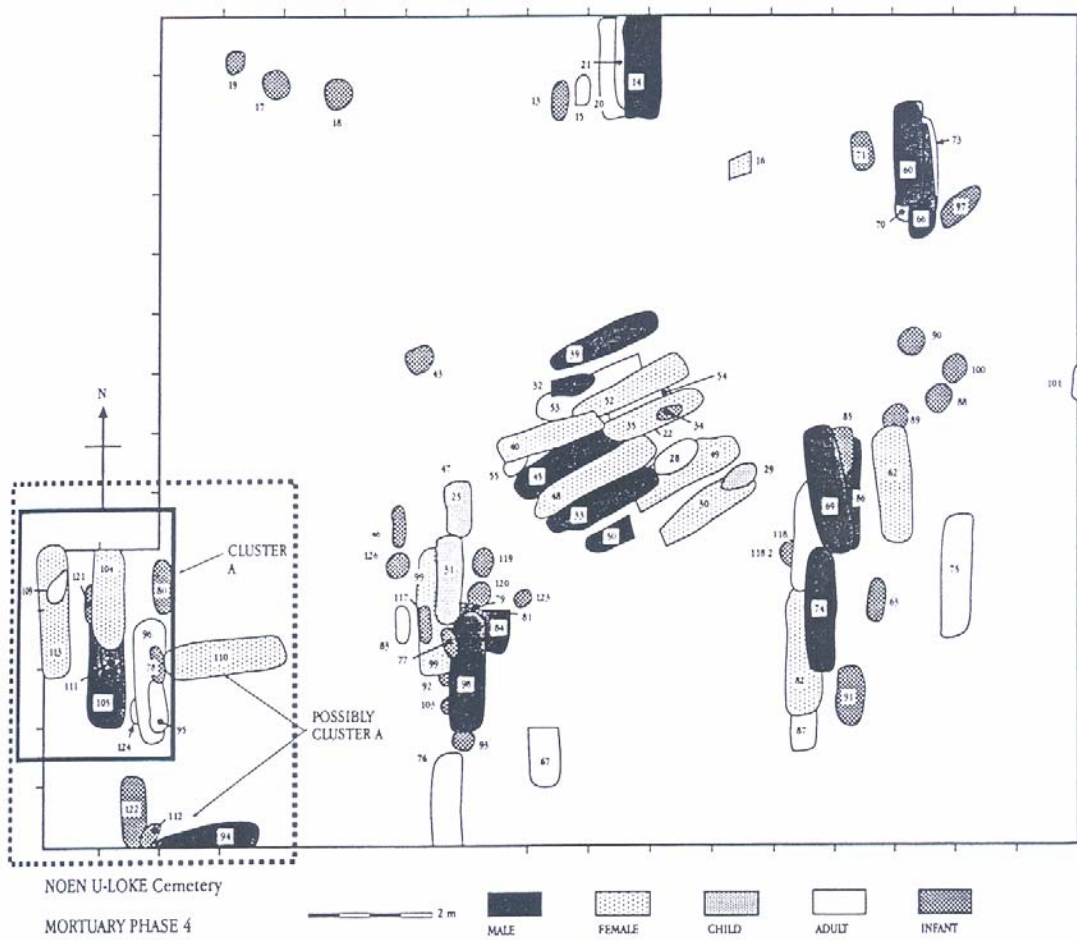


Figure 5. The diagram show position and number of each of burial in mortuary phase 4. The orientation of head of skeletons in these phase were illustrated.

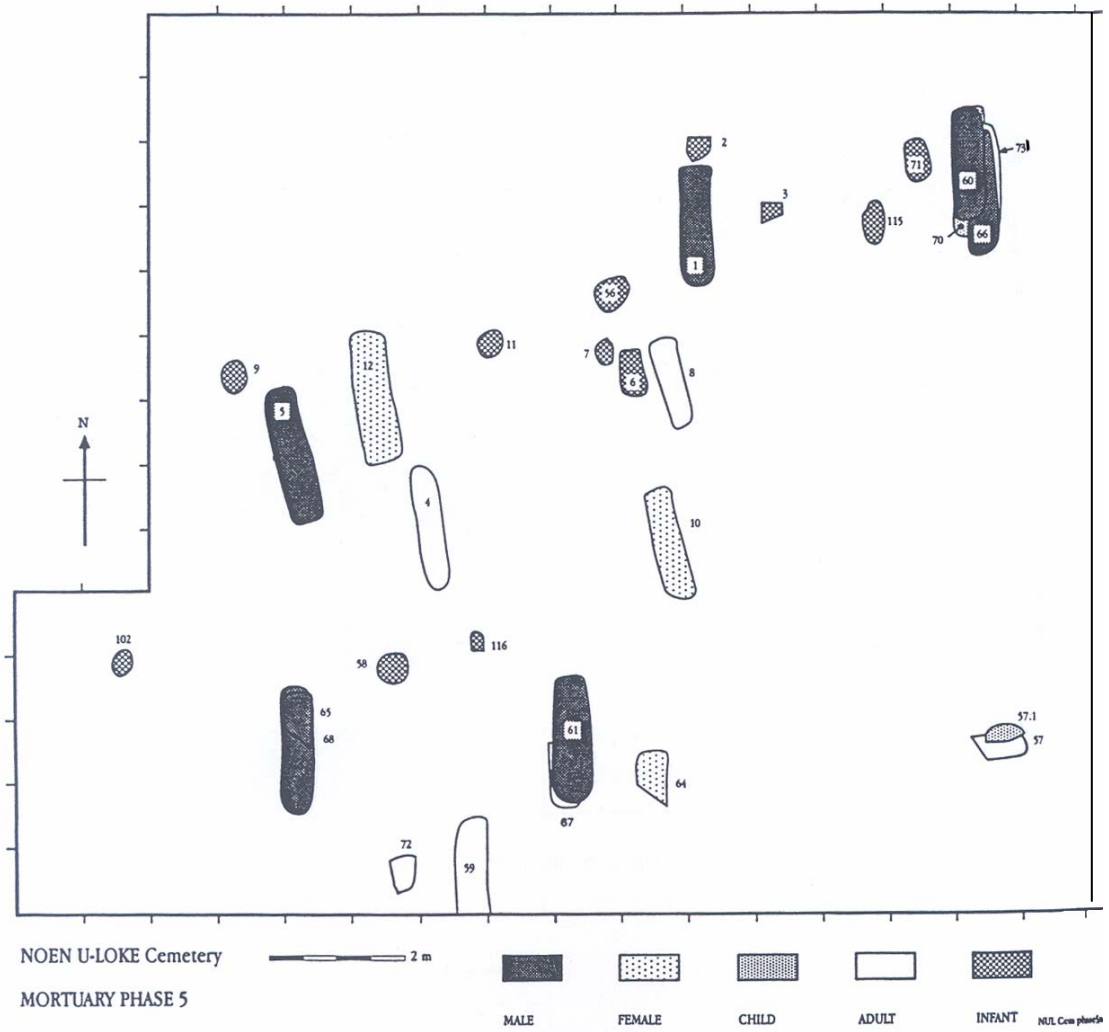


Figure 6. The diagram show position and number of each of burial in mortuary phase 5. The orientation of head of skeletons in these phase were illustrated.

provided some surprises. Wild animals were extremely rare but the overwhelming majority of remains come from quite small domestic cattle. It seems that the occupants of the site during the Iron Age concentrated on rice cultivation and cattle breeding. The Iron Age communities of the Mun River Valley witnessed a series of changes which reflect increasing social complexity latent in a transition to the state. These include larger population numbers, a concentration of settlements in favored low-lying riverine swamplands suited to rice cultivation, the production and concentration of symbols of wealth and status in the hands of individuals, the establishment of iron smelting and forging within settlements which involved a high level of skill, and the availability of a new suite of exotic goods which could have been used as emblems of status. This finding, linked with the presence of iron weapons in the latest mortuary phases, was compatible with a concentration on rice cultivation and situation of inter-community friction.

Knowledge of the prehistory of the upper Mun River Valley was also greatly increased by much residential and industrial evidence. The study of prehistoric human remain is an important aspect of archaeology to fully comprehend prehistoric culture and behavior. Human skeletons from archaeological site provide the biological anthropologist with a way to reconstruct the life histories of the prehistoric people. In order to further understanding, investigation of the relationship between the prehistoric human populations was also important. An important tool for investigation in this study was the DNA analysis.

Previous studies have shown that DNA can survive in ancient remains, and that the best subject for genetic investigations are bone and tooth samples because they are abundant and generally well preserved (4). This study addressed on tooth samples because they are often the best preserved part and some time the only part of the skeleton to be recovered from archaeological sites. For more reason, the intact teeth could prevent the inside truly ancient DNA from outside foreign DNA that causes possible contamination during the excavation. Noen U-Loke contained very well preserved material, and all the samples were collected according to the highly systematic protocol. The quality and the age of the remains provided an opportunity to identify any surviving human DNA in these remains.

Mature human had 32 teeth (Figure 7). The position of these teeth has been identified according to the International Dental Federation (FDI) nomenclature. The identification was illustrated by 2 numbers. The first number identifies the quadrant: (1= maxillary right, 2= maxillary left, 3= mandibular left, 4= mandibular right) and the second number identifies the tooth. (1= central incisor, 2= lateral incisor, 3= canine etc. up to 6= first molar, 7= second molar and 8= third molar). For example, tooth 48 is the mandibular right third molar. Human tooth is made up of four different types of tissue: cementum, enamel, dentin and pulp (Figure 8). Cementum is as hard as bone covers the outside of the root, under the gum line, and holds the tooth in place within the jaw bone. Enamel, the hardest tissue in the body, covers the dentin and forms the outermost layer of the crown. It enables the tooth to withstand the pressure of chewing and protects it from harmful bacteria and changes in temperature from hot and cold foods. Dentin surrounds the pulp. A hard yellow substance consisting mostly of mineral salts and water, it makes up most of the tooth and is as hard as bone. Pulp is the innermost portion of the tooth and consists of connective tissue, nerves, and blood vessels, which able to extract DNA from them.

Human DNA consists of nuclear DNA and extranuclear DNA, or mitochondrial DNA (Figure 9). Because of its polymorphic nature, maternal inheritance and very well known sequence information, human mitochondrial DNA has proved to be more suitable for typing ancient material and has become important in anthropological and evolutionary research (14-17). Mitochondrial DNA comes from the oocyte, and thus represents only the maternal ancestry of an individual, with little or no contribution from the father (13). Furthermore, the mitochondrial DNA resistance to extreme environmental conditions and high copy number per cell makes it easier to recover than the single copy of nuclear DNA (Figure 9). Each mitochondrion contains 5-10 copies of mitochondrial DNA, which are located in the matrix phase (Figure 10). Human mitochondrial DNA is a circular double stranded DNA consisting of 16569 base pairs (bp) in length (12). The coding regions, which code for 37 genes, and a non-coding region, are the two main parts of human mitochondrial DNA (Figure 11). The most suitable region of mitochondrial DNA for use in research of ancient remains is in the non-coding region, or displacement loop (D-loop), which is approximately 1100 bp in length and covers nucleotide numbers 16024 to 576 (21), called the hypervariable region.

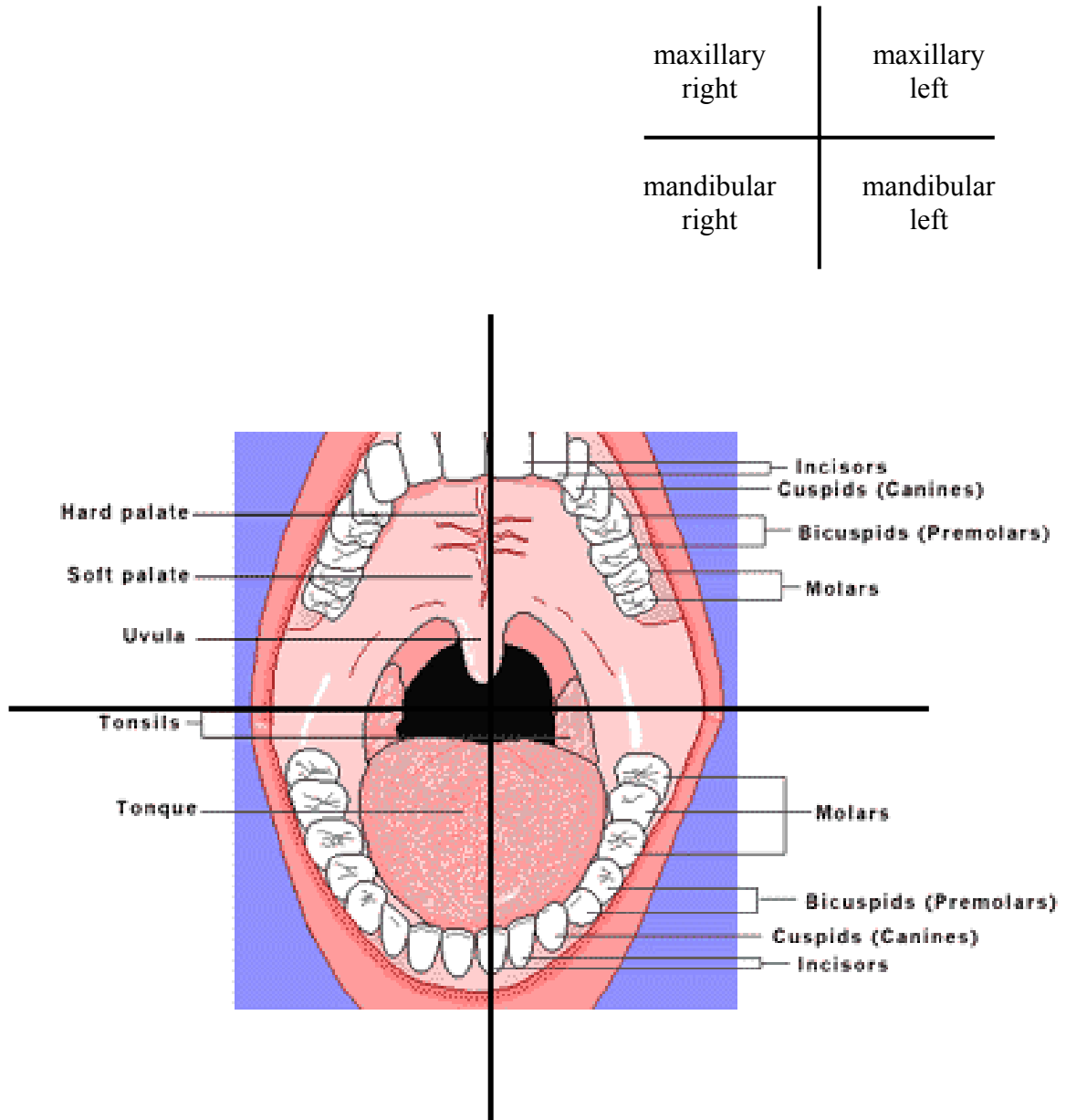


Figure 7. The mouth cavity showing position and name of each of the teeth

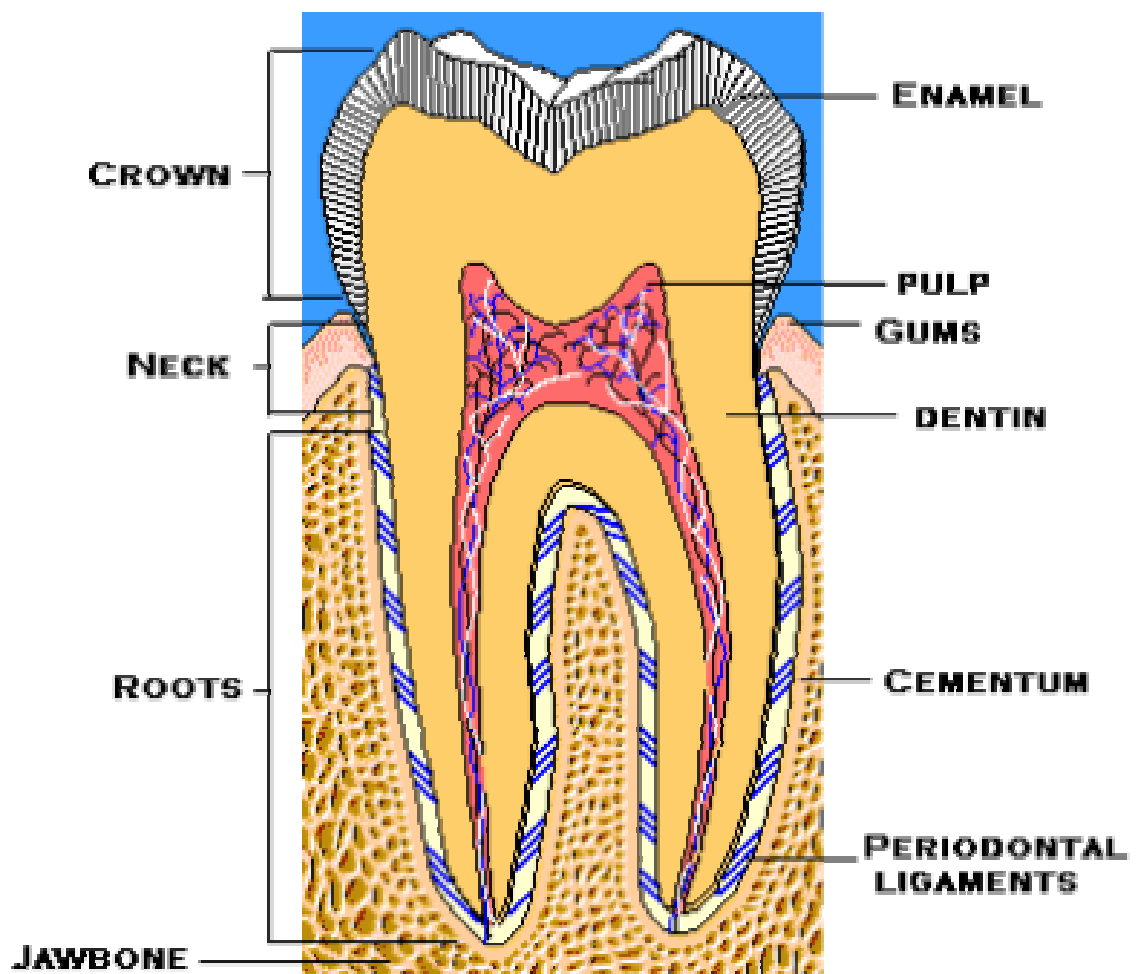


Figure 8. The component of the tooth. It is made up of four different types of tissue: cementum, enamel, dentin and pulp. Pulp is the soft tissue, containing nerves and blood vessels which able to extract DNA from them.

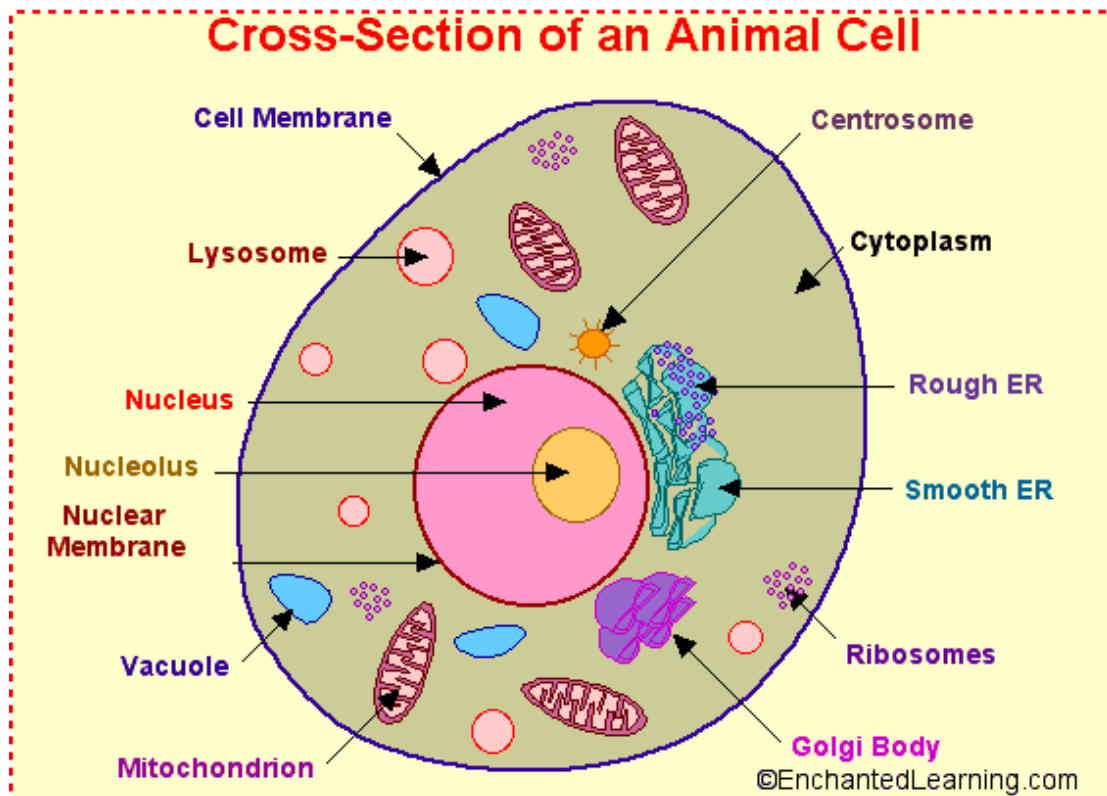


Figure 9. Cross section of an animal cell. A cell consists of a single copy of nuclear DNA in a nucleus and many copies of mitochondrial DNA in a mitochondrion. In one cell composed of high numbers of mitochondria resulting in high copies of mitochondrial DNA.

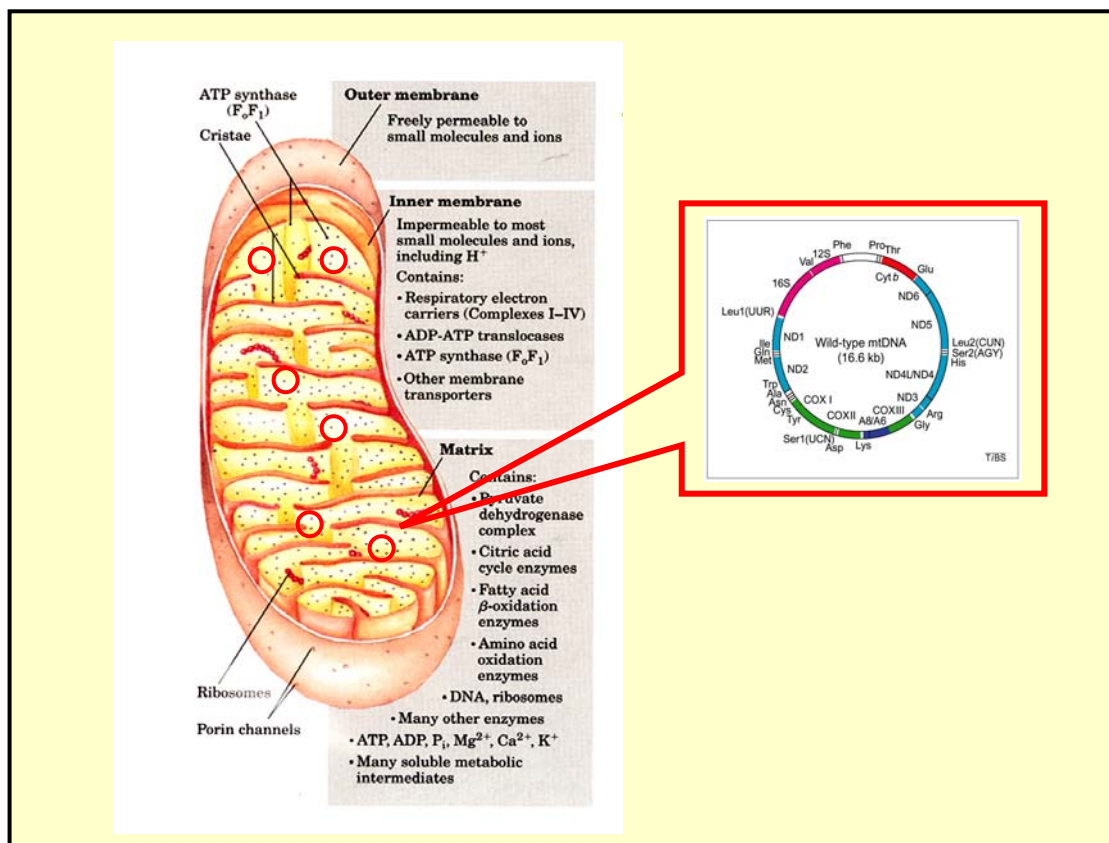


Figure 10. The component of mitochondrion and the multiple copies of mitochondrial DNA inside matrix

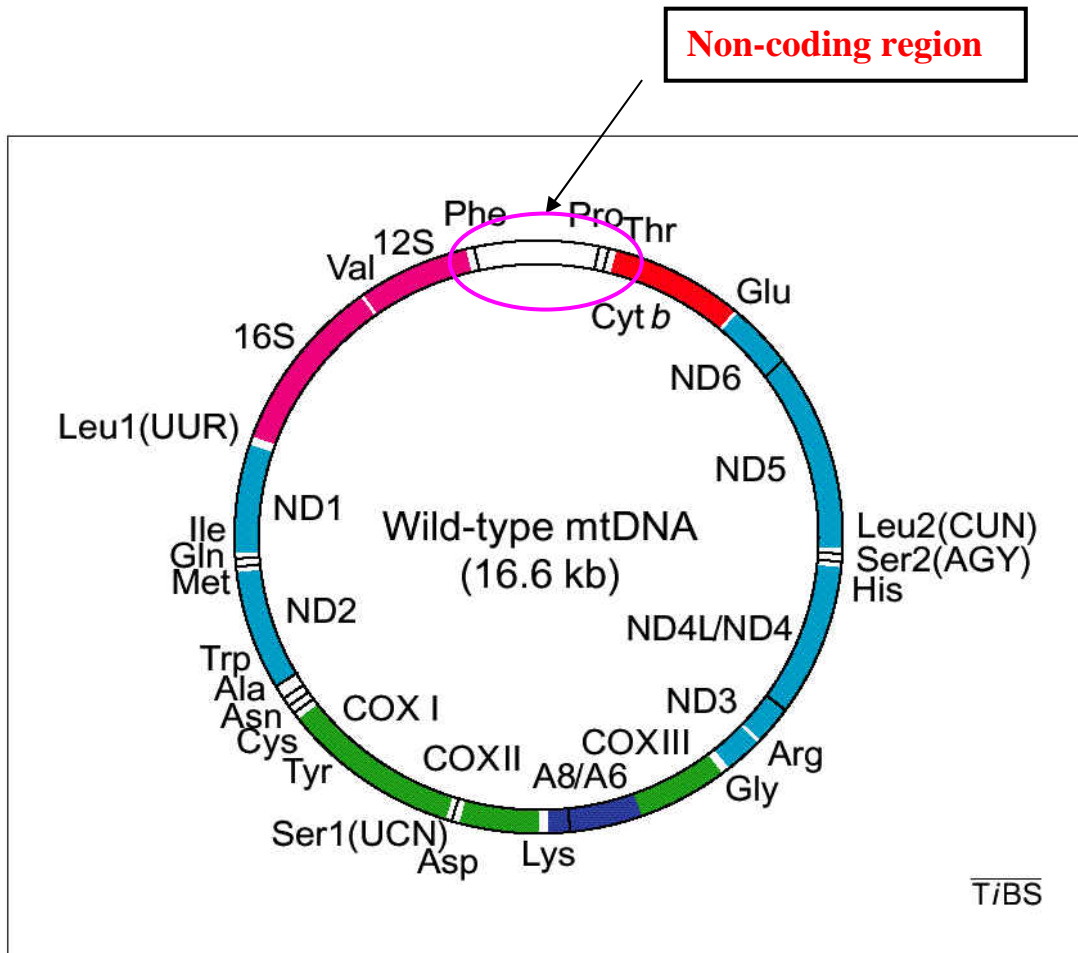


Figure 11. Mitochondrial DNA consists of coding region and non-coding region. These are the two main parts of the circular double stranded mitochondrial DNA. The non-coding region or displacement loop (D-loop) codes for no proteins but has highly mutation rate which was useful for population research.

The hypervariable region within the D-loop region separates into segment I (HVS-1) from nucleotide position 16024 to 16383 (360 bp) and segment II (HVS-2) from nucleotide position 57 to 372 (316 bp) of Cambridge Reference Sequence or CRS (22). The mutation rate of mitochondria DNA in this area is 5-10 times higher than that of nuclear DNA (23-25). This region is most suitable because of the very important feature that the nucleotide sequences here vary between individuals but have a high similarity within the maternal line. The high mutation rate combined with the maternal mode of transmission allows comparison of mitochondria sequence between maternally related individuals, and is routinely employed for identification purposes. Also, its sequence evolution is very high results in the ability to discriminate between relatively separated populations (17-20).

The previous study, using the silica-based method for DNA extraction, showed that mitochondrial DNA extracted from teeth from forensic casework provided the same mitochondrial DNA sequence in every instance (26). The same sequence patterns were also observed for each tissue type tested including samples that had undergone a variety of degrade processes. These studies demonstrated that the mitochondrial DNA extracted from teeth using silica-based method is adequate for genetic analysis. So, the present study was designed to extract DNA from ancient teeth by the same silica based method.

The story of the prehistoric people and their migration are mystery. During recent decades, a number of studies have reported the genetic structures of the modern populations and their phylogenetic relationship was debated. However, the story of the prehistoric people, their migration and the population history are still mystery and controversy. This might be due to the lack of direct genetic information about ancient people, since comparison was made only from genetic information of modern humans. Information provided by ancient mitochondrial DNA has been regarded as one of the most powerful methods to understanding and reconstructing the past from the genetic perspective (27). The molecular cloning of DNA from a quagga (28) and Egyptian mummy (29) were the first successes in the retrieval of ancient DNA sequences. However the amounts of DNA present in the old tissues were so small that the isolation was impossible (30). The development of the polymerase chain reaction (PCR) made it possible to produce essentially unlimited numbers of copies from very

few or even single original DNA copies. However, the PCR had two technical complications. The first complication was evident from the fact that when PCR was used to reexamine the cloning technique, some sequences were shown to be incorrect in the original sequences (31). The second complication was evident from work (32) showing that contemporary DNA can be contaminant. DNA contaminations give rise to erroneous DNA sequences. Had many strategies to combat these problems and authenticate the ancient DNA.

The first published criteria of authentication of ancient DNA (32) were limited. But the present criteria of authenticity have been continuously extended (33-36). A substantial list of criteria now exists 1) Extraction controls and PCR controls; 2) Repeated amplifications from the same or several extracts; 3) Inverse correlation between amplification efficiency and length of amplification; 4) Cloning of amplification products and sequencing of multiple clones; 5) Quantitation the number of amplifiable DNA molecules; 6) Biochemical assays of macromolecular preservation; 7) Exclusion of nuclear insertions of mtDNA and 8) Reproduction in a second laboratory (37-41). For the present study, the authenticity was mentioned only on the first 3 criteria from total of 8 criteria that referred. In addition, the authenticity herein present study was addressed by sequencing the products from the repeated amplification. The sequence of them were aligned and reliably. For more authenticity, the ancient DNA sequences of each of samples were aligned with DNA sequences of investigators in field-work. All amplification results and sequencing results with no authenticity were discarded from the study.

CHAPTER III

MATERIALS AND METHODS

Materials

1. Ancient samples

The second or third molar tooth of good quality was used for ancient mitochondrial DNA extraction. The twenty-six molar teeth were each collected from twenty-six adult skeletons. All of them were from excavated site at Noen U-loke Amphoe Non Sung, Nakhon Ratchasima. The excavation was undertaken by the University of Otago Department of Anthropology and the Fine Arts Department of Thailand from 1995 to 1998. This prehistoric cemetery, from seventeen remains that had been radiocarbon dated in four layers by Dr. Thomas Higham, Waikato University, New Zealand, was dated in the vicinity of the Late Bronze and Iron Age, about 3,000-1,500 years before present. The mortuary sequence was divided into five phases. The first phase was the oldest and deepest in the ground. All samples obtained in this study were from phase 2, 3, 4 and 5. An only foot bone of one skeleton was found in phase 1. One, six, nine and nine teeth samples were from phase 2, 3, 4, and 5 respectively. For more information the details of each of ancient samples are shown in Table 2 and in the book, “The Origin of the Civilization of ANGKOR”.

2. Modern human samples

The modern samples in this study consists of 1) 50 local individuals of Ban Lum Khao and Ban Nonwat, Amphoe Nonsung, Nakhon Ratchasima; 2) 20 individuals of Chao Bon, the ethnic group living in Amphoe Pugthongchai, Nakhon Ratchasima and 3) 22 Khmer individuals who live in the Thai-Cambodia border area in Chantaburi. All of these 92 individuals have given their informed consent and are not related within their groups when the pedigree was traced back at least three generations.

Table 2. The details of each of ancient teeth from Noen U-Loke, the archaeological site in Amphoe Phimai, Nakhon Ratchasima

No	Burial number	Mortuary Phase	Skeleton dates (BP)	Depth below datum (m)	Sex of skeleton	Age of skeleton at death (year)	Position of teeth*
1	26	2	2,400-2,100	3.65	♂	mid-adult	46
2	33	3	2,200-1,800	2.62	♂	25-30	38
3	41	3	2,200-1,800	3.32	?	7-11	37
4	44	3	2,200-1,800	3.21	♂	young-adult	48
5	45	3	2,200-1,800	2.70	♂	25-30	38
6	48	3	2,200-1,800	2.81	♀	25-30	47
7	50	3	2,200-1,800	2.91-3.10	♂	20-25	36
8	16	4	1,800-1,600	2.08	♀	20-25	48
9	31	4	1,800-1,600	2.18	?	~12	27
10	62	4	1,800-1,600	1.54	♀	35-40	48
11	69	4	1,800-1,600	1.82	♂	25-30	38
12	74	4	1,800-1,600	2.29	♂	25-30	48
13	99	4	1,800-1,600	2.43	♀	>40	48
14	104	4	1,800-1,600	1.84	♀	25-30	46
15	111	4	1,800-1,600	2.57	♂	25-30	38
16	113	4	1,800-1,600	3.65	♀	25-30	N/A
17	4	5	1,700-1,500	0.76	♀	15-18	37
18	5	5	1,700-1,500	0.82	♂	20-25	N/A
19	8	5	1,700-1,500	1.27	?	25-60	N/A
20	10	5	1,700-1,500	1.22	♀	>40	47
21	12	5	1,700-1,500	0.93	♀	>40	38
22	59	5	1,700-1,500	-	♀?	adult	48
23	60	5	1,700-1,500	1.09-1.39	♂	20-25	28
24	66	5	1,700-1,500	1.52	♂	35-40	48
25	68	5	1,700-1,500	1.25	♂	25-30	46
26	15	N/A	N/A	-	?	N/A	38

* The position of teeth has been identified according to the International Dental Federation (FDI) nomenclature. This means that the first number identifies the quadrant (1=maxillary right, 2=maxillary left, 3=mandibular left, 4=mandibular right). The second number identifies the tooth (1=central incisor, 2=lateral incisor, 3=canine etc up to 6=first molar, 7=second molar and 8=third molar). For example, tooth 48 is the mandibular right third molar.

3. Oligonucleotide primers

The oligonucleotide primers used in this study for the amplification of the mitochondrial hypervariable segment 1 and for DNA sequencing were synthesized by Thai Can Biotech Co. Ltd. The sequences of all primers were shown in Table 3.

4. Reagents

The reagents in this study were supplied from different companies as follows

- Taq DNA polymerase Cat.# 201203, QIAGEN, USA.
- QIAquick PCR Purification Kit Cat.# 28106 and QIAquick Gel Extraction Kit Cat.# 28704, QIAGEN, USA.
- 100 bp DNA ladder Cat.# N3231S, BioLabs Inc., USA.
- Deoxynucleotide Solution Set (dNTPs) Cat.# N0446S, BioLabs Inc., USA.
- Agarose Cat.# 1170A, Research Organics Inc., USA.
- BigDye[®] Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems, USA.
- Guanidine Thiocyanate (GuSCN) M.W. 118.16 Cat.# V2791, Promega, USA.
- Proteinase K Cat.# V3021, Promega, USA.
- Silicon dioxide (SiO₂) FW 60.08 Cat.# S5631, SIGMA, USA.
- Potassium Phosphate (K₂HPO₄) FW 174.2 Cat.# P3786, SIGMA, USA.
- ortho-Phosphoric acid 85%, MERCK, Germany
- 10% diluted Sodium hypochlorite (NaOCl), Vittayasom Sriracha Co., Ltd., Thailand
- Absolute Ethanol, MERCK, Germany
- Isopropanol, MERCK, Germany
- Sodium Acetate (NaOAc) M.W. 82.03 Cat.# US21608, Amersham Biosciences
- EDTA FW 372.2 Cat.# E5134, SIGMA, USA.
- Trisma-base FW 121.1 Cat.# T1503, SIGMA, USA.

Silica suspension was prepared according to Boom *et al.*, 1990. The details of the buffers preparation are described in Appendix II. Others standard stock solutions used in this study were prepared according to instructions in Molecular Cloning: Laboratory Manual.

5. Equipments

- PTC-150 MiniCycler™ and PTC-200 DNA Engine™ Thermal Cycler, MJ Research Inc., USA.
- ABI PRISM 377 Automated DNA Sequencer, ABI PRISM 310 Automated DNA Sequencer and ABI PRISM 3730 Automated DNA Sequencer, Applied Biosystems, USA.
- Eppendorf centrifuge 5417C, Eppendorf, Germany
- UV transilluminator, Vilber Lourmat, France
- Pipetteman, Gilson, France
- Waterbath, Memmert, Germany
- UV lamp G15T8 15 watt, General Electric Company (GE), USA.
- Agarose Gel Electrophoresis set, Hoefer, USA.
- Vortex, Scientific Industries Inc., USA.
- Gel Doc™ EQ, Bio-Rad Laboratories, Italy
- Refrigerator -20°C, Sanyo, Thailand
- Laminar Flow Hood, Lab Service Co. Ltd., Thailand
- Heraeus oven F340, W.C. Heraeus Hanau, Germany

6. Software

The software programs used in the analysis of this study were the ClustalW Software in the Bioedit version 5.0.9 (downloaded from: <http://www.mbio.ncsu.edu/Bioedit/bioedit.html>), Phylogenetic Analysis Using Parsimony program PAUP* version 4.0 (purchased from Sinaure Associates), Tree View version 1.6.6 program (downloaded from: <http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>) and Molecular Evolutionary Genetics Analysis (MEGA) program version 3.0 (downloaded from: <http://www.megasoftware.net>).

Methods

DNA extracted from ancient teeth samples and hair follicles was amplified for mitochondrial hypervariable segment 1 (HVS-1). The amplified DNA was purified and sequenced. The sequencing data from all of the samples were analyzed for nucleotide diversity and phylogenetic analysis.

1. Samples collection and preparation

1.1 Ancient teeth samples

Every step of the work with DNA from ancient remain was carried out under extremely sterile condition to prevent possible contamination (42-47). For more details see Appendix II: The Contamination Precaution.

Each of molar tooth was collected from the skull using sterile modeling mini-drill. To prevent the contamination with foreign DNA, face mask and disposable lab gloves were used at all time. The collected samples were completely sealed in the plastic bag and separately kept in dry and dark condition until use.

The tooth sample for DNA extraction was cleaned in order to get rid of all sources of contamination by soaking with 10% diluted sodium hypochlorite for 10-15 minutes. After 2-3 times wash with sterile distilled water, the tooth was exposed to UV light for at least 20 minutes per each side. The clean tooth was crushed in enclosed laminar hood with a sterile mortar/pestle and the tooth powder was aliquoted to 250 mg per tube. The tooth powder was kept in -20°C until DNA extraction procedure began.

1.2 Hair follicles

Two to three hair including root was collected from each of modern human samples. The collected hair follicles were kept in sterile eppendorf tube and stored in 4°C until DNA extraction procedure began.

2. Isolation of DNA

DNA extraction in this study was performed using 2 methods depending on the nature of the samples (ancient tooth samples or hair roots). The silica-based method was used for the ancient samples and the hair roots DNA was isolated using digestion buffer and enzyme Proteinase K.

2.1 DNA extraction and purification from ancient samples (26, 48-54)

All steps of DNA extraction and purification were done under contamination precaution (Appendix II). The extraction and purification of the ancient DNA were done using a modified method according to Boom et al., 1990 (48) and H \ddot{o} ss and P \ddot{a} äbo, 1993 (49). Tooth powder was suspended by vortexing in 500 μ l of Lysis buffer, and incubated overnight at 60°C for 16 hours with sporadic agitation. After phase separation by centrifugation at 6,000 rpm for 5 minutes, the supernatant containing DNA was transferred to a new microcentrifuge tube and kept in sterile condition at room temperature. Three hundred microliters of extraction buffer were added to the tooth powder pellet and vortexed until the pellet is re-suspended in the buffer, and incubated at 60°C for 15 minutes. This step was designed to extract the DNA adsorb in the hydroxyapatite of the teeth according to Persson, 1992 (50) and G \ddot{o} therstr \ddot{o} m and Lid \acute{e} n (51-52). After fifteen minutes, the supernatant containing DNA was collected by centrifugation at 12,000 rpm for 5 minutes. The buffer was collected and mixed with the supernatant containing DNA in the lysis buffer from previous step. Lysis buffer 1 ml was added into the tube together with Proteinase K at final concentration 1 μ g/ μ l and the tube was incubated further at 60°C for 2 hours. After that the DNA purification were performed by the addition of 40 μ l silica suspension. The DNA was allowed to adsorb to silica particles at room temperature in the dark with sporadic agitation for 10-15 minutes. After DNA adsorbed to the silica particle, the mixture was centrifuged at 6,000 rpm for 3 minutes to collect the silica pellet. The supernatant was discarded and the silica pellet was re-suspended in 1 ml wash buffer for a while at room temperature. After phase separation by centrifugation at 6,000 rpm for 3 minutes, the supernatant was removed and the silica pellet was re-suspended twice with 95% ethanol in order to get rid all of traces of GuSCN which can inhibit PCR reaction. The silica was left at -20°C for 15 minutes and centrifuged at 6,000 rpm for 3 minutes. The supernatant was discarded. The silica pellet was dried at room temperature for 1 hour under sterile condition. The DNA was eluted from the silica particles by the addition of 75 μ l elution buffer and incubated at 56°C for 10 minutes. Fifty microliters of supernatant was collected in a new Eppendorf tube after centrifuged at 12,000 rpm for 5 minutes. The extracted DNA was kept at -20°C and used for PCR within one week or stored at -80°C for more days since there was no

EDTA in the storage buffer to inhibit the DNA degradation (www.archaeology.su.se/arklab/dnaextr.htm).

2.2 DNA extraction from hair roots (55)

The DNA was extracted from hair roots by the addition of 100 µl digestion buffer (the component as in Appendix III) with 20 µg of proteinase K into the tube containing hair follicle. After overnight incubation at 37°C, the mixture was heated at 95°C for 8-10 minutes and centrifuged at 10,000 rpm for 1 minute. The supernatant containing DNA was collected and used for PCR amplification. The DNA solution was stored at -20°C.

3. PCR direct sequencing

Hypervariable segment 1 in the mitochondrial D-loop region (360 bp from nucleotide position 16024 to 16383 of the Cambridge Reference Sequence) was amplified by Polymerase Chain Reaction (PCR). The resulting PCR products were purified and proceed for DNA sequencing using DyeDeoxy Terminator Cycle Sequencing Kit.

3.1 PCR amplification

3.1.1 PCR amplification from ancient DNA (26, 42, 45, 56-57)

The mitochondrial hypervariable segment 1 (HVS-1) of ancient sample was amplified using nested PCR strategies into 4 overlapping fragments (F1, F2, F3 and F4). Primary PCR was performed using primer pair L15978/H16355 resulting in PCR product of 377 bp. The first overlapping fragment (F1=247 bp from nucleotide position 15978 to 16224) was amplified by nesting this PCR product with forward primer L15978 and reverse primer H16224 yielding 247 bp product. For second and third fragment, the primary PCR product were from the amplification of the mitochondrial DNA using L15978/H16224 yielding 247 bp product. The second overlapping fragment (=F2=135 bp from nucleotide position 15978 to 16112) was amplified by nesting this PCR product (L15978/H16224) with primer pair L15978/H16112 and the third overlapping fragment (=F3=161 bp from nucleotide position 16064 to 16224) was amplified using primer pair L16064/H16224. The last overlapping fragment (=F4=228 bp from nucleotide position 16190 to 16417) was amplified from nesting the PCR product of primers L16190 and H16422 (232 bp) with primers L16190 and H16417. The oligonucleotide primers used for primary PCR

and nested PCR were shown in Table 3. The position of each primer pairs and 4 overlapping fragments PCR product on mitochondrial hypervariable segment 1 were shown in Figure 12.

PCR amplification was carried out in a reaction mixture of 25 μ l containing 5 μ l of ancient DNA, 1X PCR buffer; pH8.7, 1X Q-solution, 0.2 mM each of dNTP, 20 pmol of each primer and 2 units Taq DNA polymerase for primary PCR, 1 unit for secondary PCR. All reagents except DNA and enzyme were mixed into a reaction cocktail and irradiated with UV light for 20 minutes. After the addition of DNA and enzyme, the mixture was incubated at 95°C for 5 minutes followed by 50 amplification cycles (94°C 30 second for denaturation, 30 second for primer annealing temperature according to each primer pairs (Table 3) and 72°C 45 second for extension) and a final extension step at 72°C for 5 minutes on PTC-200 DNA engine™ Thermal Cyclers. The nested PCR was performed as described above using primary PCR product as a template but only 30 amplification cycles were done. Three microliters of the second-round PCR product were mixed with loading dye and analyzed on 2% agarose gel electrophoresis in 1XTAE buffer. In order to check the fragment size and quality, 100 bp DNA ladder was loaded comparing with PCR product. The agarose gel was then stained with ethidium bromide and the product band was visualized by UV transilluminator. To prevent the contamination with foreign DNA, every step of PCR set up were carried on under contamination precaution (Appendix II). A PCR blank was done together with every sample and every PCR setup including nesting PCR of the blank from the first-round to check possible contamination. All products with any evidence of contamination were discarded.

3.1.2 PCR amplification from modern DNA

For modern human DNA, mitochondrial hypervariable segment 1 was amplified using forward and reverse primers into PCR product of 514 bp. The nucleotide sequences correspond to position 15904-15930 and 16398-16417 of the Cambridge Reference Sequence for forward and reverse primer, respectively. DNA amplification was carried on in 50 μ l volume containing 5 μ l of extracted DNA, 1X PCR buffer; pH8.7, 1.25 mM of MgCl₂, 0.2 mM of each dNTP, 20 pmol of each primer and 1 unit of Taq DNA polymerase.

Table 3. The oligonucleotide primers for first-round PCR and nested PCR amplification from ancient DNA

No. of primary PCR primer	Name of primary PCR primer	Sequence 5' to 3'	Annealing temperature (°C)	Size of product (bp)	No. of overlapping fragment	Name of nested PCR primer	Sequence 5' to 3'	Annealing temperature (°C)	Size of product (bp)
1	L15978	CAC CAT TAG CAC CCA AAG	58	378	F1	L15978	CAC CAT TAG CAC CCA AAG	58	247
	H16355	GGG ATT TGA CTG TAA TGT GC				H16224	AGG GTT GAT TGC TGT ACT TG		
2	L15978	CAC CAT TAG CAC CCA AAG	55	247	F2	L15978	CAC CAT TAG CAC CCA AAG	58	135
	H16224	AGG GTT GAT TGC TGT ACT TG				H16112	GGC TGG CAG TAA TGT ACG		
3	L16190	CCC CAT GCT TAC AAG CAA G	58	233	F3	L16064	TGA CTC ACC CAT CAA CAA C	58	161
	H16422	ATT GAT TTC ACG GAG GAT GG				H16224	AGG GTT GAT TGC TGT ACT TG		
					F4	L16190	CCC CAT GCT TAC AAG CAA G	58	228
						H16417	TTT CAC GGA GGA TGG TGG TC		

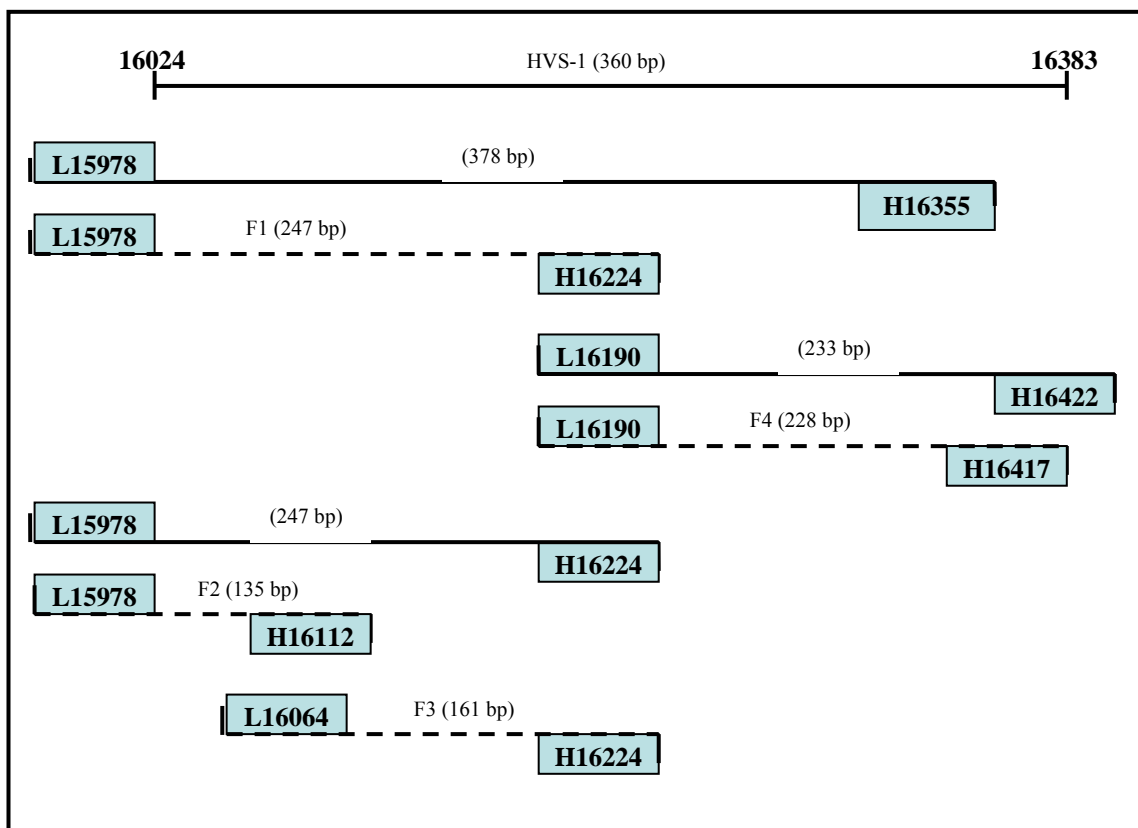


Figure 12. Position of the PCR products generated from each overlapping primer pairs on hypervariable segment 1 (HVS-1). The dark line represented primary PCR product and the dashed line represented nested PCR product with forward and reverse primer.

After start with an initial denaturation step (95°C for 5 minutes), 30 amplification cycles were performed as follows: denaturation at 94°C 1 minute, primer annealing at 58°C 1:30 minutes and 72°C 2:30 minutes for extension, and a final extension step (72°C for 10 minutes) on PTC-150 Mini CyclerTM. Amplification products were analyzed by electrophoresis in 2% agarose gel comparing with 100 bp ladder marker. The DNA was stained with ethidium bromide and visualized by UV transilluminator.

3.2 PCR purification

PCR product both from ancient and modern DNA were purified using either QIAquick PCR Purification Kit, when only specific band product was shown, or QIAquick Gel Extraction Kit, when non-specific band or smear band were showed, prior to DNA sequencing.

3.2.1 PCR purification using QIA quick PCR Purification Kit

Five volumes of buffer PB (provided in the kit) were mixed with 1 volume of the PCR product then applied the mixture in to the QIAquick spin column set. The column with collection tube was centrifuged at 12,000 rpm for 1 minute. Seven hundred and fifty microliters of buffer PE (provided in the kit) were added to spin column and centrifuged at 12,000 rpm for 1 minute. The flow-through in a collection tube was discarded and the spin column was further centrifuged at 12,000 rpm for 1 minute. The spin column was placed on to a new 1.5 ml microcentrifuge tube. DNA was eluted by the addition of 30 µl of buffer EB (provided in the kit) on the silica-gel membrane and let the column stand for 1 minute prior to centrifugation at 12,000 rpm for 1 minute. The eluted PCR product was collected and stored at -20°C until use. The fragment size and concentration of purified PCR product were analyzed by electrophoresis stained with ethidium bromide and visualized by UV transilluminator.

3.2.2 PCR purification using QIA quick Gel Extraction Kit

For more than one band PCR product, the PCR product was loaded on agarose gel and then excised specific needed band from the gel. The gel slice was weight and 3 volumes of buffer QG was added to 1 volume of gel (100 mg ~100 µl). The mixture was incubated at 50°C for 10 minute or until the gel slice was completely dissolved. One gel volume of isopropanol was added, mixed and applied to the QIAquick spin column. After centrifugation at 12,000 rpm for 1 minute

allowing the DNA to bind to silica-gel membrane, 500 µl of buffer QG were added to QIAquick column one more time. This step was to remove all traces of agarose from the sample °C for 4 minutes, for 25 cycles with an initial incubation at 96°C for 1 minute on PTC-150 Mini Cycler™ or PTC-200 DNA Engine™ Thermal Cyclers.

3.3 PCR Sequencing

3.3.1 Preparation of sequencing reaction

Nucleotide sequences were determined using BigDye™ Terminator Cycler sequencing Kit, according to the manufacture's instructions. The sequencing reaction were carried out in a 20 µl volume containing 5-50 ng purified PCR product, 5 pmol of either forward or reverse primer of the corresponding PCR product, 3 µl of 2.5X Ready Reaction Premix and 2.5 µl of 5X BigDye Sequencing Buffer. The sequencing mixture was mixed well and spin briefly and then put into thermal cycler. The thermal cycler was programmed as following protocol: 96°C for 10 seconds, 50°C for 5 seconds and 60ABI PRISM 3730 Automated DNA Sequencer for ancient DNA

3.3.2 Precipitation of cycle sequencing product

Two microliters of 3M sodium acetate (pH 5.2) and 80 µl of 95% ethanol were added to cycle sequencing product. The tube containing the mixture was mixed and placed in 4°C for 15 minutes. After centrifugation for 15 minutes at 12,000 rpm, the supernatant was discarded. The pellet was washed with 250-500 µl of 70% ethanol and centrifuged at 12,000 rpm for 5 minutes. The supernatant was discarded and the pellet was air-dried in the dark. The sequencing analysis was done on ABI PRISM 377 Automated DNA Sequencer for modern DNA, ABI PRISM 310 Automated DNA Sequencer and solution. The column with a collection tube was centrifuged at 12,000 rpm 1 minute and the solution was discarded. The DNA was washed and eluted from the membrane the same as in 3.2.1.

4. Mitochondrial haplogroup determination

The mitochondrial haplogroup of all modern samples and ancient samples were determined. Each haplotype was characterized by the group of the polymorphism of nucleotide sequence within the hypervariable segment 1 in the mitochondrial D-loop region as summarized in Table 4 (58-62).

5. Phylogenetic analysis (53, 63-66)

In this study, the hypervariable segment 1 sequences (from nucleotide position 16048 to 16383) on mitochondrial DNA of a total 419 samples were compared. All from 17 ancient samples from Noen U-Loke (NUL), 307 modern samples or 11 rational groups from several parts in Thailand (Figure 13); the Hill tribes (25 from the Lisu (HIL) and 21 from Mussur (HIM) peoples) in Chiang Mai province (northern Thailand), 25 Phuthai (PHT) in Mukdahan province (northeastern Thailand), 25 Lao Song (LAS) in Supanburi province (central Thailand), 25 Chong (CH) in Chantaburi province (eastern Thailand), 20 aboriginal Sakai (SAK) in Trang province (southern Thailand), native Thai people from Chiang Mai (CM) province 30 samples and from Khon Kaen (TH or THAT) province (northeastern Thailand) 44 samples (67), 50 native Thai people from Korat (Nakhon Ratchasima (K), northeastern Thailand), 20 Chao Bon in Nakhon Ratchasima province (CB), 22 Khmer people living in Thai-Cambodia border area (KM), and 95 modern Han samples or 7 regional groups from several parts in China (Figure 14): 17 Yunnan (YN), 16 Liaoning (LN), 13 Zhanjiang in Guangdong province (GD), 10 Wuhan in Hubei province (WH), 15 Qingdao in Shandong province (QD), 17 Xinjiang (XJ) and 7 Qinghai (QH) (68-69). The degree of nucleotide substitution were determined by ClustalW multiple alignment. Phylogenetic tree were constructed using Phylogenetic Analysis Using Parsimony PAUP* version 4.0 packaged program. The unrooted tree was drawn using the neighbor-joining (NJ) method (70) and illustrated with Tree View version 1.6.6 program.

6. Nucleotide diversity (67, 69, 71-72)

The 419 samples determined in this study were from 19 populations: 1,500-2,400 year-old human population from Noen U-Loke site (NUL), Hill tribes from Lisu (HIL) and from Mussur (HIM), Phuthai (PHT), Lao Song (LAS), Chong (CH), aboriginal Sakai (SAK), native Thai people from Chiang Mai (CM), native Thai people from Khon Kaen (TH or THAT) native Thai people from Korat (K), Chao Bon (CB), Khmer (KM), Yunnan (YN), Liaoning (LN), Zhanjiang-Guangdong (GD), Wuhan-Hubei (WH), Qingdao-Shandong (QD), Xinjiang (XJ) and Qinghai (QH).

Table 4. The particular set of the polymorphism of nucleotide sequence within the hypervariable segment 1 (HVS-1) in the mitochondrial D-loop region for haplogroup determination

Haplogroup	HVS-1 nucleotide variants
A	T16362C, G16319A, C16290T, C16223T
B	T16189C, T16217C T16519C
B*	T16140C, T16189C T16519C
C	C16327T, T16298C, C16223T
D	T16362C, C16223T, T16325C
F	T16304C
M	C16223T
P	T16357C
Q	G16129A, T16144C, C16148T, C16223T
X	T16189C, C16223T, C16278T



Figure 13. The location of each of 11 racial groups in Thailand used as the modern samples in this study



Figure 14. The location of each of 7 rational groups in China that used as modern samples in this study

Nucleotide diversity (the mean of pairwise nucleotide differences per site) and net values of nucleotide substitutions (d_A) between populations (73-74) were calculated using the 2-parameter model (75) and run on Molecular Evolutionary Genetics Analysis program version 3.0 (MEGA3). On the basis of d_A value, population tree was constructed using the NJ method.

CHAPTER IV

RESULTS

1. Ancient teeth from Archaeological site

The ancient teeth of twenty-six adult skeletons were from the excavation site at Noen U-Loke, Amphoe Non Sung, Nakhon Ratchasima. The burial numbers of each skeleton where the teeth were taken from are shown in Table 2. This archaeological site is located in the northeast of Thailand where the weather is hot and dry so that DNA preservation was generally good. This archaeological site was dated, from radiocarbon dating of seventeen remains, between 3,000-1,500 years before present (BP). The mortuary sequence was divided into five phases. Phase 1 was the deepest from the surface (3.8-4.3 meters depth below datum) and was the oldest phase, dated more than 2,500 before present (BP). Phase 2 depth was 3.65-4.12 meters, phase 3 was 2.18-3.32 meters, phase 4 was 1.54-3.65 meters and phase 5 was 0.53-1.77 meters from surface. Each phase was dated between 2,400-2,100, 2,200-1,800, 1,800-1,600 and 1,700-1,500 years before present, respectively. Twenty-six teeth samples, placed in replete jaw (Figure 15) were collected from the burials found in four phases. Nine teeth (from 4 male skeletons: 4 female skeletons: 1 can not be defined) were collected from phase 5. Nine teeth (from 5 male skeletons: 3 female skeletons: 1 can not be defined) were collected from phase 4. Six teeth samples from six skeletons (4 male skeletons: 1 female skeleton: 1 can not be defined) were collected from phase 3. One tooth from male skeleton was collected from phase 2. No available grave was found in phase 1. Sample number 15, has no information of sex, dates or phase of excavation.

2. The modern DNA from several parts in Thailand

The modern DNA was extracted from hair roots of 92 healthy individuals. Twenty-two of them are Khmer peoples living on the Thai-Cambodia border area, twenty of them are people who called themselves “Chao Bon” living in Amphoe Pugthongchai, Nakhon Ratchasima, and thirty and twenty of them live in Ban Lum



Figure 15. Ancient teeth sample from archaeological site at Noen U-Loke. a) 3rd molar in the mandibular of burial 44. b) Ancient tooth of (a) after being taken from mandible using modeling mini-drill.

Khao and Ban Non Wat at Amphoe Nonsung, Nakhon Ratchasima, respectively. Ban Lum Khao and Ban Non Wat are close to the excavation site (Figure 16). All of the peoples of Ban Lum Khao, Ban Non Wat including Chao Bon have been living in their place since they were born and their family history could be traced back more than a hundred years ago.

3. Overlapping fragments of PCR products of ancient DNA

Since ancient DNA samples are usually damaged and fragmented, therefore, sequences of less than a few hundred nucleotides were able to be amplified, and overlapping primer pairs were designed to assess the reliability of the results. Hypervariable segment 1 of the mitochondrial DNA (360 nucleotides position 16024-16383 of the Cambridge Reference Sequence) was amplified by nested-PCR using 4 pairs of overlapping primers: F1=247 bp from nucleotide position 15978 to 16224, F2=135 bp from nucleotide position 15978 to 16112, F3=161 bp from nucleotide position 16064 to 16224 and F4=228 bp from nucleotide position 16190 to 16417 (Figure 12). This study successfully amplified total 360 bp on HVS-1 of the mitochondrial DNA for 17 ancient samples, 320 nucleotides position 16064 to 16383 on HVS-1 for one more ancient sample and 194 nucleotides position 16190 to 16383 on HVS-1 for 4 more ancient samples and 4 samples could not be amplified at all (Table 5). The reliability of the results was verified by negative control and mock extraction amplification. The results are shown in Figure 17.

4. PCR product from modern DNA amplification

Hypervariable segment 1 of mitochondrial DNA was amplified using a primer pairs (L15904 forward primer and H16417 reverse primer), resulting 514 bp PCR product. The reliability of the results was verified by negative control containing all of the PCR reagents without DNA template.

5. Polymorphism on hypervariable Segment 1 in the D-loop region and haplogroup determination of the mitochondrial DNA of ancient population and modern population

Several length of sequence on the hypervariable segment 1 of all 19 populations was analyzed. The sequence of a 360 nucleotides of HVS-1 (position 16024 to 16383) of samples from 50 native Thai people from Korat (K), 20 Chao Bon (CB), 22 Khmer (KM), 17 Yunnan (YN), 16 Liaoning (LN), 13 Zhanjiang in

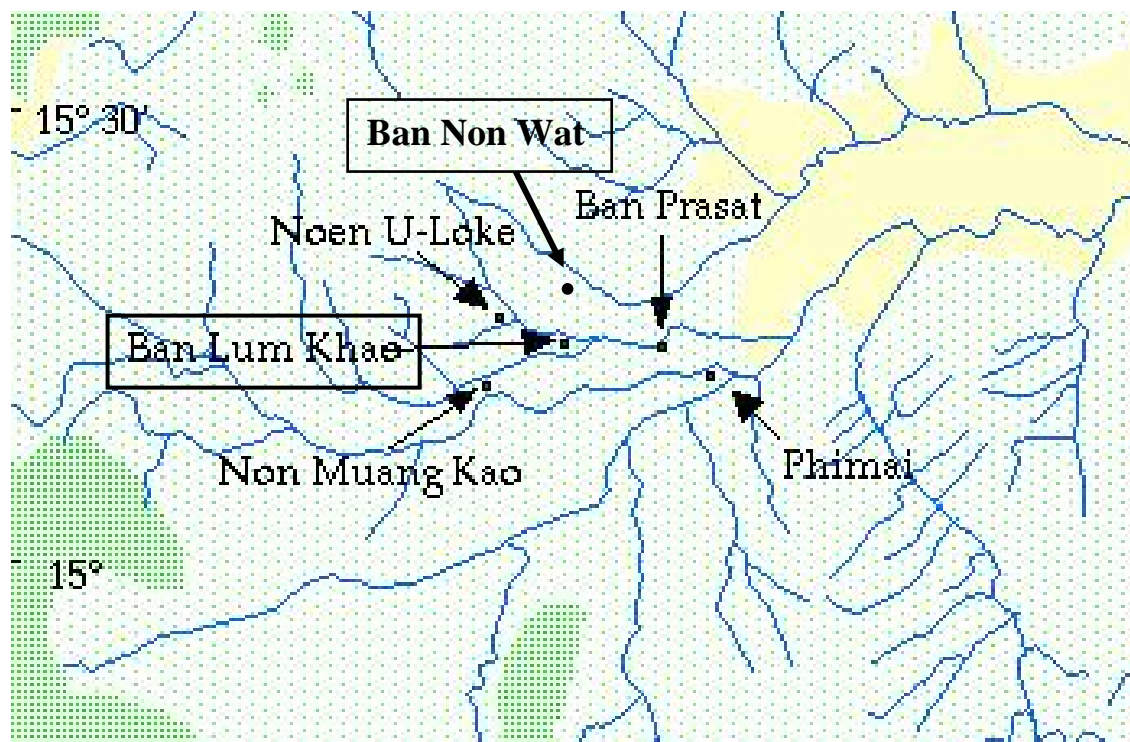


Figure 16. The location of Ban Lum Khae and Ban Non Wat at Amphoe Nonsung, Nakhon Ratchasima. Both areas are close to Noen U-Loke, the archaeological site investigated in this study.

Table 5. The amplification results of each overlapping primer pairs of all 26 ancient tooth samples.

No.	Burial number	F ₁ nt 15978 to nt 16224 (247 bp)	F ₂ nt 15978 to nt 16112 (135 bp)	F ₃ nt 16064 to nt 16224 (161 bp)	F ₄ nt 16190 to nt 16417 (228 bp)	Mitochondrial position (position on rCRS)	Size can be determined (bp)
1	26	-	+	+	+	15978-16417	440
2	33	-	+	+	+	15978-16417	440
3	41	-	-	-	-	-	-
4	44	-	-	-	+	16190-16417	228
5	45	-	-	-	+	16190-16417	228
6	48	+	-	-	+	15978-16417	440
7	50	+	-	-	+	15978-16417	440
8	16	-	-	-	-	-	-
9	31	+	-	-	+	15978-16417	440
10	62	-	+	+	+	15978-16417	440
11	69	+	-	-	+	15978-16417	440
12	74	+	-	-	+	15978-16417	440
13	99	+	-	-	+	15978-16417	440
14	104	-	-	-	+	16190-16417	228
15	111	-	+	+	+	15978-16417	440
16	113	+	-	-	+	15978-16417	440
17	4	-	-	-	-	-	-
18	5	+	-	-	+	15978-16417	440
19	8	+	-	-	+	15978-16417	440
20	10	-	-	-	-	-	-
21	12	-	-	-	+	16190-16417	228
22	59	-	-	+	+	16064-16417	354
23	60	-	+	+	+	15978-16417	440
24	66	+	-	-	+	15978-16417	440
25	68	+	-	-	+	15978-16417	440
26	15	-	+	+	+	15978-16417	440

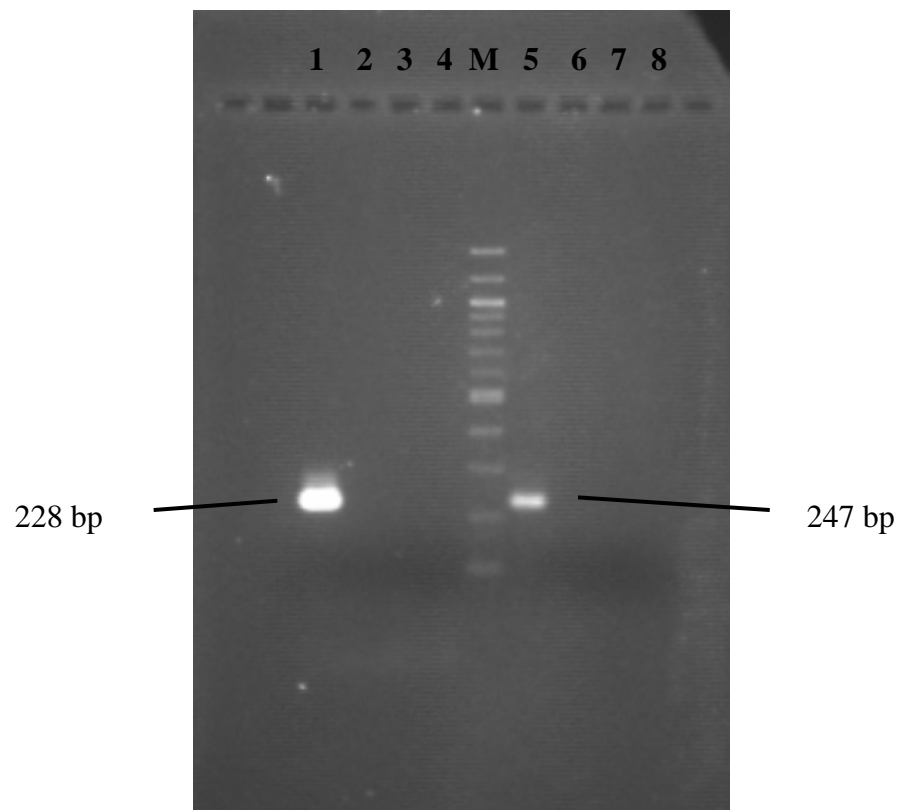


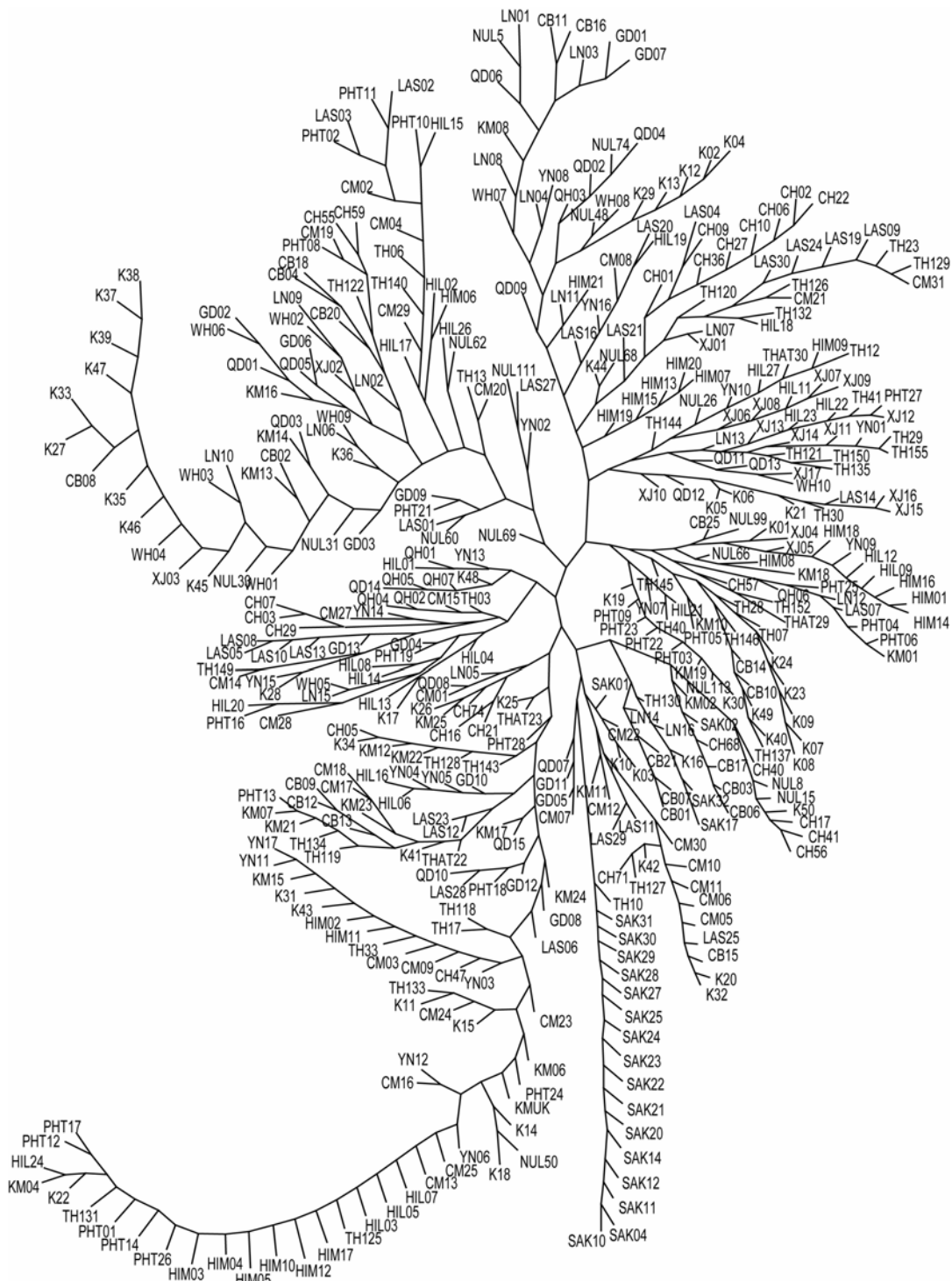
Figure 17. The amplification result of the ancient sample number 5. Lane 1 and 5 represented the sample PCR product of F4 (228 bp) and F1 (247 bp), respectively. Lane 2 and 6 represented the result of mock extraction amplification. Lane 3 and 7 represented the PCR blank from the first-round PCR in the nested PCR reaction. Lane 4 and 8 represented the negative control. Lane M represented 100 bp ladder marker.

Guangdong province (GD), 10 Wuhan in Hubei province (WH), 15 Qingdao in Shandong province (QD), 17 Xinjiang (XJ), 7 Qinghai (QH) and ancient samples from Noen U-Loke 17 samples were included in analysis of polymorphism on HVS-1 and mitochondrial DNA haplogroup determination. The rest of samples; the sequence of a 336 nucleotides of HVS-1 (position 16048 to 16383) of samples from 25 Lisu (HIL), 21 Mussur (HIM), 25 Phuthai (PHT), 25 Lao Song (LAS), 25 Chong (CH), 20 aboriginal Sakai (SAK), 30 native Thai people from Chiang Mai (CM), 44 native Thai people from Khon Kaen (TH or THAT); the sequence of a 320 nucleotides of HVS-1 (position 16064 to 16383) of samples from Noen U-Loke one more and the sequence of a 194 nucleotides of HVS-1 (position 16190 to 16383) of samples from Noen U-Loke four more, were added for analysis. However, the nucleotide that have no information of these samples were excluded from the analysis. For the construction of phylogenetic tree, only sequence of a 336 nucleotide of HVS-1 (position 16048 to 16383) of 419 samples from 7 rational groups from China, 10 rational groups from Thailand, one more from Khmer and from Noen U-Loke (17 samples from total 22 samples) were analyzed.

The 136 polymorphic sites from a total of 424 samples were determined on Table 6. The nucleotide have no information were shown by minus symbol (-). Based on the criteria for haplogroup determination, have been described by Wallace DC *et al.*, 1999 and Schurr TG and Wallace DC, 2002 (58-62) as summarized in Table 4, and additional information from phylogenetic tree (Figure 18), the results of haplogroup determination of all ancient samples and modern living peoples were shown on Table 7. All 424 samples were divided into 4 haplogroups (Table 8). Another haplogroups (A, C, D, P, Q and X) were not found in present study samples. The frequency of each of mitochondrial DNA haplogroup was determined in percentage as shown on Table 8. The most common haplogroup was haplogroup M except populations from Khmer, Guangdong and Wuhan. The common haplogroup of Khmer was haplogroup F, and of Guangdong and Wuhan was haplogroup B. Haplogroup M was only group showed in population from aboriginal Sakai and Qinghai. In the haplogroup M, Qingdao had highest value of frequency with 60%, while have no any population out from this group. For the haplogroup F Khmer were highest in frequency with 50%. Wuhan and Chong were highest in frequency within haplogroup B and B* with the percentage of 40 and 32, respectively.

Table 6 (continued). A hundred and thirty-six polymorphic sites in the HVS-1 of mtDNA for the 424 of ancient and modern samples.

Subject	Nucleotide Position																																																										
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42																	
FCR5	A	G	C	A	C	T	C	C	C	A	C	C	T	T	A	A	C	A	T	A	T	A	A	A	G	C	T	A	C	A	A	T	C	C	C	T	T	C	T	C	T	T	C	T															
CM17	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*												
CM18	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*												
CM19	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*												
CM20	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*											
CM21	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*											
CM22	G	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*										
CM23	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*										
CM24	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*									
CM25	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*								
CM27	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*							
CM28	*	*	*	*	*	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*					
CM29	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*				
CM30	*	*	*	*	*	*	*	*	*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*				
CM31	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*			
SAK01	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	*			
SAK02	*	A	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	*			
SAK04	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*		
SAK10	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*		
SAK11	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*			
SAK12	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*			
SAK14	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*			
SAK17	*	*	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*			
SAK20	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*				
SAK21	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*				
SAK22	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*					
SAK23	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*					
SAK24	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*					
SAK25	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*					
SAK27	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*					
SAK28	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*					
SAK29	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*					
SAK30	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*					
SAK31	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*					
SAK32	*	*	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*						
CH01	*	*	*	*	*	*	*	*	*	G	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	*			
CH02	*	*	*	*	*	*	*	*	*	G	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	*			
CH03	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	T	C	*	*	
CH05	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*	*			
CH06	*	*	*	*	*	*	*	*	*	G	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	*		
CH07	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	T	C	*	*	
CH09	*	*	*	*	*	*	*	*	*	G	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	*		
CH10	*	*	*	*	*	*	*	*	*	G	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	*			
CH16	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*	T	*	*		
CH17	*	*	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	T	*	*		
CH21	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*	T	*	*	
CH22	*	*	*	*	*	*	*	*	*	G	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	*	*	
CH27	*	*	*	*	*	*	*	*	*	G	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	*	*		
CH29	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	T	C	*	C	*
CH36	*	*	*	*	*	*	*	*	*	G	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	*	*
CH40	*	*	*</																																																								



Note: Noen U-Loke (=NUL), Lisu (=HIL), Mussur (=HIM), Phuthai (=PHT), Lao Song (=LAS), Chong (=CH), aboriginal Sakai (=SAK), native Thai people from Chiang Mai (=CM), native Thai people from Khon Kaen (=TH or THAT), native Thai people from Korat (=K), Chao Bon (=CB), Khmer (=KM), Yunnan (=YN), Liaoning (=LN), Guangdong (=GD), Wuhan in Hubei province (=WH), Qingdao in Shangdong province (=QD), Xinjiang (=XJ) and Qinghai (=QH).

Figure 18. The phylogenetic tree of 419 samples from 19 populations. This unrooted tree was drawn using neighbor-joining method.

Table 7. The mitochondrial haplogroup of 424 individuals determined by polymorphism on 360 bp of HVS-1 sequence data. All 424 samples included 22 ancient samples, 307 modern samples of 11 rational groups live in Thailand and 95 modern samples of 7 rational groups live in China.

Subject number	Haplogroup determination	Subject number	Haplogroup determination	Subject number	Haplogroup determination	Subject number	Haplogroup determination
NUL26	B*	K32	M	LAS10	M	HIL18	B*
NUL33	B*	K33	B*	LAS11	M	HIL19	M
NUL44	M	K34	F	LAS12	F	HIL20	M
NUL45	M	K35	B*	LAS13	M	HIL21	M
NUL48	B	K36	F	LAS14	M	HIL22	M
NUL50	F	K37	B*	LAS16	M	HIL23	M
NUL31	B*	K38	B*	LAS19	B*	HIL24	F
NUL62	B	K39	B*	LAS20	M	HIL26	B
NUL69	B	K40	M	LAS21	M	HIL27	M
NUL74	B	K41	F	LAS23	F	TH03	M
NUL99	M	K42	M	LAS24	B*	TH06	B
NUL104	F	K43	F	LAS25	M	TH07	M
NUL111	B	K44	M	LAS27	B	TH10	M
NUL113	M	K45	B*	LAS28	F	TH12	M
NUL5	B	K46	B*	LAS29	M	TH13	B
NUL8	M	K47	B*	LAS30	B*	TH17	F
NUL12	M	K48	M	HIM01	M	TH23	B*
NUL59	M	K49	M	HIM02	F	TH28	M
NUL60	B	K50	M	HIM03	F	TH29	M
NUL66	M	PHT01	F	HIM04	F	TH30	M
NUL68	M	PHT02	B	HIM05	F	TH33	F
NUL15	M	PHT03	M	HIM06	B	TH40	M
K01	M	PHT04	M	HIM07	M	TH41	M
K02	M	PHT05	M	HIM08	M	TH118	F
K03	M	PHT06	M	HIM09	M	TH119	F
K04	M	PHT08	B	HIM10	F	TH120	B*
K05	M	PHT09	M	HIM11	F	TH121	M
K06	M	PHT10	B	HIM12	F	TH122	B
K07	M	PHT11	B	HIM13	M	TH125	F
K08	M	PHT12	F	HIM14	M	TH126	B*
K09	M	PHT13	F	HIM15	M	TH127	M
K10	M	PHT14	F	HIM16	M	TH128	F
K11	F	PHT16	M	HIM17	F	TH129	B*
K12	M	PHT17	F	HIM18	M	TH130	M
K13	M	PHT18	F	HIM19	M	TH131	F
K14	F	PHT19	M	HIM20	M	TH132	B*
K15	F	PHT21	B	HIM21	M	TH133	F
K16	M	PHT22	M	HIL01	M	TH134	F
K17	M	PHT23	M	HIL02	B	TH135	M
K18	F	PHT24	F	HIL03	F	TH137	M
K19	M	PHT25	M	HIL04	M	TH140	B
K20	M	PHT26	F	HIL05	F	TH143	F
K21	M	PHT27	C	HIL06	F	TH144	M
K22	F	PHT28	M	HIL07	F	TH145	M
K23	M	LAS01	B	HIL08	M	TH146	M
K24	M	LAS02	B	HIL09	M	TH149	M
K25	M	LAS03	B	HIL11	M	TH150	M
K26	M	LAS04	B*	HIL12	M	TH152	M
K27	B*	LAS05	M	HIL13	M	TH155	M
K28	M	LAS06	F	HIL14	M	THAT22	F
K29	M	LAS07	M	HIL15	B	THAT23	M
K30	M	LAS08	M	HIL16	F	THAT29	M
K31	F	LAS09	B*	HIL17	B	THAT30	M

Note: Noen U-Loke (=NUL), Lisu (=HIL), Mussur (=HIM), Phuthai (=PHT), Lao Song (=LAS), Chong (=CH), aboriginal Sakai (=SAK), native Thai people from Chiang Mai (=CM), native Thai people from Khon Kaen (=TH or THAT), native Thai people from Korat (=K), Chao Bon (=CB), Khmer (=KM), Yunnan (=YN), Liaoning (=LN), Guangdong (=GD), Wuhan in Hubei province (=WH), Qingdao in Shandong province (=QD), Xinjiang (=XJ) and Qinghai (=QH).

Table 7 (continued). The mitochondrial haplogroup of 424 individuals determined by polymorphism on 360 bp of HVS-1 sequence data. All 424 samples included 22 ancient samples, 307 modern samples of 11 rational groups live in Thailand and 95 modern samples of 7 rational groups live in China.

Subject number	Haplogroup determination	Subject number	Haplogroup determination	Subject number	Haplogroup determination	Subject number	Haplogroup determination
SAK01	M	CH05	F	CB10	B	LN16	M
SAK02	M	CH06	B*	CB11	F	QD01	B
SAK04	M	CH07	M	CB12	F	QD02	M
SAK10	M	CH09	B*	CB13	M	QD03	B*
SAK11	M	CH10	B*	CB14	M	QD04	M
SAK12	M	CH16	M	CB15	B	QD05	B
SAK14	M	CH17	M	CB16	M	QD06	B
SAK17	M	CH21	M	CB17	B	QD07	M
SAK20	M	CH22	B*	CB18	B	QD08	M
SAK21	M	CH27	B*	CB20	M	QD09	M
SAK22	M	CH29	M	CB25	M	QD10	F
SAK23	M	CH36	B*	YN01	M	QD11	M
SAK24	M	CH40	M	YN02	B	QD12	M
SAK25	M	CH41	M	YN03	F	QD13	M
SAK27	M	CH47	F	YN04	F	QD14	M
SAK28	M	CH55	B	YN05	F	QD15	F
SAK29	M	CH56	M	YN06	F	XJ01	B*
SAK30	M	CH57	M	YN07	M	XJ02	B
SAK31	M	CH59	B	YN08	B	XJ03	B*
SAK32	M	CH68	M	YN09	M	XJ04	M
SAK33	M	CH71	M	YN10	M	XJ05	M
CM01	M	CH74	M	YN11	F	XJ06	M
CM02	B	KM01	M	YN12	F	XJ07	M
CM03	F	KM02	M	YN13	M	XJ08	M
CM04	B	KM04	F	YN14	M	XJ09	M
CM05	M	KM06	F	YN15	M	XJ10	M
CM06	M	KM07	F	YN16	M	XJ11	M
CM07	M	KM08	B	YN17	F	XJ12	M
CM08	M	KM10	M	WH01	B*	XJ13	M
CM09	F	KM11	M	WH02	B	XJ14	M
CM10	M	KM12	F	WH03	B*	XJ15	M
CM11	M	KM13	B*	WH04	B*	XJ16	M
CM12	M	KM14	B*	WH05	M	XJ17	M
CM13	F	KM15	F	WH06	B	GD01	B
CM14	M	KM16	B	WH07	B	GD02	B
CM15	M	KM17	F	WH08	B	GD03	B*
CM16	F	KM18	M	WH09	F	GD04	M
CM17	F	KM19	M	WH10	M	GD05	M
CM18	F	KM21	F	LN01	B	GD06	B
CM19	B	KM22	F	LN02	B	GD07	B
CM20	B	KM23	F	LN03	B	GD08	F
CM21	B*	KM24	F	LN04	B	GD09	B
CM22	M	KM25	M	LN05	M	GD10	F
CM23	F	KMUK	F	LN06	F	GD11	M
CM24	F	CB01	M	LN07	B*	GD12	F
CM25	F	CB02	B*	LN08	B	GD13	M
CM27	M	CB03	M	LN09	B	QH01	M
CM28	M	CB04	B	LN10	B*	QH02	M
CM29	B	CB05	M	LN11	M	QH03	M
CM30	M	CB06	M	LN12	M	QH04	M
CM31	B*	CB07	B*	LN13	M	QH05	M
CH01	B*	CB08	F	LN14	M	QH06	M
CH02	B*	CB09	M	LN15	M	QH07	M
CH03	M						

Note: Noen U-Loke (=NUL), Lisu (=HIL), Mussur (=HIM), Phuthai (=PHT), Lao Song (=LAS), Chong (=CH), aboriginal Sakai (=SAK), native Thai people from Chiang Mai (=CM), native Thai people from Khon Kaen (=TH or THAT), native Thai people from Korat (=K), Chao Bon (=CB), Khmer (=KM), Yunnan (=YN), Liaoning (=LN), Guangdong (=GD), Wuhan in Hubei province (=WH), Qingdao in Shangdong province (=QD), Xinjiang (=XJ) and Qinghai (=QH).

Table 8. Mitochondrial DNA haplogroup frequency of each population in this study.

Population	Total number	Mitochondrial haplogroup			
		B	B*	F	M
Noen U-Loke	22	7 (32%)	3 (14%)	2 (9%)	10 (45%)
Korat	50	0	9 (18%)	10 (20%)	31 (62%)
Chao Bon	20	4 (20%)	2 (10%)	4 (20%)	10 (50%)
Khmer	22	2 (9%)	2 (9%)	11 (50%)	7 (32%)
Phuthai	25	5 (20%)	0	8 (32%)	12 (48%)
Lao Song	25	4 (16%)	5 (20%)	4 (16%)	12 (48%)
Lisu	25	4 (16%)	1 (4%)	6 (24%)	14 (56%)
Mussur	21	1 (5%)	0	8 (38%)	12 (57%)
Chiang Mai	30	5 (17%)	2 (7%)	9 (29%)	14 (47%)
Khon Kaen	44	4 (9%)	5 (11%)	11 (25%)	24 (55%)
Chong	25	2 (8%)	8 (32%)	2 (8%)	13 (52%)
Sakai	20	0	0	0	20 (100%)
Yunnan	17	2 (12%)	0	7 (41%)	8 (47%)
Liaoning	16	6 (38%)	2 (12%)	1 (6%)	7 (44%)
Qingdao	15	3 (20%)	1 (7%)	2 (13%)	9 (60%)
Guangdong	13	5 (38%)	1 (8%)	3 (23%)	4 (30%)
Wuhan	10	4 (40%)	3 (30%)	1 (10%)	2 (20%)
Xinjiang	17	1 (6%)	2 (12%)	0	14 (82%)
Qinghai	7	0	0	0	7 (100%)

6. Phylogenetic tree of ancient population from Noen U-Loke

The sequence of a 360 nucleotides on HVS-1 (position 16024 to 16383) of 17 ancient samples from Noen U-Loke, the sequence of a 320 nucleotides on HVS-1 (position 16064 to 16383) of the another one ancient sample and the sequence of a 194 nucleotides on HVS-1 (position 16190 to 16383) of the another four ancient sample included in analysis of phylogenetic tree construction. The phylogenetic tree was labeled with number of burial, number of mortuary phase and mitochondrial haplogroup on each of single branch (Figure 19). Phylogenetic tree showed genetic relationship between samples. The ancient samples from Noen U-Loke burial number 59, 66 and 12 of mortuary phase 5 were genetically close to the ancient samples from Noen U-Loke burial number 99 of mortuary phase 4 and number 44 of mortuary phase 3. The ancient sample from burial number 60 of mortuary phase 5 was genetically close to the ancient sample from burial number 111 of mortuary phase 4 while the ancient sample from burial number 104 of mortuary phase 4 was genetically close to the ancient sample from burial number 50 of mortuary phase 3, and the ancient samples from burial number 69 and 4 of mortuary phase 4 were genetically close to the sample from burial number 45 of mortuary phase 3. The ancient samples from burial number 8, 68 and 5 of mortuary phase 5, burial number 62, 31 and 74 of mortuary phase 4, burial number 33 and 48 of mortuary phase 3, burial number 26 of mortuary phase 2 and burial number 15 which could not define mortuary phase, were placed on monophyletic branch indicated their close genetic relationship. Four mitochondrial haplogroups were found in ancient population from Noen U-Loke. The genetically closed samples were often classified into same haplogroup.

7. Nucleotides diversity and Population tree

The value of intrapopulation nucleotide diversity (d_x or d_y), interpopulation nucleotide diversity (d_{XY}) and net nucleotide diversity between group were calculated using average distance from pairwise computational comparison. The value of net nucleotide diversity between groups is given by $d_A = d_{XY} - (d_x - d_y) / 2$ (73-74). The within group nucleotide diversity (d_x or d_y) was ranging from 0.86% to 2.71%. Aboriginal Sakai represented low diversity population with lowest value of d_x or d_y (0.86%) and population from Liaoning showed highest value (2.71%), while Noen U-Loke population showed 1.73% value of d_x or d_y (Table 9). The value of nucleotide

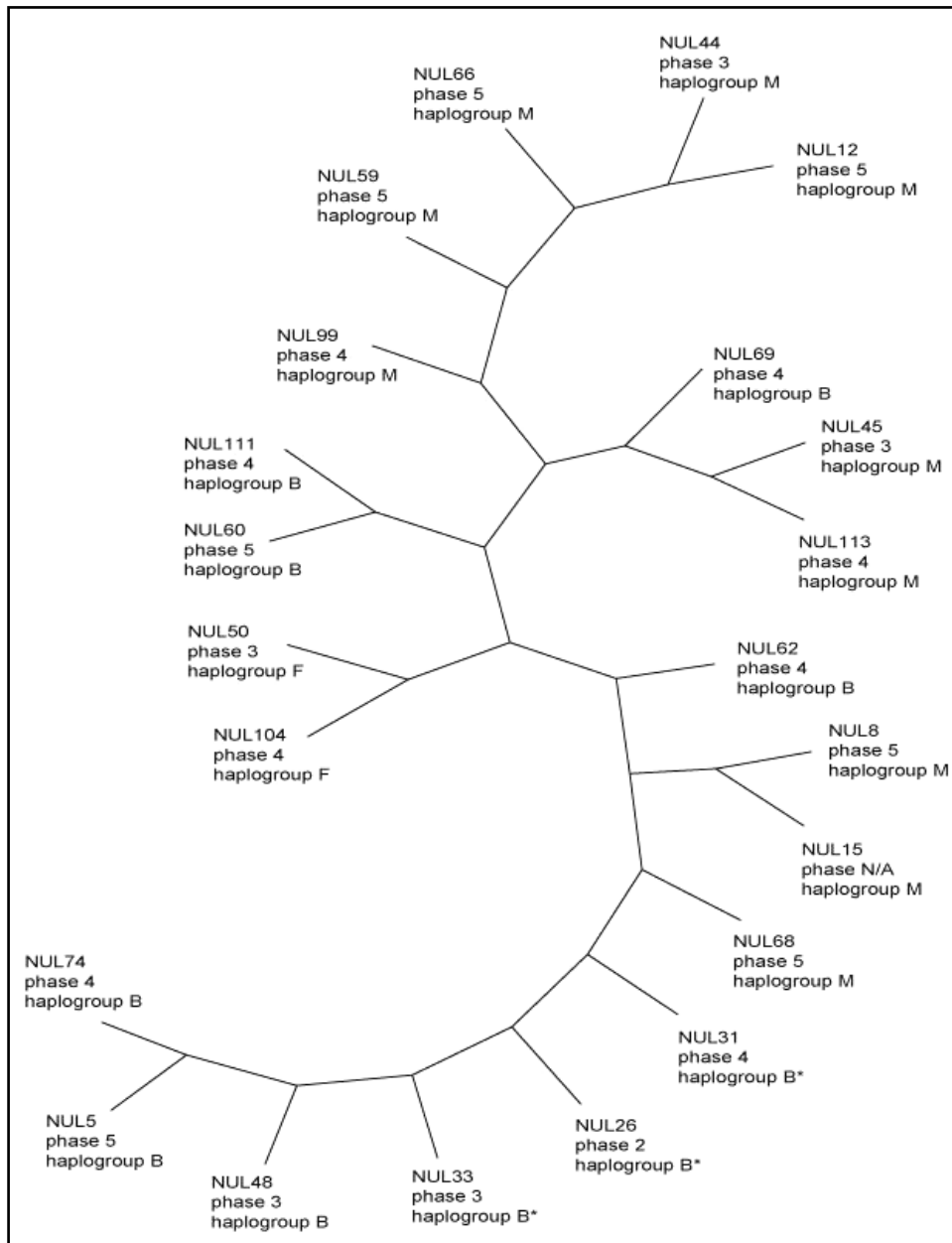


Figure 19. Phylogenetic tree of ancient population from Noen U-Loke. This tree was labeled with number of burial, number of mortuary phase and mitochondrial haplogroup on each of single branch

diversity between groups (d_{XY}) ranging from 1.89% to 2.98% (Table 9). This value showed the diversity between groups where low value represented the close genetic relationship. The value of interpopulational nucleotide diversity between ancient population from Noen U-Loke and modern population from Wuhan in Hubei province (eastern China) showed the lowest value 1.89% while the highest value of interpopulational nucleotide diversity between population from Wuhan and Sakai was 2.98%. The value of nucleotide diversity between ancient population from Noen U-Loke and Sakai was higher than another.

On the basis of net value of nucleotide diversity (d_A), population tree was constructed (Figure 20) using the neighbor-joining (NJ) method. The tree indicated that the aboriginal Sakai diverged first from the rest of the populations. The peoples of 4 Thai rational groups (Khon Kaen, Korat, Chiang Mai and Phuthai) formed a cluster with Hill tribe peoples from Lisu and Mussur, Khmer peoples and Han Chinese peoples from Yunnan province. Noen U-Loke clustered with modern Han population from eastern China (Liaoning, Wuhan in Hubei province, Qingdao in Shandong province and Zhanjiang in Guangdong province). Lao Song clustered with Chong and they were genetically closed to Chao Bon, ancient population from Noen U-Loke and modern Han population from eastern China by formed a phyletic cluster. The modern Han population from western China (Xinjiang and Qinghai) did not cluster with the modern Han population from eastern China.

Table 9. Estimates of intrapopulational nucleotide diversity (d_x or d_y) and net nucleotide diversity (d_A) among 19 rational groups.

Population	Noen U-Loke	Korat	Khmer	Chao Bon	Phuthai	Lao Song	Mussur	Khon Kaen	Lisu	Chiang Mai	Sakai	Chong	Yunnan	Wuhan	Liaoning	Qingdao	Xinjiang	Guangdong	Qinghai
Noen U-Loke	1.730	0.064	0.136	0.058	0.150	0.102	0.217	0.138	0.118	0.191	0.955	0.183	0.180	0.096	0.000	0.000	0.298	0.000	0.319
Korat	2.017	2.177	0.080	0.109	0.088	0.070	0.173	0.054	0.093	0.103	0.810	0.134	0.095	0.316	0.109	0.040	0.260	0.090	0.247
Khmer	1.924	2.093	1.847	0.144	0.001	0.121	0.069	0.037	0.057	0.041	0.911	0.241	0.008	0.488	0.221	0.128	0.386	0.073	0.356
Chao Bon	2.138	2.412	2.282	2.429	0.171	0.133	0.264	0.142	0.153	0.201	0.971	0.148	0.214	0.301	0.054	0.059	0.327	0.079	0.276
Phuthai	2.177	2.339	2.087	2.549	2.325	0.093	0.026	0.001	0.000	0.014	0.762	0.221	0.003	0.562	0.257	0.126	0.295	0.085	0.302
Lao Song	1.929	2.121	2.007	2.309	2.217	1.924	0.189	0.058	0.069	0.096	0.923	0.064	0.139	0.342	0.150	0.103	0.292	0.077	0.308
Mussur	2.022	2.203	1.934	2.420	2.129	2.091	1.882	0.089	0.013	0.074	0.825	0.317	0.014	0.636	0.310	0.203	0.385	0.131	0.334
Khon Kaen	2.083	2.223	2.041	2.437	2.244	2.100	2.110	2.161	0.017	0.013	0.758	0.157	0.002	0.503	0.218	0.106	0.187	0.089	0.271
Lisu	2.051	2.249	2.049	2.436	2.230	2.099	2.022	2.166	2.136	0.066	0.716	0.179	0.000	0.522	0.200	0.101	0.181	0.080	0.189
Chiang Mai	2.026	2.161	1.935	2.385	2.146	2.028	1.985	2.064	2.104	1.940	0.698	0.224	0.013	0.582	0.302	0.146	0.330	0.093	0.349
Sakai	2.251	2.329	2.265	2.617	2.355	2.315	2.197	2.269	2.214	2.098	0.861	0.920	0.703	1.612	1.063	0.788	0.898	0.867	0.782
Chong	2.141	2.316	2.257	2.456	2.476	2.119	2.351	2.330	2.340	2.286	2.443	2.186	0.269	0.460	0.204	0.193	0.336	0.227	0.329
Yunnan	2.171	2.310	2.058	2.556	2.292	2.228	2.082	2.209	2.182	2.110	2.261	2.489	2.253	0.572	0.281	0.141	0.247	0.101	0.241
Wuhan	1.893	2.337	2.344	2.449	2.657	2.237	2.510	2.516	2.523	2.484	2.975	2.485	2.631	1.865	0.013	0.117	0.613	0.168	0.608
Liaoning	2.185	2.551	2.498	2.622	2.773	2.466	2.604	2.652	2.622	2.625	2.847	2.651	2.761	2.299	2.707	0.000	0.356	0.012	0.293
Qingdao	1.968	2.262	2.185	2.407	2.422	2.198	2.277	2.319	2.302	2.249	2.351	2.419	2.400	2.183	2.470	2.266	0.217	0.000	0.239
Xinjiang	2.163	2.348	2.309	2.542	2.457	2.254	2.326	2.268	2.249	2.300	2.329	2.429	2.374	2.543	2.709	2.350	2.000	0.359	0.323
Guangdong	2.042	2.383	2.201	2.497	2.452	2.243	2.276	2.373	2.352	2.267	2.502	2.524	2.432	2.305	2.569	2.293	2.563	2.408	0.353
Qinghai	1.977	2.129	2.073	2.284	2.257	2.063	2.069	2.145	2.051	2.112	2.006	2.216	2.161	2.394	2.439	2.165	2.116	2.351	1.587

Note: All values are multiplied by 100. The bold face numbers refer to d_x or d_y , the number below the diagonal represent the values of d_{xy} and the number above the diagonal represent the values of d_A .

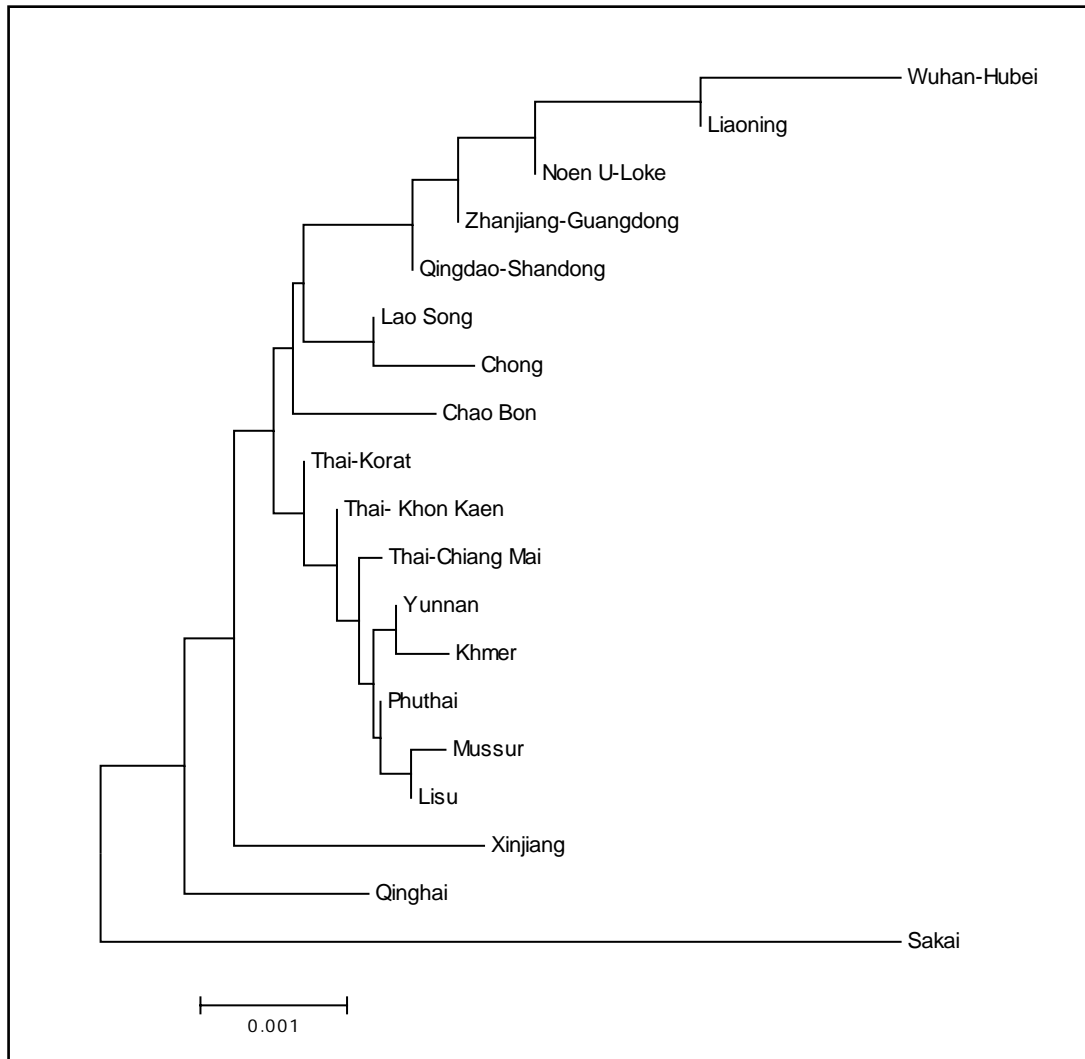


Figure 20. Population tree. The tree was constructed using neighbor-joining (NJ) method. The result showed the relationship between 19 populations of ancient peoples and modern peoples.

CHAPTER V

DISCUSSION

This study aims to develop procedures to successfully extract DNA from prehistoric human samples in Thailand and to investigate the genetic structure of ancient population in relative to modern populations.

We have developed the modified protocol suitable for ancient DNA extraction from our samples. The method originally used for ancient DNA extraction was very similar to a silica/guanidine thiocyanate method described in Boom *et al*, 1990 (48) and modified by Höss and Pääbo (49). The additional used of phosphate buffer was designed to add for DNA extraction from hydroxyapatite crystal according to Persson, 1992 (50) and Götherström and Lidén (51-52). This modified method gave us a good yield of DNA and high success rate of DNA recovery (17/26=70%). We found that the pH of buffers must be prepared accurately and fresh. For the amplification of ancient DNA extracted, we used nested-PCR approach in stead of the secondary PCR using the same primer pair as used in the previous reports. This study also used 4 overlapping primer pairs (F1 to F4) in the second-round of nested-PCR and could amplify the degraded DNA no longer than 500 bp (37). The nested-PCR method using this 4 overlapping primer pairs gave better results than one round PCR amplification.

A total of 8 criteria for authenticity were proposed by Pääbo, 2004 (37). These are 1) Extraction controls and PCR controls; 2) Repeated amplifications from the same or several extracts; 3) Inverse correlation between amplification efficiency and length of amplification; 4) Cloning of amplification products and sequencing of multiple clones; 5) Quantitated the number of amplifiable DNA molecules; 6) Biochemical assays of macromolecular preservation; 7) Exclusion of nuclear insertions of mitochondrial DNA and 8) Reproduction in a second laboratory. The authenticity herein present study was addressed by comparing the mitochondrial DNA sequence of these ancient samples with those of all investigators in the field-work and

in the laboratory. All amplification and sequencing results with suspected of no authenticity were discarded from the study. The mitochondrial DNA sequences from each of the 22 ancient teeth samples were then trusted to be derived from the excavated remains.

In this study, mitochondrial DNA of twenty-two from twenty-six teeth (85%) could be extracted and amplified. Four samples (15%) could not be amplified at all. The preservation of DNA at this archaeological site and the extraction protocol used in this study were relatively good with the 85% success rate. The amplification of seventeen from twenty-two samples gave the results of 360 nucleotides of HVS-1 (position 16024 to 16383). Five of twenty-two samples yield the PCR product that can be sequenced less than 360 nucleotides of HVS-1. One from these five samples gave the results of 320 nucleotides (position 16064 to 16383) and another four gave the results of 194 nucleotides (position 16190 to 16383).

From the hypervariable segment 1 (nt 16024 to nt 16383) analysis, the 26 polymorphic sites from 22 ancient samples were investigated. The polymorphic was found between nucleotide position 16086 to 16381. The frequency of each haplogroup determined in Noen U-Loke population were 32% for haplogroup B, 14% for haplogroup B*, 9% for haplogroup F and 45% for haplogroup M.

The phylogenetic tree showed relationship between the ancient samples from Noen U-Loke. The samples that clustered within same branch were from several mortuary phase and often classified into same haplogroup (Figure 19). The results from phylogenetic tree and haplogroup classification indicated a probably of genetically related between samples within the branch which were from several phases and dated. The diversity within ancient population from Noen U-Loke seems to be low with intrapopulation nucleotide diversity value 1.73%. Low diversity addressed the close relation between peoples within population. Genetic information was passed from generations to generations or from ancestor in mortuary phase 2 to their descendants in mortuary phase 3, 4 and 5. This suggested that the Noen U-Loke area was used by a relative population without or very few migrations from different genetic population through 900 years (2,400 – 1,500 BP).

The hypervariable segment 1 from a total of 424 samples were also analyzed, the 136 polymorphic sites from (nt 16024 to nt 16383) were detected. The highly

polymorphic region of the samples was between nucleotide positions 16190 to 16383 suggesting that this position was suitable for this kind of study. The modern Han population from eastern China, Liaoning, has the mitochondrial DNA haplogroup frequency close to Noen U-Loke population with the haplogroup frequency, 38% for haplogroup B, 12% for haplogroup B*, 6% for haplogroup F and 44% for haplogroup M. This result suggested that the modern Han population from Liaoning was genetically closed to ancient population from Noen U-Loke.

The lowest value 1.89% of interpopulational nucleotide diversity was showed between ancient population from Noen U-Loke and Wuhan in Hubei province of eastern China indicating that this modern Han population was also genetically closed to ancient population from Noen U-Loke.

The population tree was constructed using the value of net nucleotide diversity between 19 populations in this study. The tree showed that Sakai population, moved from India and Malaysia to the southern part of Thailand (67), diverged first from the rest of the ethnic groups (Figure 20). This showed less genetic relation to another population. The other populations were clustered in 2 major groups. The first group showed close genetic relation between 1,500-2,400 year-old ancient population, modern Han population from eastern China (Fengcheng, Liaoning; Wuhan, Hubei; Qingdao, Shandong and Zhanjiang, Guangdong a Chinese term for Sino-Tibetan Chinese speakers) (76), Lao Song (Tai speakers) (76), Chong (Austro-Asiatic Mon-Khmer speakers) (76) and Chao Bon (Austro-Asiatic Mon-Khmer speakers) (76). The second group showed close genetic relationship between Hill tribe peoples from Lisu (Sino-Tibetan Tibeto-Burman speakers) (76), Hill tribe peoples from Mussur (Sino-Tibetan Miao-Yao speakers) (76), Khmer peoples (Austro-Asiatic Mon-Khmer speakers) (76), modern Thai population from Khon Kaen, Korat, Chiang Mai and Phuthai (Tai speakers) (76), and modern Han population from Khunming, Yunnan (Sino-Tibetan Chinese speakers) (76). The modern Han population from western China (Yili, Xinjiang and Xining, Qinghai a Chinese term for Sino-Tibetan Chinese speakers) (76) diverged from others and did not cluster with the modern Han population from eastern China. The diversity of populations in Sino-Tibetan family of language was high, indicated that this language family was older than another in this study (Tai and Austro-Asiatic family) but close relation to them.

The results from phylogenetic tree construction, mitochondrial haplogroup classification, nucleotide diversity computational calculation, population tree construction and family of languages supported each others and suggested that 2,400-1,500 year-old population from Noen U-Loke was genetically closed to modern Han population from eastern China than to modern Thai population. This result indicated the ancestor of the ancient population from Noen U-Loke probably moved from eastern China more than 2,400 before.

In this study, the MT1 region in hypervariable segment 1 of mitochondrial DNA (nucleotide position 16190 to 16383) was also analyzed from a total of 419 samples same as in HVS-1 analysis. On the basis of the value of net nucleotide diversity (d_A), population tree was constructed. The results from HVS-1 analysis were not the same as the results from MT1 region analysis, indicated that the MT1 could not be a representative for completed hypervariable segment 1 (data not shown).

Suggestion for further study, 1) develop better process to extract and amplify ancient DNA in order to increase the rate and 2) expand the study to other several archaeological sites, both recovery along the Mun River Valley and other sites in the country. In this way the complete and continuous information of the prehistoric population lived in the Mun River Valley will be performed. The understanding of genetic structure, population migration, population history and genetic relationship, between prehistoric population and modern populations live in "The Present Thailand", will be increased.

CHAPTER VI

CONCLUSION

This present study has succeeded to develop procedures to extract DNA from prehistoric human remains. These modified protocols gave us a good yield of DNA and high success rate of DNA recovery. For the amplification of ancient DNA extracted, we used nested-PCR which could amplify the degraded DNA better. The ancient DNA authenticity was considered to be derived from the excavated remains. The study also investigated the genetic structure of an ancient population and their relation to modern population. The investigations indicated that the Noen U-Loke area probably was used by a relative population without or very few migrations from outside through 900 years (2,400-1,500 BP). The DNA analysis in this study suggested that the ancestor of the ancient population from Noen U-Loke probably moved from eastern China more than 2,400 before. The MT1 region in hypervariable segment 1 of mitochondrial DNA was also analyzed in this study. The analysis result showed that this region could not be a representative for completed hypervariable segment 1. Suggestion for further study are 1) develop the extraction and amplification protocol to increase the success rate and 2) expand the study to other several archaeological sites in Mun River Valley and other sites in the country for the completion and continuous information of the prehistoric population live in the Mun River Valley.

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APPENDIX

16024 TTCTTTC ATGGGGAAGC AGATTTGGGT ACCACCCAAG TATTGACTCA CCCATCAACA
 16081 ACCGCTATGT ATTCGTACA TTA CTGCCAG CCACCATGAA TATTGTACGG TACCATAAAT
 16141 ACTTGACCAC CTGTAGTACA TAAAAACCCA ATCCACATCA AAACCCCTC CCCATGCTTA
 16201 CAAGCAAGTA CAGCAATCAA CCCTCAACTA TCACACATCA ACTGCAACTC CAAAGCCACC
 16261 CCTCACCCAC TAGGATACCA ACAAACCTAC CCACCCTTAA CAGTACATAG TACATAAAGC
 16321 CATTTACCGT ACATAGCACA TTACAGTCAA ATCCCTTCTC GTCCCCATGG ATGACCCCCC
 16381 TCAGATAGGG GTCCTTGAC CACCATCCTC CGTGAAATCA ATATCCCGCA CAAGAGTGCT
 16441 ACTCTCCTCG CTCCGGGCC ATAACACTTG GGGGTAGCTA AAGTGAAGT TATCCGACAT
 16501 CTGGTTCCTA CTTCAGGGTC ATAAAGCCTA AATAGCCAC ACGTTCCCT TAAATAAGAC
 16561 ATCACGATG
 1 GATCACAGGT CTATCACCT ATTAACCACT CACGGGAGCT CTCCATGCAT TTGGTATTTT
 61 CGTCTGGGGG GTATGCACGC GATAGCATTG CGAGACGCTG GAGCCGGAGC ACCCTATGTC
 121 GCAGTATCTG TCTTTGATTC CTGCCTCATC CTATTATTTA TCGCACCTAC GTTCAATATT
 181 ACAGGCGAAC ATACTTACTA AAGTGTGTTA ATTAATTAAT GCTTGTAGGA CATAATAATA
 241 ACAATTGAAT GTCTGCACAG CCACTTTCCA CACAGACATC ATAACAAAAA ATTTCCACCA
 301 AACCCCTCCT CCCCCGCTTC TGGCCACAGC ACTTAAACAC ATCTCTGCCA AACCCAAAAA
 361 ACAAAGAACC CTAACACCAG CTAACCAGA TTTCAAATTT TATCTTTTGG CGGTATGCAC
 421 TTTTAAACAGT CACCCCCAA CTAACACATT ATTTTCCCT CCCACTCCCA TACTACTAAT
 481 CTCATCAATA CAACCCCGC CCATCCTACC CAGCACACAC ACACCGCTGC TAACCCATA
 541 CCGGAACCA ACCAAACCC AAAGACACCC CCCACA

Appendix A. The sequence of control region or displacement loop (D-loop) in the mitochondrial DNA from nucleotide position 16024 to 576 of the Cambridge Reference Sequence. The bold face shows the sequence of hypervariable segment 1 in the D-loop region (360 bp from nucleotide position 16024 to 16383).

Appendix B

The Contamination Precaution

1. The work area and equipment were frequently cleaned with 10% sodium hypochlorite and irradiated under UV light for several hours nightly.
2. Disposable laboratory gloves, filter tips, dedicated pipette, mouth mask and microcentrifuge tubes were irradiated with UV light at least 1 hour prior to use.
3. Three sets of dedicated pipette were used specifically. One for DNA extraction, one for PCR set up and one for buffer preparation. They were cleaned with 10% sodium hypochlorite and irradiated under UV light before use, together with sterile filter tips.
4. Buffers and all solutions were made with sterile deionized water and eliminated foreign DNA using autoclave or silica bead adsorption before aliquot in a laminar hood.
5. All PCR reagents were aliquot into single use amounts and sealed before stored at -20°C.
6. Mock extraction (DNA extraction performed in parallel with the ancient extracts to which no bone powder added) and PCR blank were done together with every set of sample reactions to check for possible contamination.
7. The reaction cocktail minus DNA and enzyme Taq DNA polymerase were irradiated by placing the tube directly on UV light source 20 minutes before use.

Appendix C

Buffers and Reagents Preparation

1. Buffers and Reagents for DNA extraction and purification from ancient teeth samples.

1.1 Lysis buffer

Dissolve 17.724 g Guanidine Thiocyanate (GuSCN), Cat.# V2791, Promega, USA in 5 ml of sterile deionized water.

Add 2.5 ml of 0.1 M Tris HCL (pH 6.4), Cat.# T1503, SIGMA, USA.

Dissolution was facilitated by incubation in waterbath at 65°C.

Add 2.5 ml of 0.2 M EDTA (pH 8.0), Cat.# E5134, SIGMA, USA and 1.625 ml of Triton X-100, SIGMA, USA.

Adjust to final volume 25 ml with sterile deionized water.

Dispense into 1 ml aliquot.

Add 10 µl of silica suspension in each aliquot and store at room temperature in dark for at most 2 months.

Centrifuge at 6,000 rpm for 1 minute before use.

1.2 Wash buffer

Dissolve 17.724 g Guanidine Thiocyanate (GuSCN), Cat.# V2791, Promega, USA in 5 ml of sterile deionized water.

Add 2.5 ml of 0.1 M Tris HCL (pH 6.4), Cat.# T1503, SIGMA, USA.

Dissolution was facilitated by incubation in waterbath at 65°C.

Adjust to final volume 25 ml with sterile deionized water.

Dispense into 1 ml aliquot.

Add 10 µl of silica suspension in each aliquot and store at room temperature in dark for at most 2 months.

Centrifuge at 6,000 rpm for 1 minute before use.

1.3 Proteinase K.

Dissolve 20 mg of Proteinase K, Cat.# V3021, Promega, USA in 1 ml of sterile deionized water for preparing 20 mg/ml stock concentration.

1.4 Silica suspension (100% w/v)

Suspend 3 g silicon dioxide, Cat.# S5631, SIGMA, USA in 25 ml of sterile deionized water.

Vortex and left to sediment for 24 hours.

Carefully remove 22 ml of the supernatant.

Add sterile deionized water to a total volume of 25 ml.

Vortex and left for further sediment 5 hours.

Discard 22 ml of the supernatant.

Add 30 µl of concentrated Hydrochloric acid (HCl).

Autoclave in 121°C for 20 minutes.

Dispense into small aliquots and store at room temperature in dark for at most 4 months.

1.5 Extraction buffer

Dissolve 10.89 g of Potassium Phosphate (K_2HPO_4), Cat.# P3786, SIGMA, USA in 25 ml of sterile deionized water.

Neutralize to pH 7.0 with 85% ortho-Phosphoric acid, MERCK, Germany.

Autoclave in 121°C for 20 minutes.

Expose with UV light for 15 minutes prior to use.

Note:

The pH of the mixture (300 ml of extraction buffer and 1 ml of lysis buffer) must be below than 7.5 for efficient DNA adsorption to silica particles.

1.6 95% Ethanol

Dissolve 95 ml of absolute Ethanol, MERCK, Germany in 5 ml of sterile deionized water.

Expose with UV light for 15 minutes prior to use.

1.7 Elution buffer

Dissolve 100 µl of 0.1 M Tris HCl (pH 8.0), Cat.# T1503, SIGMA, USA in 900 µl of sterile deionized water.

Autoclave in 121°C for 20 minutes and expose with UV light for 15 minutes prior to use.

Note:

The pH of the elution buffer must be above than 7.5 otherwise the DNA was not be efficiently eluted from the silica particles.

2. Buffer and Reagent for DNA extraction from hair root.

2.1 Digestion buffer

Mix 5 ml of 0.1 M Tris HCL (pH 8.0), Cat.# T1503, SIGMA, USA with 0.05 ml of 0.2 M EDTA (pH 8.0), Cat.# E5134, SIGMA, USA and 10 ml of 0.5% Tween 20.

Adjust to final volume 10 ml with distilled water.

Autoclave in 121°C for 20 minutes.

2.2 Proteinase K.

Dissolve 20 mg of Proteinase K, Cat.# V3021, Promega, USA in 1 ml of sterile deionized water for preparing 20 mg/ml stock concentration.

3. Reagents for DNA amplification

3.1 10X PCR buffer

3.2 10X Q-Solution

3.3 25 mM MgCl₂

3.4 10 mM dNTP, Cat.# N0446S, BioLabs Inc., USA.

dATP, dCTP, dGTP, dTTP

3.5 5 units/µl Tag DNA polymerase, Cat.# 201203, QIAGEN, USA.

4. Reagents for agarose gel electrophoresis

4.1 50X Tris acetate buffer (TAE)

Dissolve 121.1 g of Trisma-base, Cat.# T1503, SIGMA, USA in 28.55 ml of Glacial acetic acid and 125 ml of 0.2 M EDTA (pH8.0), Cat.# E5134, SIGMA, USA.

Adjust to final volume 500 ml with distilled water.

4.2 2% agarose gel.

Dissolve 2 g agarose gel, Cat.# 1170A, Research Organics Inc., USA, in the 100 ml of 1X TAE.

4.3 Ethidium bromide

Dissolve 1 g ethidium bromide in 100 ml of distilled water.

4.4 Loading dye

0.25% bromophenol blue

40% (w/v) sucrose in water

4.5 100 bp ladder marker, Cat.# N3231S, BioLabs Inc., USA.

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