ลำดับนิวคลีโอไทด์ของยีนพาราไทรอยด์ฮอร์โมนในลิงโลกใหม่และลิงโลกเก่า

นางสาวกันยา อนุกูลธนากร

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THE NUCLEOTIDE SEQUENCES OF PARATHYROID GENE IN NEW WORLD AND OLD WORLD MONKEYS

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การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อ 1) ศึกษาข้อมูลพื้นฐานของลำดับนิวคลีโอไทด์และลำดับกรดอะมิโนของยีนพาราไทรอยด์ฮอร์โมนในสัตว์กลุ่มไพรเมท (ในลิงโลกใหม่และลิงโลกเก่า) และ 2) หาความสัมพันธ์เชิงวิวัฒนาการของสัตว์ในกลุ่มไพรเมทจากข้อมูลที่ได้ โดยศึกษาลำดับนิวคลีโอไทด์จำนวน 500-553 คู่เบสของยีนพาราไทรอยด์ฮอร์โมน (PTH gene) ในสัตว์กลุ่มไพรเมท 8 ชนิด คือ *Cebus apella, Callithrix jacchus, Macaca fascicularis, Papio hamadryas, Presbytes obscura, Hylobates lar, Gorilla gorilla* และ *Pan paniscus* โดยวิธี direct sequencing จากนั้นนำลำดับนิวคลีโอไทด์ที่ได้ไปอนุมานลำดับกรดอะมิโนโดยโปรแกรม DNASIS และนำทั้งลำดับนิวคลีโอไทด์และลำดับกรดอะมิโนที่ได้ไปหาความสัมพันธ์เชิงวิวัฒนาการโดยวิธี Neighbor-Joining โดยใช้โปรแกรม PHYLIP และวิธี Parsimony โดยใช้โปรแกรม PAUP

พบว่าลำดับนิวคลีโอไทด์ของ PTH gene ประกอบไปด้วย intron 1 ส่วน และ exon 2 ส่วน โดย intron ที่ได้ใน *C. apella* และ *C. jacchus* มีขนาดเท่ากับ 102 คู่เบส และไพรเมทที่เหลืออีก 6 ชนิดมีขนาดเท่ากับ 103 คู่เบส สำหรับ exon ทั้ง 2 ส่วนสามารถถอดและแปลรหัสได้เป็น prepro-PTH ที่ประกอบไปด้วยส่วน “pre” 25 กรดอะมิโน, ส่วน “pro” 6 กรดอะมิโน และส่วน “PTH” 84 กรดอะมิโน เมื่อทำการเปรียบเทียบลำดับนิวคลีโอไทด์และลำดับกรดอะมิโนของ PTH gene ระหว่างคนและไพรเมททั้ง 8 ชนิด พบว่ามีค่าความคล้ายคลึงสูงถึง 95.38-99.80% และ 93.04-100.00% ตามลำดับ จากการวิเคราะห์ความสัมพันธ์ทางพันธุกรรมเชิงวิวัฒนาการโดยอาศัยลำดับนิวคลีโอไทด์ พบว่าสามารถแบ่งไพรเมททั้ง 8 ชนิดออกเป็น 2 กลุ่มใหญ่ ได้ในระดับ Infraorder คือ 1) กลุ่มลิงโลกใหม่ หรือ Infraorder Platyrrhini ที่ประกอบไปด้วย *C. apella* และ *C. jacchus* 2) กลุ่มลิงโลกเก่า หรือ Infraorder Catarrhini ที่สามารถแบ่งออกเป็น 2 กลุ่มย่อยได้ในระดับ Superfamily คือ 2.1) กลุ่มลิงโลกเก่า หรือ Superfamily Cercopithecoidea ประกอบด้วย *M. fascicularis, P. hamadryas* และ *P. obscura* และ 2) กลุ่มเอพและมนุษย์ หรือ Superfamily Hominoidea ประกอบด้วย *H. lar, G. gorlla* และ *P. paniscus* ดังนั้น จากการศึกษาในครั้งนี้สามารถสรุปได้ว่า PTH gene เป็นยีนอนุรักษ์ที่มีการเปลี่ยนแปลงน้อยมากในสัตว์กลุ่มไพรเมท

ภาควิชา.....ชีววิทยา..... ลายมือชื่อนิสิต.....

สาขาวิชา.....สัตววิทยา..... ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา...2543..... ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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AMINO ACID SEQUENCE
KANYA ANUKULTHANAKORN : THE NUCLEOTIDE SEQUENCES OF PARATHYROID GENE IN NEW WORLD AND OLD WORLD MONKEYS. THESIS ADVISOR: ASSIST. PROF. DR. SUCHINDA MALAIVIJITNOND, THESIS CO-ADVISOR: PROF. DR. OSAMU TAKENAKA, Ph.D. 108 pp. ISBN 974-346-107-8.

The aims of this study are 1) to determine the nucleotide and deduced amino acid sequences of parathyroid gene in primates, new world and old world monkeys, and 2) to construct the phylogenetic tree of those primates based on the determined sequences. The nucleotide sequences of the parathyroid hormone (PTH) gene in 8 species of primates; *Cebus apella, Callithrix jacchus, Macaca fascicularis, Papio hamadryas, Presbytes obscura, Hylobates lar, Gorilla gorilla*, and *Pan paniscus*, were analyzed. The sequences of 500-553 bp were determined by direct sequencing method. The nucleotide sequences were deduced to amino acid sequences by DNASIS program. Thus, the phylogeny was estimated from those nucleotide and amino acid sequences using a Neighbor-Joining method (PHYLIP program) and Parsimony method (PAUP program).

The PTH gene contained one intron which separated two exons that coded the sequences of prepro and PTH, respectively. The intron in *C. apella* and *C. jacchus* was 102 bp long, whereas it contained 103 bp in the other 6 species of primates. The prepro-PTH consisted of 25 amino acids of the "pre" sequence, 6 amino acids of the "pro" sequence, and 84 amino acids of the "PTH". Comparison of nucleotide and amino acid sequences of PTH gene between human and 8 species of primates showed strong homology of 95.38-99.80%, and 93.04-100.00%, respectively. Phylogenetic analysis of the nucleotide sequences revealed that these 8 species of primates were divided into 2 groups; 1) New world monkeys or Infraorder Platyrrhini consisted of *C. apella* and *C. jacchus*, and 2) Old world monkeys or Infraorder Catarrhini which could be subdivided into 2 groups of 2.1) Old world monkeys or Superfamily Cercopithecoidea consisted of *M. fascicularis, P. hamadryas, P. obscura*, and 2.2) Apes and man or Superfamily Hominoidea consisted of *H. lar, G. gorilla* and *P. paniscus.* From this study, it may conclude that PTH gene is conserved among primates.

ภาควิชา.....ชีววิทยา..... ลายมือชื่อนิสิต.....
สาขาวิชา.....สัตววิทยา..... ลายมือชื่ออาจารย์ที่ปรึกษา.....
ปีการศึกษา.....2543..... ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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Abbreviation

PTH	=	Parathyroid hormone
cAMP	=	Cyclic adenosine monophosphate
CT	=	Calcitonin
RER	=	Rough endoplasmic reticulum
prepro-PTH	=	Prepro-parathyroid hormone
hPTH	=	Human parathyroid hormone
bPTH	=	Bovine parathyroid hormone
cPTH	=	Canine parathyroid hormone
rPTH	=	Rat parathyroid hormone
mRNA	=	Messenger ribonucleic acid
PKA	=	Protein kinase A
PKC	=	Protein kinase C
VIP	=	Vasoactive intestinal polypeptide
PTHrP	=	Parathyroid hormone related peptide
GRF	=	Growth hormone-releasing factor
CRF	=	Corticotropin-releasing factor
PACAP	=	Pituitary adenylate cyclase stimulating peptide
sPTH	=	Synthetic parathyroid hormone
OVX	=	Ovariectomized
μg	=	Microgram
kg	=	Kilogram
BW	=	Body weight
U	=	Unit
HRT	=	Hormone replacement therapy
FDA	=	Food and Drug Administration

EDTA	=	Ethylenediamine tetraacetic acid
NaCl	=	Sodium chloride
$AgNO_3$	=	Silver nitrate
NH_4OH	=	Ammonium hydroxide
mg	=	Milligram
ml	=	Milliliter
rpm	=	Round per minute
DNA	=	Deoxyribonucleic acid
µl	=	Microliter
SDS	=	Sodium dodecyl sulfate
RNAase A	=	Ribonuclease A
$^{\circ}C$	=	Degree celsius
PCR	=	Polymerase Chain Reaction
FITC	=	Fluoresceinisothiocyanate
PAGE	=	Polyacrylamide gel electrophoresis
TEMED	=	N,N,N',N'-tetramethyethylenediamine
APS	=	Ammonium persulfate
V	=	Volt
ds	=	Double strand
ss	=	Single strand
dNTPs	=	Deoxyribonucleotide triphosphates
ddNTP's	=	Dideoxyribonucleotide triphosphates
DMSO	=	Dimethyl sulfoxide
MPC	=	Magnetic particle collector
WB	=	Washing buffer
bp	=	Base pair

Chapter I

Introduction

Parathyroid hormone or parathormone (PTH) is an important hormone regulating the calcium and phosphate concentrations in plasma. It is synthesized and secreted by parathyroid glands. The cells of parathyroid glands which synthesize PTH are called chief cells. The four parathyroid glands have been found in pairs on the dorsal surface of each two lobes of the thyroid gland in human (Figure 1.1), and the parathyroid glands have a highly blood supply. The synthesis and secretion of PTH are controlled by plasma calcium concentrations. PTH secretion is increased in response to a drop of plasma calcium ion concentration while high calcium concentrations suppress the production (Rhoades and Pflanzer, 1996).

The effects of PTH are to increase calcium concentrations and to decrease phosphate concentrations in plasma. PTH has a direct effect on bone and kidneys, and an indirect effect on intestine (Figure 1.2). At the bone, PTH probably activates bone resorption by having a direct action on osteoblasts which secondarily stimulate osteoclasts, the cell responsible for bone resorption (Royer and Kemper, 1990). At the renal tubules of the kidneys, PTH increases reabsorption of calcium, but inhibits reabsorption of phosphate. In intestine, PTH increases the absorption of calcium and phosphate by its effect on increasing the metabolism of vitamin D precursor to form the highly active 1,25-dihydroxyvitamin D_3 in the proximal tubules of the kidney. PTH exerts its effects on its target cells both by increasing intracellular cyclic adenosine monophosphate (cAMP), and intracellular calcium (Mundy, 1994).

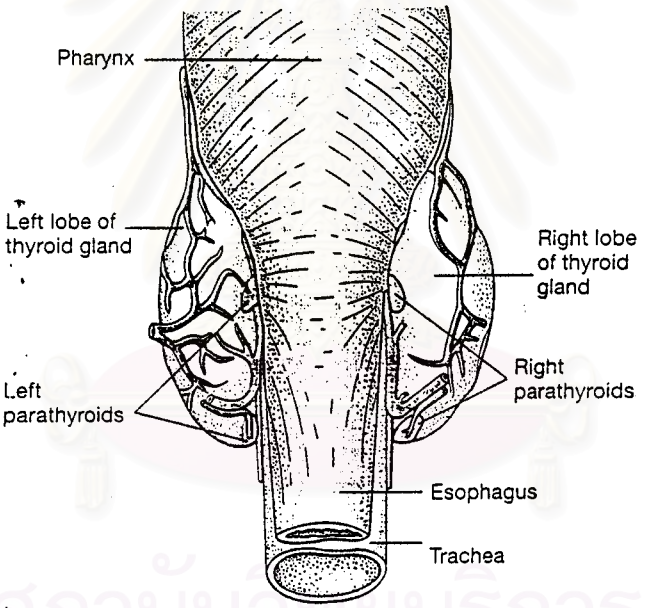


Figure 1.1 Anatomical locations of the four parathyroid glands in human. The view depicts the dorsal surface of the thyroid gland (Rhoades and Pflanzer, 1996).

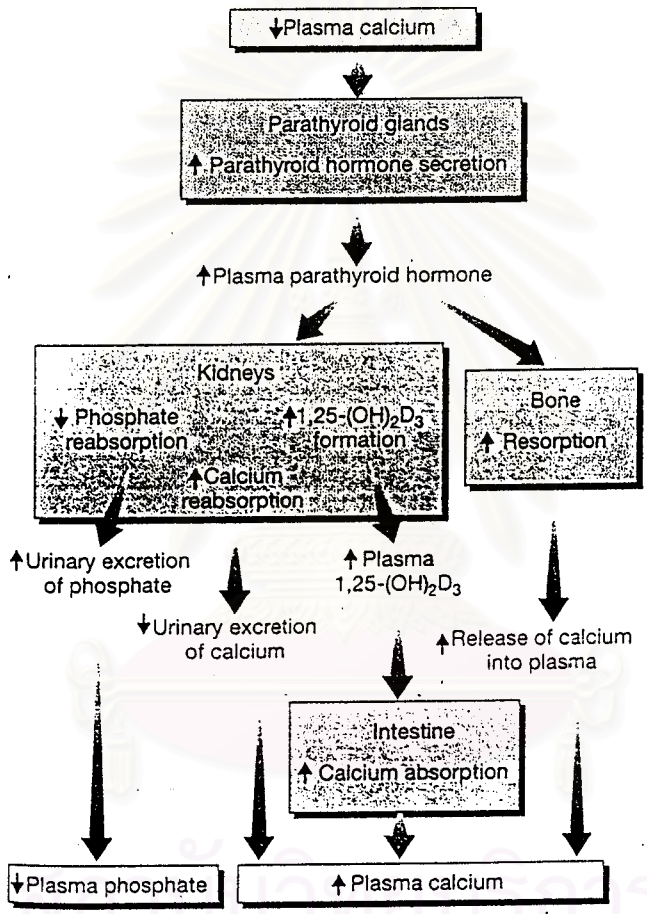


Figure 1.2 Diagram showing changes that occur in response to a decrease in plasma calcium. The end result is that plasma calcium concentration is restored to its normal value (Rhoades and Pflanzer, 1996).

Due to its main effect on bone, PTH has several effects which lead to increased net bone resorption of bone remodeling. Bone remodeling consists of bone resorption and bone formation. Bone cells are classified into three primary cell types: osteoblasts, osteocytes, and osteoclasts (Figure 1.3). The cell involved in the remodeling of bone are osteoblasts and osteoclasts. Osteoblasts are small cubodial cells that are responsible for synthesizing new bone and then mineralizing it, and osteoclasts are large multinucleated cells that are responsible for breaking down bone. Osteoclasts are unique cells that are the only cell type in the body known to have the capability of resorbing bone. Bone is remodeled by a specific sequence of cellular events which always begins with osteoclastic bone resorption, followed by new bone formation (Figure 1.4). There are a balance in normal health between the processes of bone resorption and bone formation. In contrast, an imbalance of this process can cause an osteoporosis. Osteoporosis is characterized by decrease trabecular (or cancellous) bone mass in which both the mineral and the matrix decreased to the same extent, but a gross abnormality of bone composition is not changed. Osteoporosis is divided into two types: primary and secondary osteoporosis. Primary osteoporosis is that occurrs without any associations with other diseases, but secondary osteoporosis occurred in association with other conditions. Primary osteoporosis is subdivided into type I (postmenopausal osteoporosis), and type II (senile osteoporosis). The deficiency of estrogen may be the cause of type I, whereas the impaired calcium absorption from gastrointestinal tract (G-I tract) and osteoblasts function may be the cause of type II (Mundy, 1994).

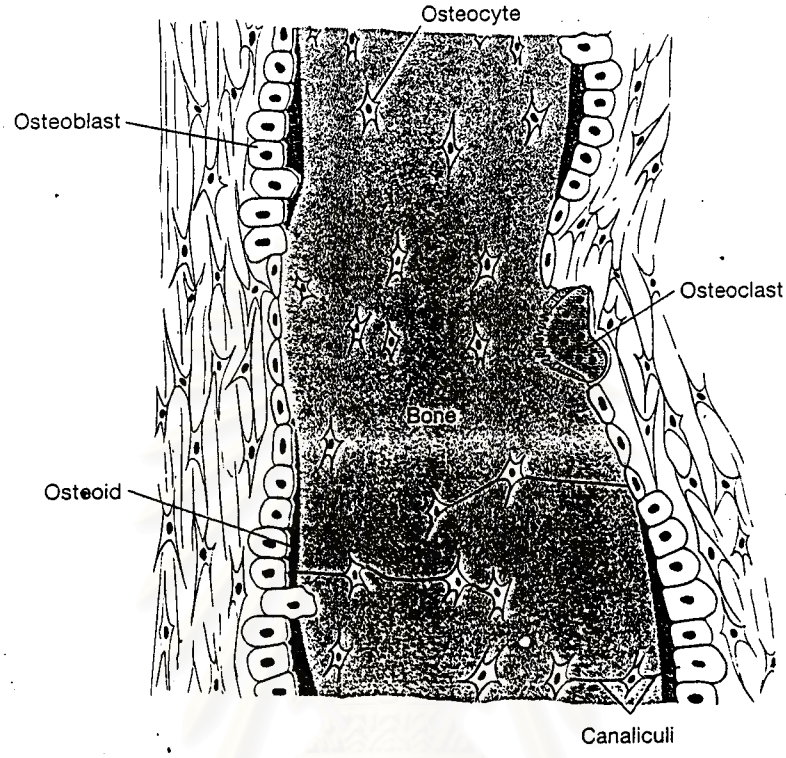


Figure 1.3 Diagram illustrating the relationships among different types of bone cells. Osteoblasts line the surface of bone and secrete osteoid into the space adjacent to the bone. For the sake of simplicity, only a few of the canaliculi that interconnect osteocytes and osteoblasts are shown near the bottom of the figure. An osteoclasts activity engaged in bone resorption is represented by the large cell on the right. Note the ruffled appearance of the osteoclast membrane on the side next to the bone surface (Rhoades and Pflanzer, 1996).

In women, there is an additional marked acceleration of bone loss at the time of the menopause. The withdrawal of estrogen or estrogen deficiency leads to increase bone resorption. Thus, the abnormality increment of PTH can additionally cause an osteoporosis in postmenopausal women whose ovaries cease the secretion of estrogen. Estrogen secretion from the ovaries in premenopausal women can protect against bone loss. Similarly in men, osteoporosis can exhibit in hypogonadism, a patient with an impairment of gonad function and secretes the low levels of plasma androgen. Because androgens secreted from gonad can be converted to estrogen and also protect against bone loss in man (Rhoades and Pflanzer, 1996). Thus, the decline in bone mass occurs in both men and women, but it is more pronounced in women, makes skeleton more fragile in the elderly and is the reason why older people develop fractures of susceptible bones following trivial injuries.

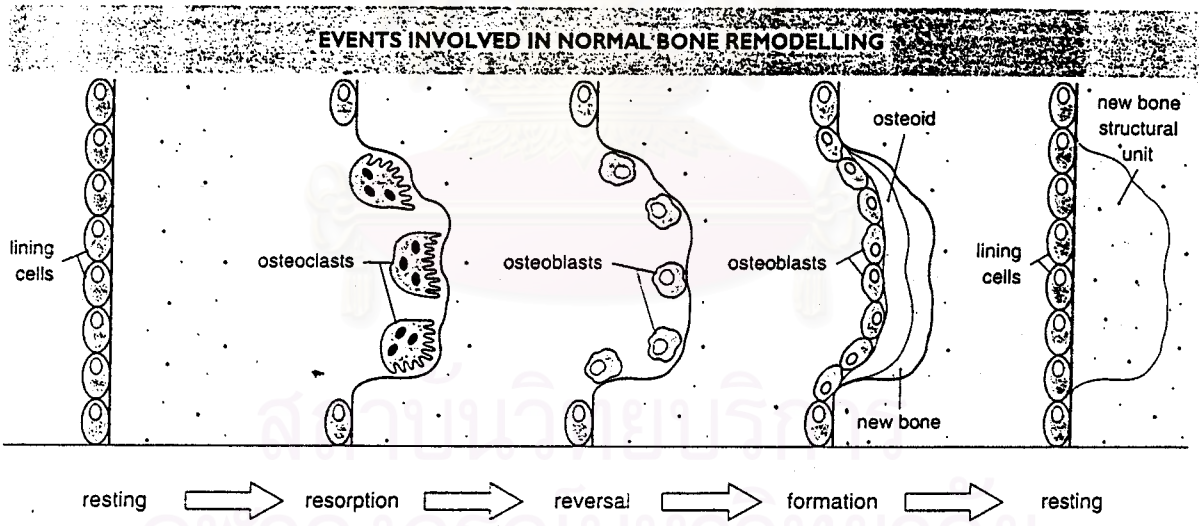


Figure 1.4 The consequence of events involved in normal bone remodeling on a trabecular bone surface (Mundy, 1994).

Pathogenesis of osteoporosis that occurs after menopause is related to a decrease in gonadal steroids. Common sites of fractures among postmenopausal women are the vertebrae, forearm, and hip (Hadley, 1996). This disease is a major public health concern in the United States and other countries (Rhoades and Pflanzer, 1996). The prevention of osteoporosis might be realized if estrogen treatment is continued after menopause. Virtually all the available evidence indicates that the use of estrogen lowers the risk of postmenopausal fractures (Hadley, 1996). However, estrogen treatment increases the risk for the development of endometrial (uterine) cancer. Then, the other therapeutic agents are being searched for this osteoporosis symptom (Rhoades and Pflanzer, 1996).

In patients with established osteoporosis, therapies that are available include drugs that inhibit bone resorption (anti-resorptive agent), and bone formation (anabolic agent). Calcitonin (CT), bisphosphonates, and estrogen are drugs which inhibit bone resorption. Their effects on bone formation are probably much less prominent than their effects on bone resorption. Fluoride and low-dose PTH seem to stimulate bone formation. Fluoride increases bone mass, although it may be increase propensity to fracture. Low-dose PTH has an anabolic effect on the skeleton to increase bone mass, although this effect may not be consistently maintained and still in investigation (Mundy, 1994). A variety of studies over last 65 years reviewed by Dempster *et al*. (1993) and Kimmel *et al*. (1993) have established that intermittent injections of PTH could increase trabecular bone mass at supra-normal levels in rats. PTH administration also increases trabecular bone mass in dogs, sheep, ferrets, and humans (Mitlak *et al*, 1996). This means that PTH seems to hold promise as a treatment for osteoporosis, but the exact mechanism of PTH actions in stimulating bone formation is not known. Thus, other studies in PTH are being used as a single therapeutic agent may be fulfill this role of PTH.

However, the research in human is usually encumbered by prevailing ethical and legal standards. Then, many researchers turn to use animals as an experimental model. Primates, especially Apes, have the characteristic circulating patterns of principal ovarian steroids and pituitary gonadotropins, as well as dynamics of the follicular, periovulatory, and luteal phase events, are virtually indistinguishable from those of human menstrual/ovarian cycle (Dukelow and Bruggeman, 1979; Meury-Dessolle and Dang, 1985; Malaivijitnond and Varavudhi, 1995, 1997). Primates, therefore, serve as a choice for the study of osteoporosis and PTH (Thorndike, and Turner, 1998) and are hence applicable to clinical use in human. To understand the mechanism of action of PTH in primates, the nucleotide and amino acid sequences of PTH gene in these animals should be known. Although there have been many studies of the PTH gene in humans (Vasicek *et al*, 1983), cattle (Kronenberg *et al*., 1979; Weaver *et al*., 1984), dogs (Rosol *et al*., 1995), pigs (Sauer *et al*., 1974), and rats (Heinrich *et al*., 1984), a few reports have published on the PTH gene in primates (Malaivijitnond and Takenaka, 1998; Anukulthanakorn *et al*., 1999). Accordingly, this study aimed to determine nucleotide sequences and the deduced amino acid sequences in 8 species of primates, new world and old world monkeys.

Taxonomy of these experimental animals is as follows;

Order Primates

Suborder Anthropoidea

Infraorder Platyrrhini (New world monkeys)

Family Cebidae:

1. *Cebus apella*

Family Callitrichidae:

2. *Callithrix jacchus*

Infraorder Catarrhini (Old world monkeys)

Family Cercopithecidae:

3. *Macaca fascicularis*

4. *Papio hamadryas*

5. *Presbytes obscura*

Family Hylobatidae:

6. *Hylobates lar*

Family Hominidae:

7. *Pan paniscus*

8. *Gorilla gorilla*

(Ankel-Simons, 1983)

Objectives

1. To determine nucleotide and deduced amino acid sequences of the PTH gene in 8 species of primates in the Suborder Anthropoidea.

2. To establish phylogenetic trees of 8 species of Anthropoidea primates based on nucleotide and amino acid sequences analyses of the PTH gene.

Scope of work

Determine nucleotide and deduced amino acid sequences of the PTH gene in 8 species of primates (Order Primates, Suborder Anthropoidea).

Expected outcome

1. Knowing the nucleotide and amino acid sequences of the PTH gene in 8 species of Anthropoidea primates.

2. Getting the phylogenetic tree of 8 species of Anthropoidea primates: according to nucleotide and amino acid sequences of the PTH gene.

3. Applying useful information from this research to study bone metabolism of PTH using primates as the model.

Chapter II

Literature review

PTH is produced and secreted by parathyroid glands. These glands are present in all terrestrial vertebrates (Royer and Kemper, 1990). The glands are composed of two general cell types, the chief cells and the oxyphil cells. The former cell type is the source of PTH, but the function of oxyphil cells is unknown (Hadley, 1996). The synthesis and secretion of PTH are depended on the calcium ion concentration of the serum. As the extracellular fluid calcium concentrations decrease, PTH synthesis and secretion is increased. PTH is secreted by exocytosis. The membrane of the secretory granule containing the hormone fuses with the plasma membrane, which is subsequently lysed, releasing PTH into the blood stream. Interestingly, a reduction in calcium level increases exocytosis of the glands. Cyclic adenosine monophosphate (cAMP) may be involved in the calcium controlled exocytosis of PTH. PTH circulates in the from of the intact 1-84 amino acid hormone, and carboxyl-terminal fragments, with a long half-life approaching several hours, are also found. The liver reduces the 1-84 amino acid PTH to a smaller fragment. The kidney also intervenes in PTH metabolism (Royer an Kemper, 1994). PTH acts directly on kidney and bone by interact with receptors on renal cortical cells and on bone mononuclear cells to increase serum calcium. Indirectly, PTH acts on the intestine to promote calcium absorption (Mallete, 1991; Heinrich *et al*., 1996).

PTH is a polypeptide chain of 84 amino acid initially synthesized as a precursor, prepro-PTH, which consists of 115 amino acids. The pre-sequence consists of 25 extra amino acids at the N-terminus of pro-PTH. It contains a core of 12

hydrophobic amino acids which makes pre-sequence serving the function of the signal sequence. This prepro-PTH is processed in the rough endoplasmic reticulum (RER) by

enzymic removal of an amino terminal sequence to the relative stable 90 amino acids, pro-PTH. Pro-PTH consists of 6 amino acids with the sequence of Lys-Ser-Val-Lys-Lys-Arg in human. Similar sequences are found in rat, pig, dog and chicken pro-PTH molecules (Baulieu and Kelly, 1990). The function of pro-sequence is unknown but the two basic residues, direct a peptidase to cleave the pro-sequence from PTH. Comparison of the pro-sequence found that it is conserved and the last two residues are always Lys-Arg. Pro-PTH is processed in the Golgi complex of the cell to the 84 amino acid mature PTH by additional proteolytic cleavage (Heinrich *et al*., 1983; Weaver *et al*., 1984; Kronenbreg *et al*., 1985). Cleavage of the pro-PTH to PTH usually occurs just before the hormone is secreted, and occurs 15 to 20 minutes after the synthesis of pro-PTH, at about the time the precursor reaches the Golgi complex in the cell. The first 34 amino acids from the amino- terminal part of mature PTH are responsible for the biological effects of PTH and for binding of PTH to its receptor (Besser and Thorner, 1994).

The complete amino acid sequences (84 amino acids) of human PTH (hPTH) was firstly reported by Keutmann *et al*. (1978) using Edman degradation method. It was shown that hPTH consists of 84 amino acids with the molecular weight of 9427. The comparison of hPTH amino acid sequence with that of bovine and porcine showed differences in 11 positions (Keutmann *et al*., 1978). Hendy *et al*. (1981) studied the nucleotide sequence of cloned cDNAs encoding human prepro-PTH. They used bovine (bPTH) cDNA clone as a probe to screen recombinant clones containing hPTH DNA (the amino acid homology of PTH gene between bovine and human is 86%). Vasicek *et al*. (1983) studied the nucleotide sequence of hPTH gene and found that

hPTH gene contains two intervening sequences which separate the gene into a 5' untranslated region (UTR), a prepro-sequence domain, and a domain containing the PTH sequence and the 3' UTR. They determined hPTH gene approximately 4200 bp long. The study using restriction endonuclease analysis of human leukocyte DNA showed that the haploid human contains one copy of the prepro-PTH gene. The PTH gene in human is located on the short arm of chromosome11 (Mallete, 1991). Kronenberg *et al*. (1979) found that the prepro-PTH is predominant in the form of messenger prepro-PTH RNA in the parathyroid gland.

The study of nucleotide and amino acid sequences of rat PTH (rPTH) gene further supports that the PTH gene is an unique and present in a single copy in the genome, as in bovine PTH (bPTH) gene. The rPTH is organized into two introns and three exons. Exon I consists of the sequence encoding the 5' UTR of rPTH mRNA, and followed by intron A of 1.6 kb length. Exon II encodes the prepeptide or leader sequence of rat prepro-PTH, and a part of the propeptide. Intron B is short (111 bp) and followed by exon III, which contains the remaining sequences of rPTH mRNA. It can be concluded that exon II of the rPTH gene encodes the small sequence of rat prepro-PTH and exon III of the rPTH gene encodes the biologically active secreted PTH peptide of 84 amino acids in length. Comparison of amino acid sequence of rat prepro-PTH with bovine and human prepro-PTH showed that the hormone sequences were highly conserved in two regions of known functions near the amino terminus and in a third region near the carboxyl terminus (Heinrich *et al*., 1984).

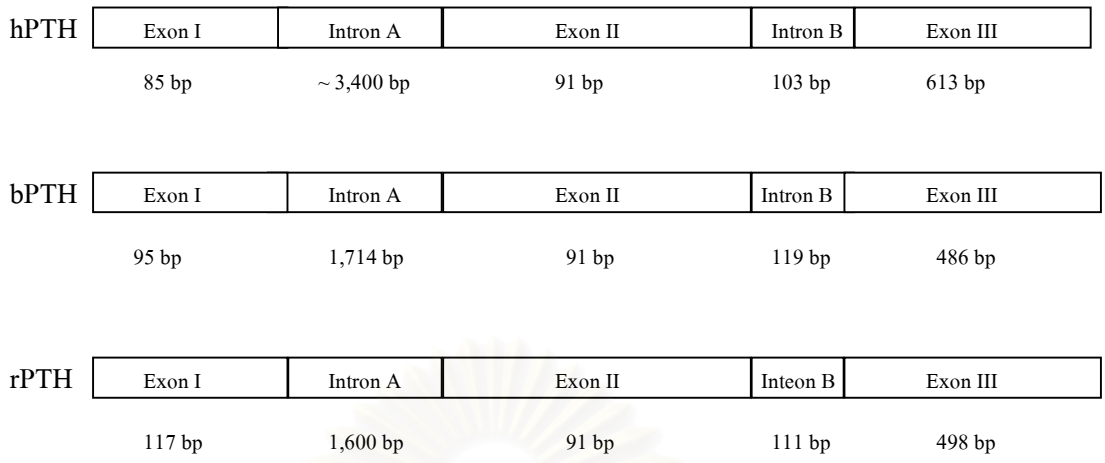


Figure 2.1 Physical maps of PTH gene in human; h (Vasicek *et al.,* 1983), bovine; bPTH (Weaver *et al.,* 1984), and rat; rPTH (Heinrich *et al.,* 1984). The PTH gene contains two introns which separate three exons that code primarily for : (i) the 5' untranslated region (5' UTR), (ii) the pre-sequence and the first four amino acids of the pro-sequence of prepro-PTH, and (iii) the remainder of the pro-sequence, PTH and the 3' untranslated region (3' UTR).

In porcine, the amino acid sequence was highly similar to bPTH because they are different in only 7 of 84 amino acids (Sauer *et al*., 1974). Recently, nucleotide and amino acid sequences of canine PTH (cPTH) and cPTH-related peptide were reported (Rosol *et al*., 1995). The result showed that the partial 5' UTR was 67% homology to exon I of hPTH gene, and the partial 3' UTR was 82% homology to the 3' UTR of exon III of hPTH. Comparison of coding regions (exon II and exon III) of cPTH with hPTH showed 90% homology of their molecules. The mature cPTH was 88% homologous to hPTH with two substitutions in the first 40 amino acids at position 7 and 16, but these substitutions were not unique to cPTH because of its occurrence in bovine (position 7 and 16) and porcine (position 16).

Recently, Malaivijitnond and Takenaka (1998) studied nucleotide sequences and deduced amino acid sequences of five species of macaque in Thailand. They determined 600 bp of macaque PTH gene and found that the intron which separate two exons primarily containg for the pre-sequence of macaque prepro-PTH and PTH. Comparison of nucleotide sequences between macaque PTH and human PTH showed a high homology of 97.4%, and compared amino acid sequences showed only three amino acids substitution, at the 35^{th}, 58^{th}, and 76^{th} position of PTH. The results showed the 100% homology of nucleotide sequences of PTH coding regions among five species of macaques. Anukulthanakorn *et al*. (1999) studied nucleotide sequences of two groups of macaques; silenus-sylvanus (*Macaca silenus, M. sylvanus, M. maurus,* and *M. nigra*) and fascicularis (*M. fascicularis*), and found 100% homology of nucleotide sequences between these two groups. It may conclude that PTH is very conserved among macaques and less evolved from human.

The major regulator of PTH is the serum calcium levels. The low calcium increases PTH synthesis by post-transcriptional mechanism in addition to its effects on transcription. Post-transcriptional regulation of gene expression provides a means whereby protein synthesis can be regulated rapidly in response to acute changes in stimuli, whereas responses to long-term changes in the cellular environment can be mediated through changes in gene transcription. The post-trascriptional regulation may occur including changes in processing of nuclear RNA, mRNA stability, and translation. The mechanism of post-transcriptional regulation have generally demonstrated a role for RNA-protein interactions, particularly in the noncoding region of mRNA. UV cross-linking studies revealed two proteins in the cytosol of parathyroid cells which bound specifically to the 5' UTR of PTH mRNA with molecular masses of 66 and 68 kD, while protein with apparent molecular masses of

48 and 70 kD bound to the 3' UTR. *In vitro* translation assays indicated that the parathyroid cell cytosol contained factor that inhibits translation of PTH mRNA . Translation of RNA was containing only the 5' UTR of PTH mRNA but the RNA containing the 3' UTR was reduced the translation. The 5' UTR is directly involved in the initiation of translation with the Kozak model of translation predicting that ribosomes interact with the cap site of mRNA and scan along the 5' UTR , melting any protein structure until reaching an AUG codon to initiate translation. The 3' UTR regulates protein synthesis both by blocking initiation or modifying translation efficiency. This inhibition may be due to conformational changes in the mRNA which reduces access of ribosome, or it may be due to differences in the half-life of PTH mRNA when associated with the ribosomes (Vadher *et al*. , 1996).

PTH stimulates multiple intracellular signals (cAMP, inositol phosphates, and calcium) and activates both proteinkinase A and C (PKA and PKC) by binding with specific receptors in two major target tissues, bone and kidney (Abou-Samara *et al*., 1992). The PTH receptor has seven membrane-spanning domains and constitutes a new family of G-protein coupled receptors along with receptors for secretin, calcitonin (CT), vasoactive intestinal polypeptide (VIP), and glucagon-like protein 1 (Schneider *et al*.,1993). Members of this family show the following common features; an extracellular amino terminal extension that is intermediate in length between the catecholamine receptors and glycoprotein hormone receptors, conserved extracellular cysteine residues, and highly conserved first cytoplasmic loop and seventh membrane spanning regions (Kong *et al*., 1994). The hPTH receptor consists of 593 amino acids. Schneider *et al*. (1993) found 90% and 78% identity between hPTH receptor and the rat and opossum counterparts, respectively.

The mRNA encoding the PTH/PTH-related peptide (PTHrP) receptor is particularly abundant in kidney and bone. The receptor has a very wide spread distribution, and has been demonstrated in most of the tissue with PTH receptor (Usdin Gruber, and Bonner, 1995). The study of Kong *et al*. (1994) showed that the PTH/PTHrP receptor gene is highly conserved in mammalian species. They studied the PTH/PTHrP gene in rat, mouse, and human and found that the gene extended over approximately 22 kb and encoded by at least 15 exons , 14 introns. The PTH/PTHrP receptor gene in the three species contain GT-rich regions that are commonly found 20-30 bp downstream of polyadenylation signals but it does not contain a typical polyadenylation signal (AATAAA) that allow cleavage and polyadenylation of the receptor transcript, that is commonly found in mammalian genes. They concluded that the PTH/PTHrP receptor shares significant homology with receptors for CT, secretin, growth hormone-releasing factor (GRF),corticotropin-releasing factor (CRF), glucagon, glucagon-like peptide, gastric inhibitory peptide, pituitary adenylate cyclase stimulating peptide (PACAP), VIP, and an insect diuretic hormone. The main features of this novel G-protein-coupled receptor family are the conserved extracellular cysteine residues and highly conserved sequences in the first cytoplasmic loop, and the third and seventh membrane-spanning domains. Kong *et al.* proposed that the organization of this gene may be a prototype for all the genes of the PTH/PTHrP receptor family. The study of Usdin *et al.* (1995), identified a new PTH-recognizing receptor, which referred to the PTH2 receptor. Unlike the PTH/PTHrP receptor, PTH2 receptor recognized PTH but not PTHrP. When PTH binds to its receptor , it activates two signal transduction system : the cAMP-dependent PKA and the phospholipase C-activated calcium/PKC pathways.

The expression of PTH receptor is a major phenotype feature of the osteoblast lineage. The effect of PTH on osteoblast function is complex, as it effects both bone resorption and formation. In stimulating bone resorption, PTH activates cells in the osteoblast lineage to produce cytokine that regulate osteoclastogenesis and bone resorption. In addition, PTH may stimulate differentiated osteoclasts in a manner that favors bone resorption. The effects of PTH on osteoblast proliferation are either stimulatory or inhibitory depending upon the osteoblastic model (Onishi and Hruska, 1997).

Due to its main action on bone remodeling (bone resorption and formation), PTH contributed with bone diseases, particularly osteoporosis. The bone disease osteoporosis which is a disease of inappropriate bone fracture, occurs most frequently in older estrogen-depleted women or postmenopausl women (Kimmel *et al*.,1993). Postmenopausal osteoporosis is characterized by progressive bone loss, which begins after natural or surgical menopausal and causes spontaneous fractures. Two basic of treatment have been used to cure bone loss, inhibition of bone resorption and stimulation of bone formation. Anti-resorptive agents, such as estrogen, can transiently increase bone mass and prevent further bone loss, but the mechanism of the protective effect of estrogen on bone resorption is not fully understood. An anabolic agents, such as fluoride and intermittent PTH administration, are capable of improving are capable of improving vertebral bone mass (Meng *et al*., 1996). The therapeutic efficiency of estrogen, bisphosphonate risedronate (NE-58095), and synthetic hPTH (sPTH 1-34) for restoration of lost of bone mass in osteopenic was studied in ovariectomized (OVX) rats by Wronski *et al*. (1993). The result showed that the treatment of OVX rats with estrogen or NE-58095 alone depressed bone turnover and prevented cancellous bone loss during the treatment period. While, OVX rats treated

with 80 μg/kg BW PTH alone showed a marked stimulation of bone formation which resulted in the augmentation of cancellous bone mass 2-flold greater than the vehicle-treated control rats. Treatments of OVX rats with PTH plus estrogen, and PTH plus NE-58095 had effectively reversed cancellous osteopenia in OVX rats, but the effect did not appear to be more beneficial to the estrogen-deplete skeleton than the treatment of PTH alone. This results indicated that PTH is a powerful stimulator of bone formation and completely restores lost cancellous bone in osteopenic OVX rats.

In 1993, Kimmel *et al.* studied the effect of recombinant hPTH (1-84) and synthetic hPTH (sPTH 1-34) on the skeleton of estrogen-depleted osteopenic rats or ovariectomized rats. OVX rats were treated with hPTH (1, 1.55, 15.5, or 155 μg/kg W.day) or sPTH (0.55, 5.5, or 55 μg/kg BW.day) by daily subcutaneous injection for 70 days. They found that trabecular bone was thickness in OVX rats treated with 15.5 and 155 μg /kg BW.day hPTH, and 5.5 and 55 μg/kg BW.day sPTH, but it did not increase trabecular number. On the molar basis, sPTH was more potent than hPTH. Additionally, the study of Meng *et al*. (1996) was also corresponded with the studies of Wronski *et al*. (1993), and Kimmel *et al.* (1993). Meng *et al.* (1996) studied the effect of rPTH (1-34) on OVX rats. A dose of 20 μg/kg/day of rPTH was administered by subcutaneous injection for 8 weeks to OVX rats. The result showed that cancellous bone volume increased gradually over 8 weeks of treatment as did the bone formation rate, as determined by an increase in both total mineralization of surface and mineral apposition rate. This bone formation was accompanied by an increase in trabecular thickness without increment in trabecular number. In the study of Mitlak *et al*. (1996) demonstrated that PTH increased both osteoblast and osteoclast function during daily PTH therapy in OVX rats, and the response depended on both the dose of PTH and the duration of therapy. These finding propose the choice of using

PTH as a new hormone replacement therapy (HRT) in oophorectomized and postmenopausal women with established osteoporosis. In 1997, Lindsay *et al*. demonstrated an interesting result that PTH has potentially cured the osteoporosis in postmenopausal women. The treatment group was postmenopausal women with osteoporosis taking both of HRT (estrogen) and hPTH (1-34; 400 U/25 μg daily sc.), and the controls, women taking HRT only. They found that patients taking HRT and hPTH had continuous increased in vertebral bone-mineral density during 3 years, whereas there was no significant change in the control group The total increase 13.0% in vertebral bone-mineral density ($p<0.001$); 2.7% at the hip ($p=0.05$); and 8.0% in total-body bone mineral ($p=0.002$). No loss of bone mass was found at any skeletal site and increased bone mass was associated with a reduction in the role of vertebral fracture, which was significant when fractures were taken as a 15% reduction in vertebral height ($p=0.04$). This result indicated that PTH has a pronounced anabolic effect on the central skeleton in patients on HRT.

Since the therapeutic options for osteoporosis are limited. In the USA only three agents are approved by Food and Drug Administration (FDA); estrogen, salmon calcitonin (CT), and alendronate (biphosphonate derivative). The effects of these agents are primarily reduce bone turnover, mainly by reducing the activation of new remodeling units within the skeleton (Lindsay *et al*., 1997). However, the copious data about PTH administration on bone formation is only gained from rats, a few reports have been published on the PTH effect in human. To get useful information, the experiments of PTH administration on bone formation in humans should be performed. Then, PTH could be used as a new HRT for postmenopausal osteoporotic women in the future. However, the study of bone metabolism in human may be encumbered by the ethical and legal standards. Recently, many researchers turn to use primates, e.g.

cynomolgus monkeys (*Macaca fascicularis*), as an animal model for bone metabolism study (Thorndike and Turner, 1998). However, the basic knowledge of PTH gene in primates is not well understood. There are few published reports of PTH gene in primates (Malaivijitnond and Takenaka, 1998; Anukulthanakorn *et al.*, 1999). It is not covered all species of popularly used primates. Thus, this study aims to determine the nucleotide sequences and deduced amino acid sequences of PTH gene in primates.

Primates, from the Latin "primus' meaning the first, is the term describing the highest order of mammals (Birdsell, 1972; Harlow and Persons, 1974). Primates are higher mammals usually adapted for climbing in trees. The eyes sockets are always surrounded by a closed bony ring . The clavicles are present. The first finger or toe (thumb or big toe), and at least one pair of digits, is possible and able to grasp. Fingers and toe usually have nails, but less frequent claw-like nails. The occipital lobe is well developed (it comprises the visual area), containing the fissura calcerina. The caecum is well developed. The penis is usually suspended. The testes lie within the scrotum. There is usually one pair of mammae or teats in the chest region (Fiedler *et al*., 1972). All primates (except man) are native tropical and sub-tropical countries, and are arboreal in habit. The primate's senses of hearing, touching, and vision are in general better developed than is the sense of smell. Primates; especially Apes are Man's closet relative (Harlow and Parsons, 1974).

The primates are classified into Order Primates, consists of lemur, lorises, monkeys, gibbon, apes, and man. According to Lekagul and McNeely (1977), taxonomy of primates is as follows:

Order Primates

Suborder Prosimii

Infraorder Lemuriformes

Superfamily Lemuroidea

Family Lemuridae (6 genera)

Family Indriidae (3 genera)

Family Daubentoniidae (1 genera)

Infraorder Lorisiformes

Family Lorisidae (5 genera)

Infraorder Tarsiiformes

Family Tarsiidae (1 genera)

Suborder Anthropoidea

Infraorder Platyrrhini

Superfamily Ceboidea

Family Cebidae (10 genera)

Family Callithricidae (2 genera)

Infraorder Catarrhini

Superfamily Cercopithecoidea

Family Cercopithecidae (15 genera)

Superfamily Hominoidea

Family Hylobatidae (3 genera)

Family Pongidae (3 genera)

Family Hominidae (1 genus)

Infraorder Platyrrhini is so-called new world monkeys. They distribute in the vast forest which range from the western mountain ranges of South America to the Atlantic, and from the southern edge of Maxico to the northern edge of the Argentina. They divided into 26 species. They differ markedly from the old world monkeys (Infraorder Catarrhini) (Harlow and Parsons, 1974). Their nostrils is wide, or ceboida, after their principle genus Cebus. They are descended from Eocene lamura whose remains have found in North America. It can be concluded that they and old world monkeys evolved separately but in parallel fashion to attain the structural grade represented by the monkeys. There is nothing remotely approaching an apelike level of evolution among them, so that it may be safely concluded that neither man nor the great ape could have evolved from the grade in the confirms of new world. The majority of the larger ceboids are characterized by prehensile grasping tails. The latter are generally diurnal in their activity and more or less omnivorous (Birdsell, 1972). New world monkeys are diurnal except night monkey (Aotes).

Infraorder Catarrhini is so-called old world monkeys. They include 60 species. They are in many respects higher in the evolutionary tree than are their relatives of the new world monkeys (Harlow and Persons, 1974). The old world monkeys contain two structural grades, the cercopithecoids or monkeys, and the hominoids, which include both the apes and man. In the old world monkeys, baboons widely inhabiting Africa and macaques in Asia are unique in their use of habitat. Almost all of primate species are arboreal. However, baboons and macaques can access the resources on the ground. Due to their adaptability, they can be accustomed to the captive condition and, therefore, they are used frequently as experimental animal models. The cercopithecoids have developed their own unique ecological niche over 30 million years period and so do not serve as models for early men (Birdsell, 1972).

Thus, in this study the 8 species of primates in Suborder Anthropoidea, new world and old world monkeys, were selected to determine nucleotide and deduced amino acid sequences. Hopefully, these results could provide considerable useful information of using primates as an animal model for studying about the bone metabolism related to PTH, and applying the knowledge to clinical use in human.

Chapter III

Materials and Methods

3.1 **Materials**

3.1.1 **Animals**

Eight species of animals in Order Primates, Suborder Anthropoidea were used in this study (Table 3.1).

Table 3.1 The scientific name, common name (Grzimek, 1975; Ankel-Simons, 1983; Corbet and Hill, 1991), and animal number of 8 species of primates used in this study.

Scientific name	Common name	Animal number
1. *Cebus apella* (Linneaus, 1758)	Black-capped capuchin or brown capuchin	4 individuals
2. *Callithrix jacchus* (Linneaus, 1758)	Common marmoset or tufted-ear marmoset	2 individuals
3. *Macaca fascicularis* (Raffles, 1821)	Crab-eating macaque or long-tailed macaque or cynomolgus macaque	2 individuals
4. *Papio hamadryas* (Linneaus, 1758)	Hamadryas baboon	2 individuals
5. *Presbytes obscura* (Reid, 1837)	Dusky leaf monkey or spectacled leaf monkey	3 individuals
6. *Hylobates lar* (Linneaus, 1771)	White-handed gibbon or common gibbon	3 individuals
7. *Gorilla gorilla* (Savage & Wyman, 1847)	Gorilla	4 individuals
8. *Pan paniscus* (Schwarz, 1929)	Pygmy chimpanzee	3 individuals

All specimens were kindly supported by Prof. Dr. Osamu Takenaka from Department of Cellular and Molecular Biology, Primate Research Institute, Kyoto University, Japan. They have been collected under CITES regulation and kept as DNA solution.

3.1.2 **Chemicals and instruments**

Chemicals and instruments are in an appendix IV.

3.2 Methods

3.2.1 **Blood collection**

Each animal except gorilla was given 10 mg/kg of ketamine hydrochloride intramuscularly. After an injection for 5-10 minutes, while the animal was anesthetized, a blood sample (3 ml/kg BW, maximum 10 ml) was collected by femoral venepuncture and immediately mixed with 0.1 ml of heparin and used for DNA extraction. In the case of gorilla, some tissue specimens were collected from a dead body during pathological examination at the rehabilitation center in Congo and sent them to Japan, under CITES regulation.

3.2.2 **DNA extraction**

The DNA sample was extracted from the buffy-coat by Phenol-Chloroform extraction (Hashimoto *et al*., 1996). The protocol was as follows:

1) Add a 4 to 5-volumes of 0.2% NaCl-1mM EDTA Na_3 to the blood sample in a polypropylene tube (50 ml) with a plastic cap.
2) Mix the contents of the tube, and centrifuge at 2,500 rpm for 10 minutes.
3) Discard the supernatant.
4) Repeat step 1 through 3 again.
5) Add 0.5 ml of 1 x STE, mix throughly by pasture pipette and transfer into another tube containing 2 ml of 1 x STE, 200 μl of 10% SDS, and 50 μl of 5 mg/ml of proteinase K.
6) Incubate or tilt at 37-55 °C for 2-4 hours with occasional shaking gently.
7) Add a 1/10 volume of 5 M NaCl, ฝ volume of TE-saturated phenol, and ฝ volume of CIAA (chloroform : isoamyl alcohol; 24 : 1) to the mixture.
8) Tilt at room temperature for 2 hours, and centrifuge at 2,500 rpm for 20 minutes.
9) Transfer the upper layer to a dialysis bag, and immerse the bag in 1 x TE overnight.
10) Transfer the solution to a polypropylene tube (15 ml) with a plastic cap, and add 10 μl of 10 mg/kg of ribonuclease A (RNAaseA).
11) Incubate at 37 °C for 30 minutes.
12) Add 60 μl of 5 M NaCl, and 50 μl of 5 mg/ml of proteinase K to the mixture .

13) Incubate or tilt at 55 °C for 2 hours.

14) Repeat steps 7 through 9.

15) Transfer that DNA sample to a sterile vial, add several drops of chloroform to prevent bacterial contamination, and store at 4 °C.

3.2.3 **PCR primers for amplification and sequence determination**

The primers used for PCR amplification and sequence determination in this study were designed according to the primers used for the macaque PTH (mPTH) gene sequence (Malaivijitnond and Takenaka, 1998). The PCR primers for amplification were primers 446 and 448, and for sequence determination were primers 451 and 452. The primer 446 is a biotinylated primer, whereas primers 451 and 452 are FITC labeled primers (Japan Bioservice, Japan). The position and nucleotide sequence of each primers showed in Figure 3.1 and Table 3.2.

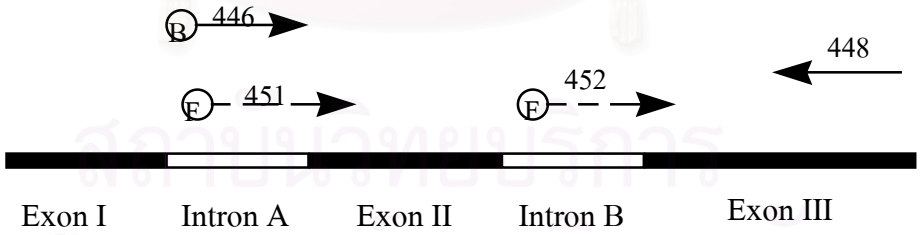


Figure 3.1 The region of the PTH gene of primates and primers used for PCR amplification and sequence determination. B and F show biotin (B) and fluoresceinisothiocyanate (FITC, F) labeling, respectively. Arrows show the direction from 5' to 3'.

Table 3.2 The nucleotide sequences of primers used for PCR amplification and sequence determination.

Number	Primer position*	Primer sequence
446	(-109) - (-90)	5'-GCTTCTCGTGAAAACCAACC-3'
448	(496) - (516)	5'-TTAGCAGCATGTATTGTTGCC-3'
451	(-104) - (-83)	5'-TCGTGAAAACCAACCCAATTAG-3'
452	(166) - (186)	5'-CCTCTCTGTTTCTCTTCTTCC-3'

* Following the nucleotide number for human (Vasicek *et al*., 1983)

3.2.4 **Amplification of PTH gene by PCR method**

PCR for amplification was carried out using a thermal cycler of Perkin Elmer Cetus, model 480. The total volume of the PCR mixture was 50 μl. In sterile 0.5 ml microfuge tube, the solution was mixed in the following order:

10xAmplification buffer	5.0 μl
25 mM $MgCl_2$	4.0 μl
2.5 mM dNTP	10.0 μl
DMSO	5.0 μl
Primers 446 + 448 (50 p moles/μl)	2.0 μl
DNA template (200 ng) + Deionized water	22.0 μl
Taq DNA polymerase (5 U/μl)	2.0 μl
Total	50.0 μl

Before the addition of *Taq* polymerase, the reaction mixture was heated for 6 minutes at 94 °C to denature the DNA completely. The reaction mixture was overlaid

with 30 μl of mineral oil to prevent evaporation of the sample during repeated cycled of heating and cooling. The main cycle of amplification was denaturation at 94 °C for 1 minute, annealing at 50 °C for 2 minutes, and extension at 72 °C for 3 minutes, for 30 cycles. After the amplification was finished, the mineral oil was removed from the DNA sample by extraction with 30 μl of CIAA. In brief, some mineral oil on the upper layer part was removed , added 30 μl of CIAA, mix throughly, and centrifuge at 10,000 rpm for 1 minute. The aqueous phase (upper part), which contained the amplified DNA, has been transferred to a fresh tube. Then, the amplified DNA was examined by 5% PAGE.

3.2.5 **Determination of the PCR product using 5% polyacrylamide gel electrophoresis (PAGE)**

The PCR product was checked by 5% PAGE, and the protocol was as follows;

1) Assemble plates and spacers for casting gel. Spacers are clamped in place to secure the separation of the plates and determine the gel thickness.
2) Prepare 5% gel by mixing the solution in the following order:

30% acrylamide solution	3.33 ml
5 x TBE	4.00 ml
Distilled water	12.5. ml
TEMED	16.7 μl
10% APS	167 μl

Mix gently by swirling. Dispense mixture into the chamber and fill the space entirely.

3) Promptly insert a comb into the top of the chamber.
4) Allow the acrylamide to polymerized for 45-60 minutes at room temperature.
5) When polymerization in complete, carefully remove an underneath spacer and a comb, and mount gel in electrophoresis apparatus.
6) Fill the upper and lower tank with 1 x TBE, and submerge the upper slot.
7) Immediately rinse out the wells with 1 x TBE.
8) Remove any bubbles from the space under the gel in the lower tank, and upper slot, to remove unpolymerized acrylamide.
9) Mix 1.5 μl of PCR product or 1 μl of 100-bp DNA Ladder with 0.5 μl of 5 x loading buffer containing dye. Load the mixture into the wells using a micropipette.
10) Connect the electrodes to a power pack.
11) Run the gel by applying a voltage of 180 V for 1.30 hours or until the marker dye has migrated to the desired distance.
12) Turn off the power supply and remove the gel from the apparatus.
13) Separate the plates by inserting a spatula between them in one corner and gently twisting to separate a corner. With the gel adhering to one plate, carefully remove the other plate.
14) Detect the positions of bands of DNA in the polyacrylamide gel by silver staining.

3.2.6 **Silver staining**

The bands of DNA were detected in the polyacrylamide gel by silver staining. The protocol was as follows;

1) Gently transfer the gel to the solution of 200 ml of 0.1% CTAB in the tray, carefully shake the gel for 20 minutes, and then discard the solution.
2) Add 200 ml of distilled water, shake very gently the gel for 20 minutes and discard the solution . Repeat this step.
3) Add 200 ml of 0.3% NH_4OH, shake very gently the gel for 15 minutes, and discard the solution.
4) Add the solution of 200 ml of 320 mg $AgNO_3$, 0.08 ml of 10 N NaOH, and 0.8 ml of 30% ammonia. Shake the gel for 15 minutes and discard the solution.
5) Add the solution of 200 ml of 4 g Na_2CO_3 and 100 μl of formaldehyde solution. Shake the gel until the bands of DNA on the polyacrylamide gel are appeared and discard the solution.
6) Add 200 ml of 0.1% glacial acid, shake the gel for 15 minutes and discard the solution.
7) Immerse the gel in 20% glycerol overnight.
8) Wrap the gel with plastic.

Note: All steps were performed at the room temperature.

3.2.7 **Preparation of the single stranded DNA (ssDNA) for sequencing**

The ssDNA of PTH gene was prepared from PCR product (dsDNA) using the avidin-coated magnetic beads (Dyna beads) in combination with biotinylated primer (primer 446). The protocol was followed Hashimoto *et al*. (1996).

1) Pipette the PCR product produced with a biotinylated primer (primer 446) into the upper chamber of microfuge tube (Microcon 100, Amicon Inc.) with a molecular weight cut off at 100 K dalton to pass through.
2) Add 400 μl of distilled water and centrifuge at 3,000 rpm for 10 minutes. This step removes the biotinylated primer which has not been incorporated into the amplified products, the reagents used in PCR, dNTP's, DMSO and $MgCl_2$, and other salts.
3) Repeat step 2 until the small amount of the solution in the upper chamber of the microfuge tube was remained.
4) Reverse the upper chamber and fix into the recovery container and centrifuge at 3,000 rpm for 1 minute.
5) Pipette 20 μl of magnetic beads suspension into a 0.5 ml microfuge tube and place the tube on the magnetic particle collector (MPC). Beads are adsorbed on the wall nearby the magnet and remove the clear supernatant.
6) Wash the beads by adding 80 μl of 1 x WB (washing buffer) into microfuge tube, vortex mix, and centrifuge for a few seconds.
7) Place the tube on MPC and remove the clear supernatant.
8) Repeat step 6 and 7.

9) Pipette the DNA sample from the recovery container at step 4 and measure its volume by adjusting the pipette, and add to the microfuge tube containing the beads.
10) Add the equal volume of 2 x WB to microfuge tube.
11) Rotating mix for 30-60 minutes, and centrifuge for a second.
12) Place the microfuge tube on MPC and remove the upper layer.
13) Add 60 μl of 1 x WB, vortex mix, centrifuge for a second and place the microfuge tube on MPC.
14) Discard the clear supernatant.
15) Alkalinize dsDNA by adding 12 μl of 0.1 N NaOH into microfuge tube, and incubate for 10 minutes at room temperature, and place the microfuge tube on MPC.
16) Transfer the supernatant into a fresh microfuge containing 6 μl of 0.2 N HCl and 1.5 μl of 1 M Tris-HCl pH 8.0, and keep at 4 °C for sequencing.
17) Add 10 μl of 1 x TE to the microfuge tube of step 16, and keep at 4 °C for sequencing.

3.2.8 **Preparation of sequencing gel**

1) Prepare the glass plates required to accommodate the sequencing reactions, and wipe them with cotton pad and small amount of absolute ethanol.
2) Assemble the plates and spacer for coating gel similarly to the preparation of 5% PAGE for determining the PCR product.

3) Prepare the sequencing gel as follows;

3.1) Add

acrylamide (acrylamide : bisacrylamide; 19 : 1)	1.5 g
urea	15 g
10 x TBE	3 ml to a 100-ml beaker

3.2) Mix the solution by stirring and adjust the volume to 30 ml with deionized water, and filter the solution through a Millipore filter of 0.22 μm

3.3) Add 150 μl of 10% APS and 15 μl of TEMED, and mix the solution by rapid swirling.

4) Draw the solution into a 50-ml syringe.

5) Introduce the nozzle of the syringe into the space between the two glass plates, and slowly expel the acrylamide solution from the syringe, filling the space almost the top.

6) Insert the comb into the open top end between the two plates, with the teeth pointing up, and the flat side of the comb in the gel mix.

7) Allow the acrylamide to polymerize for 6 hours, and mouth the gel in electrophoresis apparatus similarly to the preparation of 5% PAGE for examining the PCR product.

8) Remove the comb, and reinsert comb with the teeth about 1 mm into the bottom of the rectangular well formed in the polymerized gel.

9) Load 2.5 μl of the sequencing reaction onto adjacent slot of the gel using a 10-μl hypodermic syringe.

10) Run the gel at constant power of 20 W for 10 hours.

11) Determine the sequence by a Shimadzu fluorescent automatic sequencer DSQ-1.

3.2.9 **Cycle sequencing**

The protocol was as follows;

1) Prepare 4 sets of 0.2 ml microfuge tube and add ddNTPs in each of tube as follows;

Set 1; red labeled tubes	=	add 1.5 μl of ddATP/tube
Set 2; blue labeled tubes	=	add 1.5 μl of ddGTP/tube
Set 3; yellow labeled tubes	=	add 1.5 μl of ddCTP/tube
Set 4; white labeled tubes	=	add 1.5 μl of ddTTP/tube

2) Prepare the mother mixture solution in the following order:

Sequencing primer 451 or 452 (2 pmoles/μl)	0.83 μl
3.5 x Buffer	2.06 μl
Deionized water	4.98 μl
DNA template	4.50 μl
Taq DNA polymerase	0.83 μl
Total	13.20 μl

3) Pipette 3.0 μl of the mixture solution to each tube of step 1.

4) Amplify the PTH gene by the thermal cycler of Perkin Elmer Cetus 9600 in the following sequences;

- Pre-heat at 95 °C for 5 minutes.
- Denaturation at 95 °C for 30 seconds, annealing at 53 °C for 50 seconds, and extension at 72 °C for 80 seconds, the main cycle of amplification, for 20 cycles.
- Denaturation at 94 °C for 30 seconds, and extension at 72 °C for 60 seconds, for 20 cycles.
- Extension at 72 °C for 10 minutes.

5) Add 2.25 μl of the chain-termination solution in each tube. This sequencing reaction can be used to load to the gel immediately or can keep at -20 °C for a few days.

6) Load 2.5 μl of the sequencing reaction onto adjacent slot of the gel using a 10-μl hypodermic syringe.

3.2.10 **Data analysis**

The nucleotide sequences determined with a Shimadzu fluorescent automatic sequencer DSQ-1 were deduced to amino acid sequences by DNASIS program version 3.0 (Hitashi Software Engineering Co.LTD.). The nucleotide and amino acid sequences were aligned using the Clustal X. The phylogenetic trees were constructed by 2 methods. One was obtained from genetic distance data (Kimura's 2 parameters) based on the Neighbor-Joining method using the computer software, PHYLIP version 3.572c. The other was constructed by a Parsimony method using the computer software, PAUP version 3.0s. Human nucleotide and amino acid sequences of PTH gene was used as an ingroup reference (Vasicek *et al*., 1983). Nucleotide and amino acid sequences of bovine PTH gene were used as an outgroup reference (Weaver *et al*., 1984). Constraints of phylogenetic trees were determined using the bootstrapping approach.

Chapter IV

Results

4.1 DNA amplification of PTH gene

The PCR products of 8 primate species using primers 446 and 448 were approximately 650 bp when compared with 100-bp DNA ladder (Figure 4.1). According to GibcoBRL manual (Hsieh *et al*., 1991; Stellwagen, 1983), the 100-bp DNA ladder consists of 15 blunt-ended fragments between 100 and 1500 bp in multiples of 100 bp and an additional fragment at 2072 bp. Moreover, part of 600 bp band may migrate anomalously slowly in polyacrylamide gel. This band may appear as an extra band near the 700 bp band. However, the 600-bp band is approximately 2 to 3 times brighter than the other ladder bands, and it can provide internal orientation. From this knowledge, it can conclude that the 600 bp band appeared into 2 bands lower than 700 bp band in this study. In addition, the primers 446 and 448 used in this study designed from human PTH nucleotide sequence, and covered 626 bp of those human sequence.

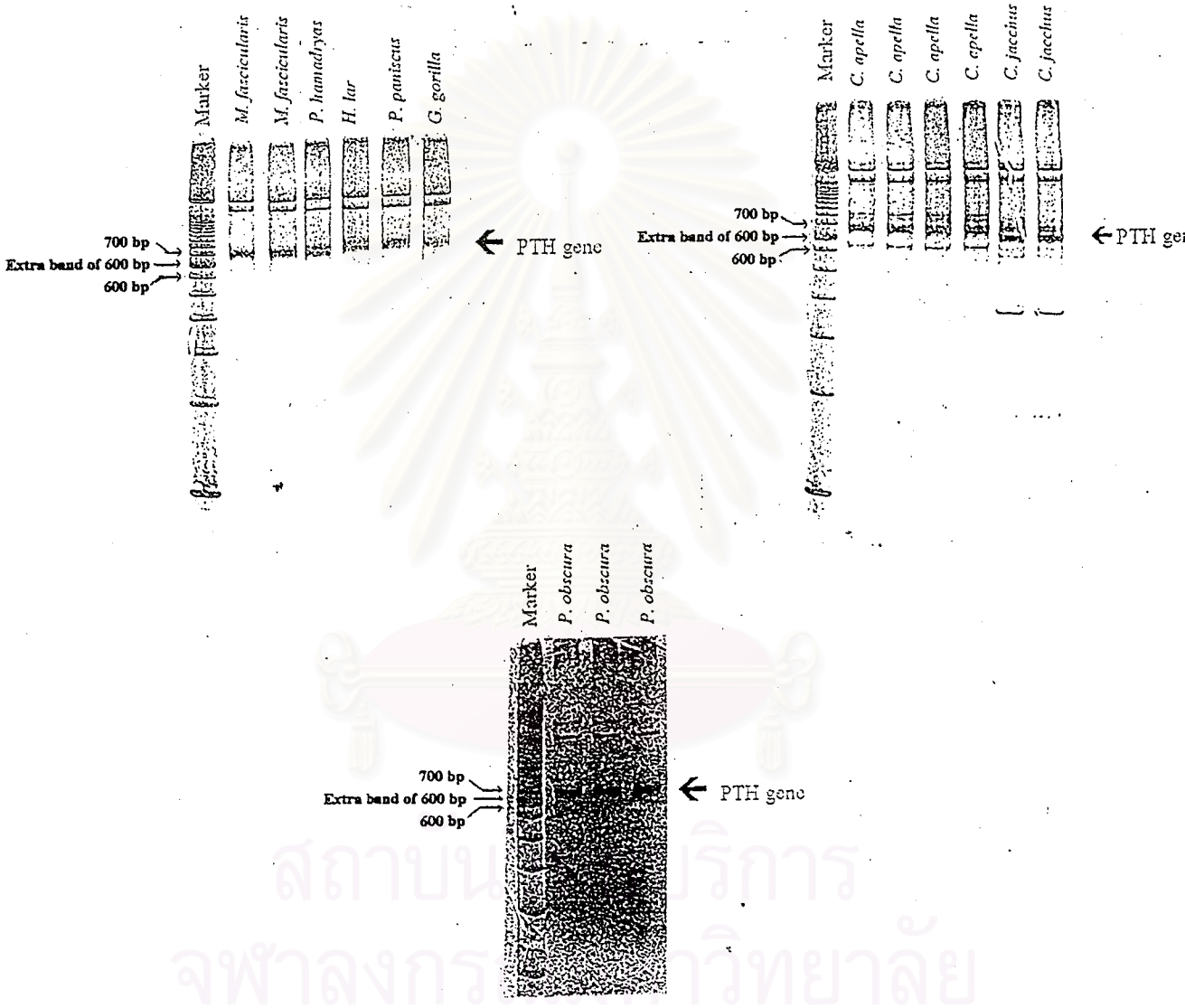


Figure 4.1 The arrows show the expected band of PTH gene; approximately 650 bp fragment, on 5% PAGE. The marker was a 100 bp DNA ladder.

4.2 ssDNA of PTH gene

To ensure that ssDNA of PTH gene prepared using the avidin-coated magnetic bead and a biotinylated primer has not been lost, the produced ssDNA of PTH gene was examined by 5% PAGE. On the gel, the expected band of ssDNA of PTH gene may be appeared into 2 locations by their structural difference (Figure 4.2).

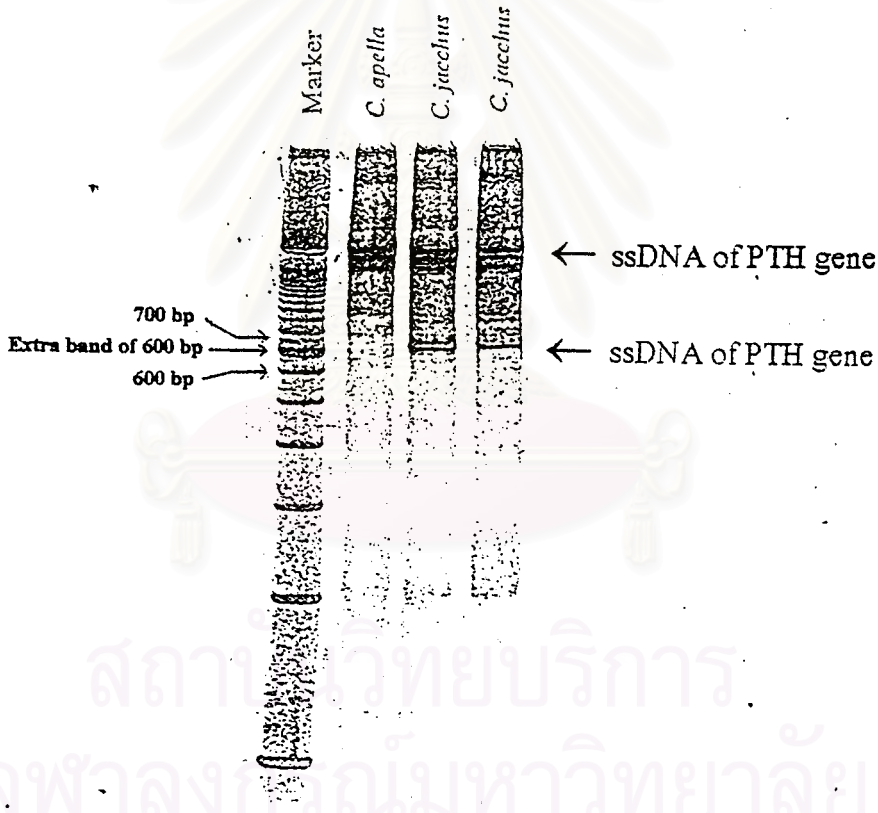


Figure 4.2 The expected band of ssDNA of PTH gene (indicated by a large arrow) on 5% PAGE, and the marker was 100 bp DNA ladder.

4.3 **The nucleotide and deduced amino acid sequences of PTH gene**

The nucleotide and deduced amino acid sequences of PTH gene in 8 species of primates show in Figure 4.3 - 4.10. The nucleotide sequences of all specimens could be determined in different number in range 500 to 553 bp of nucleotide (Table 4.1).

Table 4.1 The number of determined nucleotide sequences of PTH gene in 8 species of primates.

Scientific name	Number of determined sequences (bp)	Number of coding sequences (bp)		
		Exon II	Intron	Exon III
1) *Cebus apella*	511	91	102	316
2) *Callithrix jacchus*	548	91	102	319
3) *Macaca fascicularis*	500	91	103	281
4) *Papio hamadryas*	553	91	103	322
5) *Presbytes obscura*	538	91	103	313
6) *Hylobates lar*	550	91	103	311
7) *Gorilla gorilla*	539	91	103	316
8) *Pan paniscus*	538	91	103	313

According to Vasicek *et al*. (1983), and Malaivijitnond and Takenaka (1998), the determined nucleotide sequences of PTH gene could be separated into 3 parts; 2 exons (exon II and III) and 1 intron which located between exon II and exon III. The exon-intron can be separated by the GT-AG rule, the splice sites of nuclear genes of many eukaryotes. The intron defined starts with the dinucleotide GT and ends with the dinucleotide AG. In *C. apella* and *C. jacchus*, the intron was 102 bp long, whereas the intron of the other 6 species contained 103 bp. Exon II contained 91 bp that

corresponds to 5 bp from the 5' untranslated region, and the region that coded for the pre-sequence and the first four amino acids of the pro-sequence of prepro-PTH. Exon III contained 281 bp in *M. fascicularis*, 311 bp in *H. lar*, 313 bp in *P. obscura* and *P. paniscus*, 316 bp in *C. apella* and *G. gorilla*, 319 bp in *C. jacchus*, and 322 bp in *P. hamadryas*. Exon III coded for the remaining of the pro-sequence, mature PTH, and the 3' untranslated region. An 115 amino acid polypeptide, prepro-PTH, was fully deduced. At the N-terminus, a typical 25-amino acid of pre-sequence was followed by the 6-amino acid of pro-sequence, and then by the 84-amino acid of mature PTH. The last two residues of pro-sequence were Arg-Arg in *C. apella*, but it was Lys-Arg in the other 7 species of primates.

4.4 Individual differences

The nucleotide sequences of PTH gene were determined for several (2-4) individuals in each species (Table 3.1). Those sequences were in fairly agreement with each other within one species.

```
                      -30                 -10        1
             :        :        :        :        :.        :
             tctttttaaatacctccattttgcttttgcttttttagTGAAG ATG ATA CCT GCA
                                                         Met Ile Pro Ala

                                                     50
              :           :           :           :           :
AAA GAC ATG GCT AAA GTA ATG ATT GTC ATG TTG GCA ATT TGT TTT CTT ACA
Lys Asp Met Ala Lys Val Met Ile Val Met Leu Ala Ile Cys Phe Leu Thr

                                                100
              :           :           :         :         :         :
AAA TCG GAT GGG AAA TCT GTT AA gtaagtactgttttgcctgggaactggattttaat
Lys Ser Asp Gly Lys Ser Val Ly

                                150
              :         :         :         :         :         :
gttggctttatcatttagaagtggggagctaatgggaaatggccccctctgtttctcttcttcccagG
                                                                   S

                200
:               :           :           :           :           :
AAG AGA TCT GTG AGT GAA ATA CAG CTT ATG CAC AAT CTG GGA AAA CAT CTG
Lys Arg Ser Val Ser Glu Ile Gln Leu Met His Asn Leu Gly Lys His Leu

                250
                :           :           :           :           :
AAC TCG ATG GAG AGA GTA GAA TGG CTA CGT AAG AAG CTG CAG GAT GTG CAC
Asn Ser Met Glu Arg Val Glu Trp Leu Arg Lys Lys Leu Gln Asp Val His

              300
              :           :           :           :           :
AAT TTT GTT GCC CCT GGT ACT CCT CTA GTT CCC AGA GAT GCT GGT TCC CAA
Asn Phe Val Ala Pro Gly Thr Pro Leu Val Pro Arg Asp Ala Gly Ser Gln

             350
             :           :           :           :           :
AGG CCC CGA AGA AAG GAA GAC AAT GTC CTG GTT GAG AGC CAT GAA AAA AGT
Arg Pro Arg Arg Lys Glu Asp Asn Val Leu Val Glu Ser His Glu Lys Ser

            400
            :           :           :           :           :
CTT GGA GAG GCA GAC AAA GCT GAT GTG GAT GTA TTA ACT AAA GCT AAA TCC
Leu Gly Glu Ala Asp Lys Ala Asp Val Asp Val Leu Thr Lys Ala Lys Ser

          450                                             500
          :         :         :         :         :         :
CAA TGA AAAGGAAAACAGATATGGTCAGAGTTCTGCTCTAGACAGTGTAGGGCAACAATACAT
Gln ***
```

Figure 4.4 The 548 bp of DNA sequence and deduced amino acid sequence of PTH gene in *Callithrix jacchus* (common marmoset or tufted-ear marmoset). Nucleotides in mature messenger RNA following Vasicek *et al.*(1983) are capitalized; nucleotides in flanking and intervening DNA sequences are shown in small letters. The first nucleotide of the coding region is designated as the nucleotide 1.

```
                                                -20                   1
                                                 :           :           :  .                  :
                                              attttgcctttcctttttagTGAAG ATG ATA CCT GCA
                                                                         Met Ile Pro Ala

                                                                       50
         :              :              :              :              :
AAA GAC ATG GCT AAA GTA ATG ATT GTC ATG TTG GCA ATT TGC TTT CTT ACA
Lys Asp Met Ala Lys Val Met Ile Val Met Leu Ala Ile Cys Phe Leu Thr

                                                                 100
         .
         :              :              :              :              :              :
AAA TCA GAT GGG AAA TCT GTT AA gtaagtactgttttgcctgggaattggattttaat
Lys Ser Asp Gly Lys Ser Val Ly

                                          150
         :           :           :           :           :           :           :
gttgactttatcattttgaagtggggagctaatgggaagtggccctctctgtttctcttcttcccagG
                                                                                    s

                 200
                  :              :              :              :              :
AAG AGA TCT GTG AGT GAA ATA CAG CTT ATG CAT AAC CTG GGA AAA CAT CTG
Lys Arg Ser Val Ser Glu Ile Gln Leu Met His Asn Leu Gly Lys His Leu

              250
              :              :              :              :              :
AAG TCG ATG GAG AGA GTA GAA TGG CTG CGT AAG AAG CTG CAG GAT GTG CAC
Asn Ser Met Glu Arg Val Glu Trp Leu Arg Lys Lys Leu Gln Asp Val His

           300
           :              :              :              :              :
AAT TTT ATT GCC CTT GGA GCT CCT CTA GCT CCC AGA GAT GCT GGT TCC CAG
Asn Phe Ile Ala Leu Gly Ala Pro Leu Ala Pro Arg Asp Ala Gly Ser Gln

          350
          :              :              :              :              :
AGG CCC CGA AAA AAG GAA GAC AAT ATC TTG GTA GAG AGC CAT GAA AAA AGT
Arg Pro Arg Lys Lys Glu Asp Asn Ile Leu Val Glu Ser His Glu Lys Ser

      400
      :              :              :              :              :
CTT GGA GAG GCA GAC AAA GCT GAT GTG GAT GTA TTA ACT AAA GCT AAA TCC
Leu Gly Glu Ala Asp Lys Ala Asp Val Asp Val Leu Thr Lys Ala Lys Ser

    450
    :           :           :
CAA TGA AAATGAAAATAGATATGGTCAGAGT
Gln ***
```


Figure 4.5 The 500 bp of DNA sequence and deduced amino acid sequence of PTH gene in *Macaca fascicularis* (crab-eating macaque or long-tailed macaque or cynomolgus macaque). Nucleotides in mature messenger RNA following Vasicek *et al.* (1983) are capitalized; nucleotide in flanking and intervening DNA sequences are shown in small letters. The first nucleotide of the coding region is designated as the nucleotide 1.

```
          -40                          -10          1
           :          :          :          :          :          :
          gtattctaaaatacctccattttgcctttccttttagTGAAG ATG ATA CCT GAA
                                                      Met Ile Pro Glu

                                                  50
           :          :          :          :          :          :
AAA GAC ATG GCT AAA GTA ATG ATT GTC ATG TTG GCA ATT TGT TTT CTT ACA
Lys Asp Met Ala Lys Val Met Ile Val Met Leu Ala Ile Cys Phe Leu Thr

                                                 100
           :          :          :          :          :          :
AAA TCA GAT GGG AAA TCT GTT AA gtaagtactgttttgcctgggaattggatttttaat
Lys Ser Asp Gly Lys Ser Val Ly

                          150
           :          :          :          :          :          :          :
gttgactttatcattttgaagtggggagctaatgggaagtggccctctctgtttctcttcttcccagG
                                                                      s

               200
               :          :          :          :          :          :
AAG AGA TCT GTG AGT GAA ATA CAG CTT ATG CAT AAT CTG GGA AAA CAT CTG
Lys Arg Ser Val Ser Glu Ile Gln Leu Met His Asn Leu Gly Lys His Leu

             250
             :          :          :          :          :          :
AAC TCG ATG GAG AGA GTA GAA TGG CTG CGT AAG AAG CTG CAG GAT GTG CAC
Asn Ser Met Glu Arg Val Glu Trp Leu Arg Lys Lys Leu Gln Asp Val His

            300
            :          :          :          :          :          :
AAT TTT ATT GCC CTT GGA GCT CCT CTA GCT CCC AGA GAT GCT GGT TCC CAG
Asn Phe Ile Ala Leu Gly Ala Pro Leu Ala Pro Arg Asp Ala Gly Ser Gln

           350
           :          :          :          :          :          :
AGG CCC CGA AAA AAG GAA GAC AAT ATC TTG GTT GAG AGC CAT GAA AAA AGT
Arg Pro Arg Lys Lys Glu Asp Asn Ile Leu Val Glu Ser His Glu Lys Ser

         400
         :          :          :          :          :          :
CTT GGA GAG GCA GAC AAA GCT GAT GTG GAT GTA TTA ACT AAA GCT AAA TCC
Leu Gly Glu Ala Asp Lys Ala Asp Val Asp Val Leu Thr Lys Ala Lys Ser

       450                                                    500
       :          :          :          :          :          :          :
CAA TGA AAATGAAAATAGATATGGTCAGAGTTCTGCTCTAGACAGTGTAGGGCAACAATACATGCT
Gln ***
```

Figure 4.6 The 553 bp of DNA sequence and deduced amino acid sequence of PTH gene in *Papio hamadryas* (hamadryas baboon). Nucleotides in mature messenger RNA following Vasicek *et al.* (1983) are capitalized; nucleotide in flanking and intervening DNA sequences are shown in small letters. The first nucleotide of the coding region is designated as the nucleotide 1.

```
                    -30                 -10          1
                     :                   :           :.          :
          taaaatacctccattttgcctttccttttagCGAAG ATG ATA CCT GCA
                                               Met Ile Pro Ala

                                                  50
                     :                   :       :          :
AAA GAC ATG GCT AAA GTA ATG ATT GTC ATG TTG GCA ATT TGT TTT CTT ACA
Lys Asp Met Ala Lys Val Met Ile Val Met Leu Ala Ile Cys Phe Leu Thr

                                              100
                     :                   :       :          :
AAA TCA GAT GGG AAA TCT GTT AA gtaagtactgttttgcctgggaattggatttaaat
Lys Ser Asp Gly Lys Ser Val Ly

                               150
                     :         :       :          :          :
gttgactttatcattttgaagtggggagctaatgggaagtggccctctctgtttctcttcttcccagG
                                                                   s

            200
             :                   :       :          :          :
AAG AGA TCT GTG AGT GAA ATA CAG CTT ATG CAT AAC CTG GGA AAA CAT CTG
Lys Arg Ser Val Ser Glu Ile Gln Leu Met His Asn Leu Gly Lys His Leu

          250
           :                   :       :          :          :
AAC TCG ATG GAG AGA GTA GAA TGG CTG CGT AAG AAG CTG CAG GAT GTG CAC
Asn Ser Met Glu Arg Val Glu Trp Leu Arg Lys Lys Leu Gln Asp Val His

         300
          :                   :       :          :          :
AAT TTT GTT GCC CTT GGA GCT CCT CTA GCT CCC AGA GAT GCT GGT TCC CAG
Asn Phe Val Ala Leu Gly Ala Pro Leu Ala Pro Arg Asp Ala Gly Ser Gln

        350
         :                   :       :          :          :
AGG CCC CGA AAA AAG GAA GAC AAT ATC TTG GTT GAG AGC CAT GAA AAA AGT
Arg Pro Arg Lys Lys Glu Asp Asn Ile Leu Val Glu Ser His Glu Lys Ser

      400
       :                   :       :          :          :
CTT GGA GAG GCA GAC AAA GCT GAT GTG GAT GTA TTA GCT AAA GCT AAA TCC
Leu Gly Glu Ala Asp Lys Ala Asp Val Asp Val Leu Ala Lys Ala Lys Ser

     450                                                  500
      :                   :       :          :          :
CAA TGA AAATGAAAACAGATATGGTCAGAGTTCTGTTCTAGACATTATAGGGCAACA
Gln ***
```

Figure 4.7 The 538 bp of DNA sequence and amino acid sequence of PTH gene in *Presbytes obscura* (dusky leaf monkey or spectacled leaf monkey). Nucleotides in mature messenger RNA following Vasicek *et al.* (1983) are capitalized; nucleotide in flanking and intervening DNA sequences are shown in small letters. The first nucleotide of the coding region is designated as the nucleotide 1.

```
        -40                          -10          1
         :          :         :        :        : .          :
atattaaaaatattttaaaaacctccattttgcttgtccttttagTGAAG ATG ATA CCT GCA
                                                  Met Ile Pro Ala

                                                50
         :          :         :        :        :          :
AAA GAC ATG GCT AAA GTA ATG ATT GTC ATG TTG GCA ATT TGT TTT CTT ACA
Lys Asp Met Ala Lys Val Met Ile Val Met Leu Ala Ile Cys Phe Leu Thr

                                              100
        :       .   :         :        :        :         :
AAA TCG GAT GGG AAA TCT GTT AA gtaagtactgttttgccttggaattggatttttaat
Lys Ser Asp Gly Lys Ser Val ly                                  .

                           150
       :         :         :        :        :         :         :
gttgactttatcatttcgaagtggggagctaatgggaagcggccctctctgtttctcttcttcccagG
                                                                   s

            200
            :          :         :        :          :          :
AAG AGA TCT GTG AGT GAA ATA CAG CTT ATG CAT AAC CTG GGA AAA CAT CTG
Lys Arg Ser Val Ser Glu Ile Gln Leu Met His Asn Leu Gly Lys His Leu

          250
          :          :         :        :          :
AAC TCG ATG GAG AGA GTA GAA TGG CTG CGT AAG AAG CTG CAG GAT GTG CAC
Asn Ser Met Glu Arg Val Glu Trp Leu Arg Lys Lys Leu Gln Asp Val His

         300
         :          :         :        :          :
AAT TTT GTT GCC CTT GGA GCT CCT CTA GCT CCC AGA GAT GCT GGT TCC CAG
Asn Phe Val Ala Leu Gly Ala Pro Leu Ala Pro Arg Asp Ala Gly Ser Gln

        350
        :          :         :        :          :
AGG CCC CGA AAA AAG GAA GAC AAT GTC TTG GTT GAG AGC CAT GAA AAA AGT
Arg Pro Arg Lys Lys Glu Asp Asn Val Leu Val Glu Ser His Glu Lys Ser

      400
      :          :         :        :          :
CTT GGA GAG GCA GAC AAA GCT GAT GTG GAT GTA TTA ACT AAA GCT AAA TCC
Leu Gly Glu Ala Asp Lys Ala Asp Val Asp Val Leu Thr Lys Ala Lys Ser

     450                                        500
     :          :         :        :          :
CAA TGA AAATGAAAACAGATATGGTCAGAGTTCTGCTCTAGACAGTGTAGGGCAA
Gln ***
```

Figure 4.8 The 550 bp of DNA sequence and deduced amino acid sequence of PTH gene in *Hylobates lar* (white-handed gibbon or common gibbon). Nucleotides in mature messenger RNA following Vasicek *et al.* (1983) are capitalized; nucleotide in flanking and intervening DNA sequences are shown in small letters. The first nucleotide of the coding region is designated as the nucleotide 1.

```
                    -30                 -10          1
                     :         :         :          : .          :
                taaaatacctccattttgcttgtccttttagTGAAG ATG ATA CCT GCA
                                                     Met Ile Pro Ala

                                                  50
          :            :                :          :            :
AAA GAC ATG GCT AAA GTT ATG ATT GTC ATG TTG GCA ATT TGT TTT CTT ACA
Lys Asp Met Ala Lys Val Met Ile Val Met Leu Ala Ile Cys Phe Leu Thr

                                           100
         :            :           :       :         :          :
AAA TCG GAT GGG AAA TCT GTT AA gtaagtactgttttgccttggaattggatttttaat
Lys Ser Asp Gly Lys Ser Val Ly

                          150
       :          :        :         :         :         :          :
gttgactttatcatttcgaagtggggagctaatgggaagtggccctctctgtttctcttcttcccagG
                                                                    s

             200
              :           :         :          :          :
AAG AGA TCT GTG AGT GAA ATA CAG CTT ATG CAT AAC CTG GGA AAA CAT CTG
Lys Arg Ser Val Ser Glu Ile Gln Leu Met His Asn Leu Gly Lys His Leu

           250
            :          :         :          :          :
AAC TCG ATG GAG AGA GTA GAA TGG CTG CGT AAG AAG CTG CAG GAT GTG CAC
Asn Ser Met Glu Arg Val Glu Trp Leu Arg Lys Lys Leu Gln Asp Val His

          300
           :          :         :          :          :
AAT TTT GTT GCC CTT GGA GCT CCT CTA GCT CCC AGA GAT GCT GGT TCC CAG
Asn Phe Val Ala Leu Gly Ala Pro Leu Ala Pro Arg Asp Ala gly Ser Gln

      350
       :          :         :          :          :
AGG CCC CGA AAA AAG GAA GAC AAT GTC TTG GTT GAG AGC CAT GAA AAA AGT
Arg Pro Arg Lys Lys Glu Asp Asn Val Leu Val Glu Ser His Glu Lys Ser

     400
      :          :         :          :          :
CTT GGA GAG GCA GAC AAA GCT GAT GTG AAT GTA TTA ACT AAA GCT AAA TCC
Leu Gly Glu Ala Asp Lys Ala Asp Val Asn Val Leu Thr Lys Ala Lys Ser

    450                                          500
     :          :         :          :          :
CAG TGA AAATGAAAACAGATATTGTCAGAGTTCTGCTCTAGACAGTGTAGGGCAACA
Gln ***
```

Figure 4.9 The 538 bp of DNA sequence and deduced amino acid sequence of PTH gene in *Pan paniscus* (pygmy chimpanzee). Nucleotides in mature messenger RNA following Vasicek *et al.* (1983) are capitalized; nucleotide in flanking and intervening DNA sequences are shown in small letters. The first nucleotide of the coding region is designated as the nucleotide 1.

```
                    -30                 -10          1
                     :          :        :          : .          :
                  aaatacctccattttgcttgtccttttagTGAAG ATG ATA CCT GCA
                                                     Met Ile Pro Ala

                                                  50
          :                :                   :          :          :
AAA GAC ATG GCT AAA GTT ATG ATT GTC ATG TTG GCA ATT TGT TTT GTT ACA
Lys Asp Met Ala Lys Val Met Ile Val Met Leu Ala Ile Cys Phe Val Thr

                                              100
          :          :          :          :          :          :
AAA TCG GAT GGG AAA TCT GTT AA gtaagtactgttttgccttggaattggatttttaat
Lys Ser Asp Gly Lys Ser Val Ly

                           150
       :          :          :          :          :          :          :
gttgactttatcatttcaaagtggggagctaatgggaagtggccctctctgtttctcttcttcccagG
                                                                       s

            200
            :          :          :          :          :          :
AAG AGA TCT GTG AGT GAA ATA CAG CTT ATG CAT AAC CTG GGA AAA CAT CTG
Lys Arg Ser Val Ser Glu Ile Gln Leu Met His Asn Leu Gly Lys His Leu

          250
          :          :          :          :          :          :
AAC TCG ATG GAG AGA GTA GAA TGG CTG CGT AAG AAG CTG CAG GAT GTG CAC
Asn Ser Met Glu Arg Val Glu Trp Leu Arg Lys Lys Leu Gln Asp Val His

         300
         :          :          :          :          :          :
AAT TTT GTT GCC CTT GGA GCT CCT CTA GCT CCC AGA GAT GGT GGT TCC CAG
Asn Phe Val Ala Leu Gly Ala Pro Leu Ala Pro Arg Asp Gly Gly Ser Gln

        350
        :          :          :          :          :          :
AGG CCC CGA AAA AAG GAA GAC AAT GTC TTG GTT GAG AGC CAT GAA AAA AGT
Arg Pro Arg Lys Lys Glu Asp Asn Val Leu Val Glu Ser His Glu Lys Ser

      400
      :          :          :          :          :          :
CTT GGA GAG GCA GAC AAA GCT GAT GTG AAT GTA TTA ACT AAA GCT AAA TCC
Leu Gly Glu Ala Asp Lys Ala Asp Val Asn Val Leu Thr Lys Ala Lys Ser

     450                                                  500
     :          :          :          :          :          :
CAG TGA AAATGAAAACAGATATGGTCAGAGTTCTGCTCTAGACAGTGTAGGGCAACAATA
Gln ***
```

Figure 4.10 The 539 bp of DNA sequence and deduced amino acid sequence of PTH gene in *Gorilla gorilla* (gorilla). Nucleotides in mature messenger RNA following Vasicek *et al.* (1983) are capitalized; nucleotide in flanking and intervening DNA sequences are shown in small letters. The first nucleotide of the coding region is designated as the nucleotide 1.

4.5 The homology of nucleotide sequences and amino acid sequences of PTH gene

Comparison of the DNA sequences of PTH gene between bovine (Weaver *et al*., 1984) and 8 species of primates (Figure 4.11) showed strong homology of 79.68 - 82.11% as can be shown by Table 4.2. Comparison of the amino acid sequences of PTH gene between bovine (Weaver *et al*., 1984) and 8 species of primates in this study (Figure 4.12) showed high homology of 80.86 - 86.08% as can be seen in Table 4.2.

Comparison of the DNA sequences of PTH gene between human (Vasicek *et al*., 1983) and 8 species of primates (Figure 4.11) showed the strong homology of 95.38 - 99.8% as in Table 4.2. Comparison of the amino acid sequences of PTH gene between human and 8 species of primates showed the strong homology of 93.04 - 100.00% as can be seen in Table 4.2.

Comparison of the amino acid sequences of 8 species of primates with human sequence showed the position differences as follows: 7 amino acids in *Cebus apells* (at the 12^{th}, 39^{th}, 42^{th}, 49^{th}, 53^{th}, 60^{th}, and 76^{th} positions of PTH), 5 amino acids in *Callithrix jacchus* (at the 37^{th}, 39^{th}, 42^{th}, 53^{th}, and 76^{th} positions of PTH), 3 amino acids in *Papio hamadryas* and *Macaca fascicularis* (at the 35^{th}, 58^{th}, and 76^{th} positions of PTH), 2 amino acids in *Presbytes obscura* (at the 58^{th}, and 79^{th} positions of PTH), 1 amino acid in *Hylobates lar* (at the 76^{th} position of PTH), 1 amino acid in *Gorilla gorilla* (at the 46^{th} position of PTH), and no differece in *Pan paniscus.*

Table 4.2 The percentage of homology of nucleotide and amino acid sequences of PTH gene among human (Vasicek *et al.*, 1983), bovine (Weaver *et al.*, 1984) and 8 species of primates. Above diagonal means the percentage of homology of nucleotide sequences and below diagonal means the percentage of homology of amino acid sequences.

Species	*H. sapiens*	*P. paniscus*	*G. gorilla*	*H. lar*	*M.fascicularis*	*P. hamadryas*	*P. obscura*	*C. apella*	*C. jacchus*	Bovine
H. sapiens	-	99.80	99.23	99.03	97.50	97.50	97.69	95.38	95.76	86.34
P. paniscus	100	-	99.42	99.23	97.50	97.50	97.69	95.38	95.76	86.34
G. gorilla	98.26	98.26	-	98.84	97.11	97.11	97.30	95.00	95.38	86.53
H. lar	99.13	99.13	97.39	-	97.88	97.88	98.07	95.76	96.15	86.34
M.fascicularis	97.39	97.39	95.65	98.26	-	99.23	98.65	95.00	95.38	86.34
P. hamadryas	96.52	96.52	94.78	97.38	99.13	-	98.65	95.00	95.76	86.34
P. obscura	97.39	97.39	95.65	98.26	98.26	97.39	-	95.38	95.76	86.15
C. apella	93.04	93.04	91.30	93.91	92.17	91.30	92.17	-	97.11	85.00
C. jacchus	95.65	95.65	93.91	96.52	94.78	93.91	94.78	95.65	-	85.19
Bovine	85.21	85.21	85.21	86.08	84.34	83.47	85.21	80.00	82.60	-

```
                        10          20          30          40          50
H. sapiens          NTATTAAAAA  TATTTTAAAA  TACCTCCATT  TTGCTTATCC  TTTT-AGTGA
P. paniscus         NNNNNNNNNN  NNNNNTAAAA  TACCTCCATT  TTGCTTGTCC  TTTT-*****
G. gorilla          NNNNNNNNNN  NNNNNNNAAA  TACCTCCATT  TTGCTTGTCC  TTTT-*****
H. lar              ATATTAAAAA  TATTTTAAAA  -ACCTCCATT  TTGCTTGTCC  TTTT-*****
M. fascicularis     NNNNNNNNNN  NNNNNNNNNN  NNNNNNNATT  TTGCCTTTCC  TTTT-*****
P. hamadryas        NNNNNNNNNG  TATTCTAAAA  TACCTCCATT  TTGCCTTTCC  TTTT-*****
P. obscura          NNNNNNNNNN  NNNNNTAAAA  TACCTCCATT  TTGCCTTTCC  TTTT-**C**
C. apella           NNNNNNNNNN  NNNNNNNNNN  NNNNNNNNNN  NNNNNNNNNN  NNNNN*****
C. jacchus          NNNNNNNNNN  TCTTTTTAAA  TACCTCCATT  TTGCTTTTCT  TTTT-*****
Bovine              NNNNNNNNNN  NNNNNNNNNN  NNNNNNNNTT  TTCTTTTTCT  TTTTT***T*

                        60          70          80          90          100
H. sapiens          AGATGATACC  TGCAAAAGAC  ATGGCTAAAG  TTATGATTGT  CATGTTGGCA
P. paniscus         **********  **********  **********  **********  **********
G. gorilla          **********  **********  **********  **********  **********
H. lar              **********  **********  **********  *A********  **********
M. fascicularis     **********  **********  **********  *A********  **********
P. hamadryas        **********  **A*******  **********  *A********  **********
P. obscura          **********  **********  **********  *A********  **********
C. apella           **********  **********  **********  *A********  *********C
C. jacchus          **********  **********  **********  *A********  **********
Bovine              *T*****GT*  **********  ****T***G*  *A********  ****C*T**C

                        110         120         130         140         150
H. sapiens          ATTTGTTTTC  TTACAAAATC  GGATGGGAAA  TCTGTTAAGT  AAGTAC----
P. paniscus         **********  **********  **********  **********  ******----
G. gorilla          *********G  **********  **********  **********  ******----
H. lar              **********  **********  **********  **********  ******----
M. fascicularis     *****C****  **********  A*********  **********  ******----
P. hamadryas        **********  **********  A*********  **********  ******----
P. obscura          **********  **********  A*********  **********  ******----
C. apella           **********  **********  **********  **********  ******----
C. jacchus          **********  **********  **********  **********  ******----
Bovine              **C*******  **G***G***  A********G  **********  ******CATA

                        160         170         180         190         200
H. sapiens          ---TGTTTTG  CCTTG-----  ------GAAT  TGGATTTTTA  ATGTTGACTT
P. paniscus         ---*******  *****-----  ------****  **********  **********
G. gorilla          ---*******  *****-----  ------****  **********  **********
H. lar              ---*******  *****-----  ------****  **********  **********
M. fascicularis     ---*******  ***G*-----  ------****  **********  **********
P. hamadryas        ---*******  ***G*-----  ------****  **********  **********
P. obscura          ---*******  ***G*-----  ------****  ********A*  **********
C. apella           ---*******  ***G*-----  ------****  ********-*  ******G***
C. jacchus          ---*******  ***G*-----  ------***C  ********-*  ******G***
Bovine              GCC****C**  *A*G*TGAGG  TCAGGG****  **********  *G****G***

                        210         220         230         240         250
H. sapiens          TATCATTTCG  AAGTGGGGAG  CTAATGGGAA  GTGGCCCTCT  CTGTTTCTCT
P. paniscus         **********  **********  **********  **********  **********
G. gorilla          ********A*  **********  **********  **********  **********
H. lar              **********  **********  **********  *C********  **********
M. fascicularis     ********T*  **********  **********  **********  **********
P. hamadryas        ********T*  **********  **********  **********  **********
P. obscura          ********T*  **********  **********  **********  **********
C. apella           ********A*  **********  **********  A******C**  **********
C. jacchus          ********A*  **********  **********  A******C**  **********
Bovine              ***G****G*  ***A*****A  *******--*  ***AT*****  ***A*C**G*
```

	260	270	280	290	300
H. sapiens	TCTTCCCAGG	AAGAGATCTG	TGAGTGAAAT	ACAGCTTATG	CATAACCTGG
P. paniscus	**********	**********	**********	**********	**********
G. gorilla	**********	**********	**********	**********	**********
H. lar	**********	**********	**********	**********	**********
M. fascicularis	**********	**********	**********	**********	**********
P. hamadryas	**********	**********	**********	**********	*****T****
P. obscura	**********	**********	**********	**********	**********
C. apella	**********	*G********	**********	**********	**C*******
C. jacchus	**********	**********	**********	**********	**C**T****
Bovine	**CCT*****	******G***	**********	****T*****	**********

	310	320	330	340	350
H. sapiens	GAAAACATCT	GAACTCGATG	GAGAGAGTAG	AATGGCTGCG	TAAGAAGCTG
P. paniscus	**********	**********	**********	**********	**********
G. gorilla	**********	**********	**********	**********	**********
H. lar	**********	**********	**********	**********	**********
M. fascicularis	**********	**********	**********	**********	**********
P. hamadryas	**********	**********	**********	**********	**********
P. obscura	**********	**********	**********	**********	**********
C. apella	C*********	**********	**********	**********	******A***
C. jacchus	**********	**********	**********	*******A**	**********
Bovine	*C********	**G***C***	**A*****G*	**********	***A*****A

	360	370	380	390	400
H. sapiens	CAGGATGTGC	ACAATTTTGT	TGCCCTTGGA	GCTCCTCTAG	CTCCCAGAGA
P. paniscus	**********	**********	**********	**********	**********
G. gorilla	**********	**********	**********	**********	**********
H. lar	**********	**********	**********	**********	**********
M. fascicularis	**********	********A*	**********	**********	**********
P. hamadryas	**********	********A*	**********	**********	**********
P. obscura	**********	**********	**********	**********	**********
C. apella	**********	**********	**********	A*********	T*********
C. jacchus	**********	**********	*****C***T	A*********	T*********
Bovine	**********	****C*****	**********	***T**A***	**TA******

	410	420	430	440	450
H. sapiens	TGCTGGTTCC	CAGAGGCCCC	GAAAAAAGGA	AGACAATGTC	TTGGTTGAGA
P. paniscus	**********	**********	**********	**********	**********
G. gorilla	**G*******	**********	**********	**********	**********
H. lar	**********	**********	**********	**********	*********8
M. fascicularis	**********	**********	**********	*******A**	*****A****
P. hamadryas	**********	**********	**********	*******A**	**********
P. obscura	**********	**********	**********	*******A**	**********
C. apella	*********T	G*C******A	***G******	**********	C***C*****
C. jacchus	**********	**A*******	***G******	**********	C*********
Bovine	**G*A*****	*****A**T*	**********	**********	C*********

	460	470	480	490	500
H. sapiens	GCCATGAAAA	AAGTCTTGGA	GAGGCAGACA	AAGCTGATGT	GAATGTATTA
P. paniscus	**********	**********	**********	**********	**********
G. gorilla	**********	**********	**********	**********	**********
H. lar	**********	**********	**********	**********	*G********
M. fascicularis	**********	**********	**********	**********	*G********
P. hamadryas	**********	**********	**********	**********	*G********
P. obscura	**********	**********	**********	**********	*G********
C. apella	**********	**********	**********	**********	*G********
C. jacchus	**********	**********	**********	**********	*G********
Bovine	*****C*G**	**********	**A*******	**********	*G********

```
                          510         520         530         540         550
H. sapiens           ACTAAAGCTA  AATCCCAGTG  AAAATGAAAA  CAGATATTGT  CAGAGTTCTG
P. paniscus          **********  **********  **********  **********  ******TCTG
G. gorilla           **********  **********  **********  *******-**  ******TCTG
H. lar               **********  *******A**  **********  *******G**  ******TCTG
M. fascicularis      **********  *******A**  **********  T******G**  ******NNNN
P. hamadryas         **********  *******A**  **********  T******G**  ******TCTG
P. obscura           G*********  *******A**  **********  *******G**  ******TCTG
C. apella            **********  *******A**  ****G*****  *******G**  ******TCTG
C. jacchus           **********  *******A**  ****G*****  *******G**  ******TCTG
Bovine               *T********  **C*******  **------**  *******GA*  ****TCACTG

                          560         570         580
H. sapiens           CTCTAGACAG  TGTAGGGCAA  CA-TACATGC  TG
P. paniscus          CTCTAGACAG  TGTAGGGCAA  CANNNNNNNN  NN
G. gorilla           CTCTAGACAG  TGTAGGGCAA  CA-TANNNNN  NN
H. lar               CTCTAGACAG  TGTAGGGCAA  NNNNNNNNNN  NN
M. fascicularis      NNNNNNNNNN  NNNNNNNNNN  NNNNNNNNNN  NN
P. hamadryas         CTCTAGACAG  TGTAGGGCAA  CAATACATGC  TN
P. obscura           CTCTAGACAG  TGTAGGGCAA  CANNNNNNNN  NN
C. apella            CTCTAGACAG  TGTAGGGCAA  CAATACATNN  NN
C. jacchus           CTCTAGACAG  TGTAGGGCAA  CAATANNNNN  NN
Bovine               TTNNNNNNNN  NNNNNNNNNN  NNNNNNNNNN  NN
```

Figure 4.11 Alignment of the nucleotide sequences of PTH gene in 8 species of primates. The nucleotide sequence of bovine (Weaver *et al*., 1984) is used to be an outgroup, and the nucleotide sequence of human (Vasicek *et al*., 1983) is used to be an ingroup references. The stars (*) indicate the homology of the nucleotides, N indicates the indeterminate sequences, and dashes (-) indicate gaps.

```
                    1                                                  50
H. sapiens          MIPAKDMAKVMIVMLAICFLTKSDGKSVKKRSVSEIQLMHNLGKHLNSME
P. paniscus         **************************************************
G. gorilla          ******************V***********************************
H. lar              **************************************************
M. fascicularis     **************************************************
P. hamadryas        ***E**********************************************
P. obscura          **************************************************
C. apella           ****************************R***********A*******
C. jacchus          **************************************************
Bovine              *MS****V***********AR*********A*****F*******S***

                    51                                                 100
H. sapiens          RVEWLRKKLQDVHNFVALGAPLAPRDAGSQRPRKKEDNVLVESHEKSLGE
P. paniscus         **************************************************
G. gorilla          *************************G************************
H. lar              **************************************************
M. fascicularis     ***************I**********************I***********
P. hamadryas        ***************I**********************I***********
P. obscura          ***************************************I***********
C. apella           ******************T**V******D***R******A*********
C. jacchus          ****************P*T**V**********R***************
Bovine              ********************S*****GS***************Q*****

                    110         115
H. sapiens          ADKADVNVLTKAKSQ
P. paniscus         ***************
G. gorilla          ***************
H. lar              ******D********
M. fascicularis     ******D********
P. hamadryas        ******D********
P. obscura          ******D**A*****
C. apella           ******D********
C. jacchus          ******D********
Bovine              ******D**I***P*
```

Figure 4.12 Alignment of the amino acid sequences of prepro-PTH gene in 8 species of primates. An amino acid sequence of bovine (Weaver *et al*., 1984) is used to be an outgroup, and an amino acid sequence of human (Vasicek *et al*., 1983) is used to be an ingroup reference. The stars (*) indicate identical amino acids..

4.6 Phylogenetic analysis of the nucleotide sequences of PTH gene

The phylogenetic tree based on the nucleotide sequences of PTH gene was constructed using Neighbor-Joining method of PHYLIP program (version 3.572c) (Figure 4.13 A). Eight species of primates could be classified into 2 groups as follows;

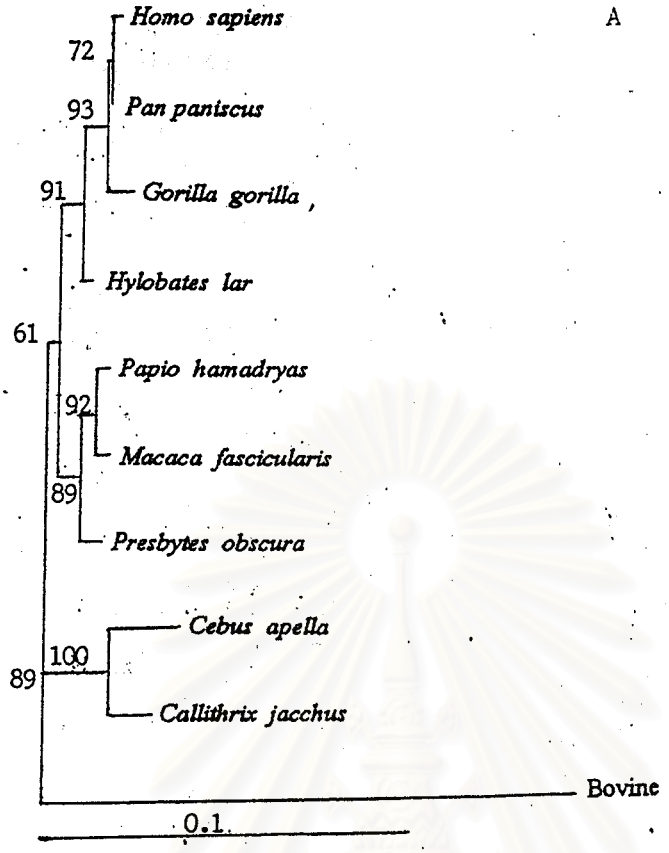
Group 1 consisted of *Cebus apella* and *Callithrix jacchus.*

Group 2 consisted of the remaining 6 species of primates, and could be further subdivided into 2 groups.

Group 2.1 consisted of *Presbytes obscura*, *Macaca fascicularis*, and *Papio hamadryas* .

Group .2 consisted of *Hylobates lar*, *Gorilla gorilla*, *Pan paniscus* and ingroup reference (*Homo sapiens*)

Phylogenetic analysis of the nucleotide sequences of PTH gene using Parsimony method of PAUP program (version 3.0s) revealed that these 8 species of primates were also divided into 2 main groups, and in group2 could be subdivided into 2 groups similarly to the result from Neighbor-Joining method of PHYLIP program (Figure 4.13 B).



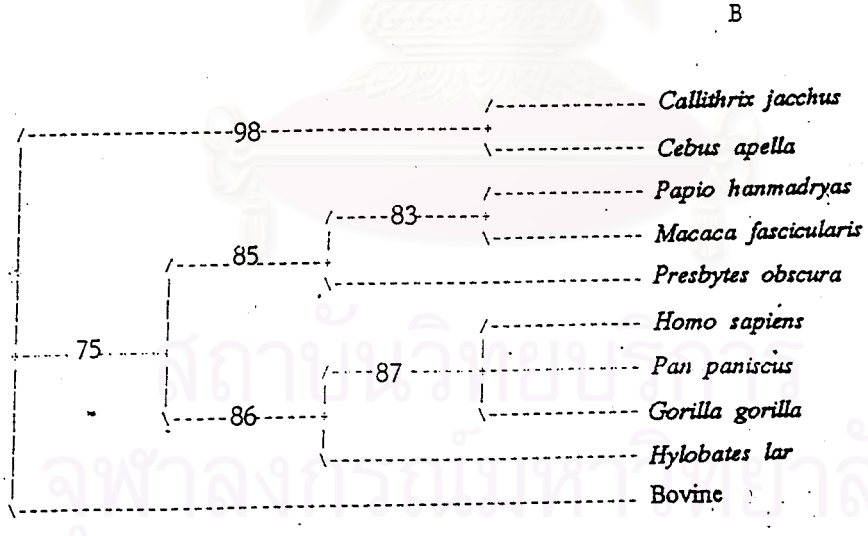


Figure 4.13 The phylogenetic trees of nucleotide sequences inferred genetic distance data by PHYLIP program (version 3.572c) (A), and PAUP program (version 3.0s)(B).The number on branches of the tree indicate the bootstrapping analysis.

4.7 Phylogenetic analysis of the amino acid sequences of PTH gene

The phylogenetic tree based on amino acid sequences of PTH gene was constructed using Neighbor-Joining method of PHYLIP program(version 3.572c) (Figure 4.14 A). Eight species of primates could be classified into 2 groups as follows;

Group 1 consisted of 7 species of primates, and could be further subdivided into 2 groups.

Group 1.1 consisted of 6 species, and could be subdivided into 2 groups.

Group 1.1.1 consisted of *Cebus apella* and *Callithrix jacchus.*

Group 1.1.2 consisted of *Presbytes obscura, Macaca. fascicularis*, *Papio hamadryas*, and *Hylobates lar.*

Group 1.2 consisted of *Pan paniscus* and ingroup reference (*Homo sapiens*).

Group 2 consisted of *Gorilla gorilla.*

Phylogenetic analysis of the amino acid sequences of PTH gene using Pasimony method of PAUP program (version 3.0s) (Figure 4.14 B). Eight species of primates could be classified into 5 groups as follows;

Group 1 consisted of *Cebus apella* and *Callithrix jacchus.*

Group 2 consisted of *Presbytes obscura, Papio hamadryas*, and *Macaca fascicularis* .

Group 3 consisted of *Hylobates lar.*

Group 4 consisted of. *Gorilla gorilla.*

Group 5 consisted of *Pan paniscus.*

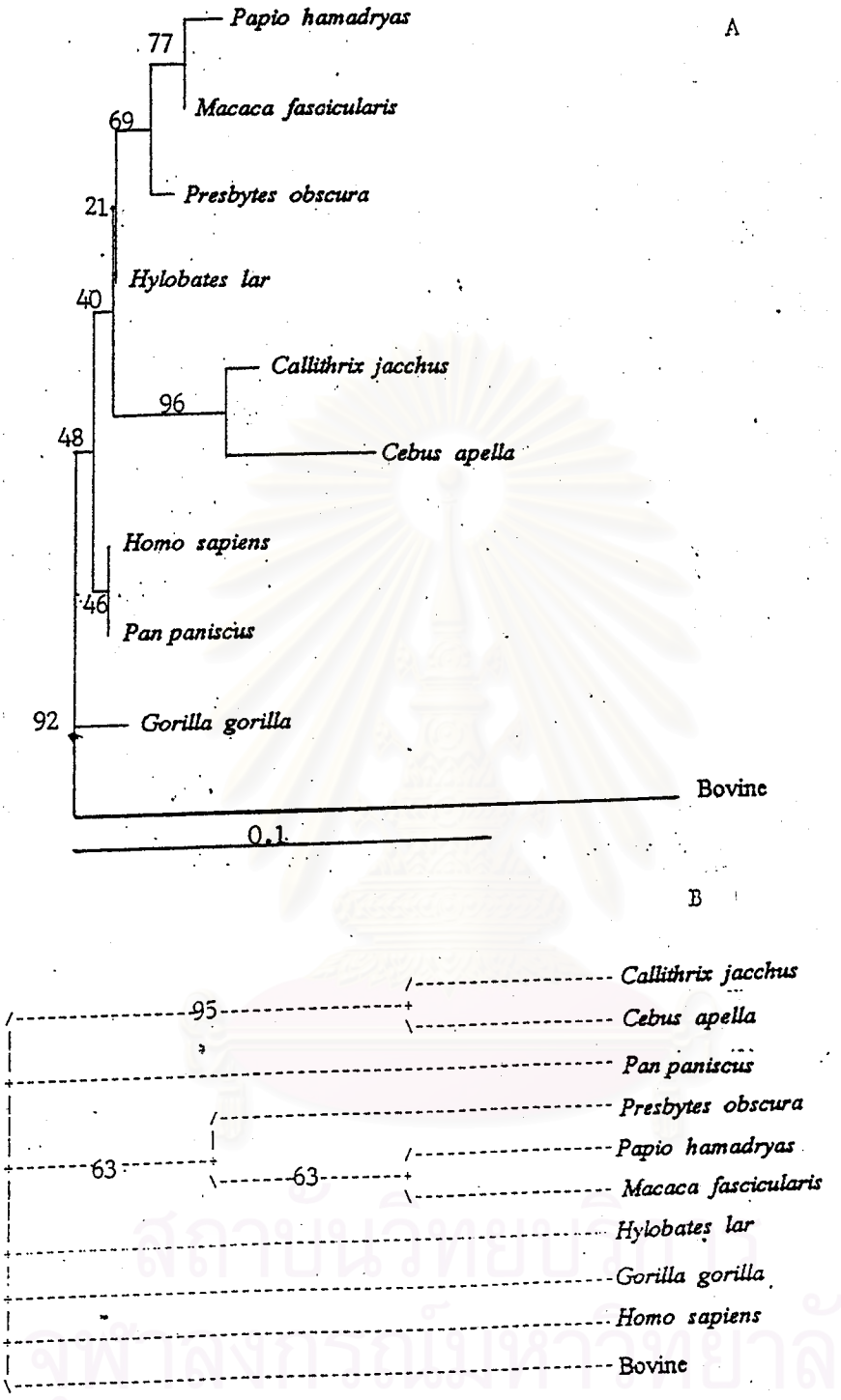


Figure 4.14 The phylogenetic trees of amino acid sequences inferred genetic distance data by PHYLIP program(version 3.572c) (A), and PAUP program (version 3.0s) (B). The number on branches of the tree indicate the bootstrapping analysis.

Chapter V

Discussion

The primers 446 and 448 were designed from hPTH gene (Vasicek *et al*., cited in Malaivijitnond and Takenaka, 1998), covered 626 bp of hPTH which consisted of 2 exons and 1 intron. Thus, the expected DNA fragment of amplified PTH gene in 8 species of primates should be located between the 600-700 bp. In this study, the expected bands of PTH gene of 8 investigated species were approximately 650 bp fragment when compared with 100 bp DNA ladder (marker). Then, the amplified PTH gene was used to prepare ssDNA for sequencing because ssDNA is needed to use to be a template for sequencing. However, some DNA was lost during the preparation, thus the produced ssDNA has to be reexamined for the remaining amount using 5% PAGE. On the gel, the expected band of ssDNA of PTH gene may be appeared into 2 locations by their structural difference. The lower band may be the ssDNA in primary structure , and the upper band may be the ssDNA in secondary structure or hairpin (stem-loop) structure. Single-stranded forms of DNA tend to fold back upon themselves whenever possible to form double-stranded helices with loops. These antiparallel duplex structure are called hairpin with a stem and loop. The stem has the paired bases and the loop has unpaired bases (Atherly *et al*., 1999). To ensure that both bands are ssDNA of PTH gene, the DNA hybridization procedure should have been done.

The nucleotide sequences of protein coding region in PTH gene in 8 species of primates were the same in number of nucleotide, 345 bp. However, the determined sequences range from 500-553 bp. Because the start and end of the sequence data could be red at different position in each species due to the bands of both regions were not clear. According to Vasicek *et al*. (1983), and Malaivijitnond and Takenaka (1998), the nucleotide sequences of all species were separated into 3 parts, 2 exons

(exon II and III) and 1 intron by GT-AG splicing rule. Lewin (1997) suggested that the intron defined the start with dinucleotide GT and end with dinucleotide AG, according to GT-AG rule. The left is 5' splice site or donor, and the right is 3' splice site or acceptor. From GT-AG rule, the intron is 102 bp in *C. apella* and *C. jacchus* but it is 103 bp in the other 6 species in this study. Comparison of these introns with human sequences (Vasicek *et al*., 1983) showed the identical with the intron B or second intervening DNA sequence which contains 103 bp and located between exon II and exon III.

According to Vasicek *et al*. (1983), the pro-sequence of hPTH consisted of 6 amino acids, and the last two amino acids (Lys-Arg) are important for cleavage the pro-sequence to form mature PTH. In this study, the last two basic residues of pro-sequence is Lys-Arg in 7 species of primates which similar to those human, except in *C. apella* is Arg-Arg. However, the Arg is also a basic residue. Thus, it may suggest that this change does not have any effect on cleavage pro-sequence from mature PTH. Nussbaum *et al*. (1980) indicated that the amino acid 25^{th} to 34^{th} of mature PTH are the most important for receptor binding of PTH, and comparison of this region between human and 8 species of primates shows 100 % homology.

For antibody binding site, RIA utilizing an antiserum directed against the the C-terminal provides the best indication of parathyroid function (Royer and Kemper, 1990). In 1995, Usami *et al*. could measure the serum monkey PTH (African green monkey; *Cercopithecus aethiops*) levels by the RIA technique using the antibody to human PTH-C (the amino acid 46^{th} to 84^{th}). According to the taxonomy classification

of Burton (1995), *C. aethiops* is classified into the same Subfamily of *M. fascicularis* (Subfamily Cercopithecinae). In addition, Malaivijitnond et al (1999) recently reported that the amino acid sequence of *C. aethiops* showed 100% homology with those of *M. fascicularis*, no changes of amino acids between these two species. In this study, the amino acid sequence of PTH gene in *M. fascicularis* showed 3 positions (at the 35^{th} , 58^{th}, 76^{th} position of PTH) different from human amino acid sequence. Therefore, it may conclude that changes in the amino acid in C-terminal (at the 58^{th} and 76^{th} position of PTH) do not have any affect on the hormone-antibody binding.

Comparison of nucleotide sequences and amino acid sequences between bovine (Weaver *et al*., 1984) and 8 species of primate has been found that the nucleotide sequences showed higher homology than those of amino acid sequences, because changes in nucleotide sequences are usually in the position of the 3^{rd} codon, and do not have any effect on the amino acid in the present sequences. Comparison of nucleotide sequences and amino acid sequences between human (Vasicek *et al*., 1983) and 8 species of primates showed a very high homology (95.38 - 99.80 % of nucleotide sequences, and 93.04 - 100.00 % of amino acid sequences, respectively), especially between human and chimpanzee (*P. paniscus*) showed 100% homology of amino acid sequence, because the evolution of human and primates are very close to each other. However, comparisons of nucleotide and amino acid sequences between bovine and 8 species of primates still revealed the high homology (85.00 - 86.53 % of nucleotide sequences, and 80.00 - 86.08% of amino acid sequences, respectively) between these sequences indicating the highly conserved sequences of PTH between distantly related taxa. According to the study of Anukulthanakorn *et al*. (1999), nucleotide sequences of PTH gene between two groups of macaques; silenus-sylvanus and fascicularis

which have an evolutionary difference for 5 million years, are 100% homology. Thus, it further supported that the PTH gene is very conserved.

Nucleotide and amino acid sequences of all primate species were used to construct phylogenetic trees based on distance (PHYLIP) and the most parsimonious approach (PAUP). The both programs were chosen to construct the phylogenetic tree because they are widely used. The first step of constructing the phylogenetic tree is aligning of sequences and giving 'cost' of the compared sequences. The alignment of protein sequences differs from alignment of nucleotide sequences in two important respects. Firstly, there are more symbols in the amino acid sequence than in nucleotide sequences because there are more amino acids (20 amino acids) than nucleotide bases (4 bases). Secondly, alignment is not simply a matter of aligning the symbols so that the greatest number match. For nucleotide sequences the cost of a mismatch between two nucleotides is often scored simply as '1', whereas a match has zero cost. Hence, nucleotide are either the same or they are different. Some methods are more sophisticated and have different costs for transitions and transversions, as these two classes of substitution often differ in frequency. However, for amino acids we need to take into account the possible pathways in which one amino acid might be replaced by another. For example, a cysteine encoded by the triple UGU can be replaced by a tyrosine (UAU) by a single nucleotide substitution at the second codon position, whereas replacing cysteine by methionine (AUG) requires three nucleotide substitutions, one at each codon position. Hence, aligning cysteine with tyrosine is less costly than aligning cysteine with methionine. The cost for every pair of possible amino acid replacements defines a cost matrix that can be used to score the alignment (Page and Holmes, 1998).

Moreover, the principles of constructing the phylogenetic tree from these 2 programs, the Neighbor-Joining method for PHYLIP and the Parsimony method for PAUP, are different. PHYLIP is a program for referring phylogenies from distance methods but PAUP is a program for referring phylogenies from discrete methods. Distance methods are based on the idea that if we knew the actual evolutionary distance between all members of a set of sequences, then we could easily reconstruct the evolutionary history of those sequences. Evolutionary distance is a tree metric and hence defines a tree. In practice, however, distances are rarely, if ever, exactly tree metrics, and hence one class of 'goodness of fit' methods to seeks the metric tree that best accounts for the 'observed' distances (i.e. the pairwise distances calculated between the sequences). The second class of method seeks the tree whose sum of branch lengths is the minimum (minimum evolution). In contrast, discrete methods operate directly on the sequences, or on functions derived from the sequences, rather than on pairwise distances. Hence, they endeavour to avoid the loss of information that occurs when sequences are converted into distance (Page and Holmes. 1998).

From phylogenetic tree based on the nucleotide sequences using Neighbor-Joining and Parsimony methods, 8 species of primates could be classified into the same 2 groups. Following the taxonomy classification of Lekagul and McNeely (1977), these two groups are in the Infraorder level; Infraorder Platyrrhini and Infraorder Catarrhini. Infraorder Platyrrhini or new world monkey includes *C. apella* and *C. jacchus*. Infraorder Catarrhini or old world monkey includes the remaining 6 species of primates which could be further subdivided into 2 groups in the Superfamily level; Superfamily Cercopithecoidea and Superfamily Hominoidea. Superfamily Cercopithecoidea in this study, includes *P. obscura, M. fascicularis* and *P. hamadryas*. Superfamily Hominoidea consisted of *H. lar, G. gorilla* and *P. paniscus* . By contrast, the phylogenetic tree based on amino acid sequences using Neighbor- Joining

and Parsimony methods showed different results. The phylogenetic tree based on the amino acid sequences using Neighbor-Joining method (PHYLIP program) could be classified the 8 species of primates into 2 groups, and using Parsimony method (PAUP program) could be classified the 8 species of primates into 5 groups. In addition, the phylogenetic tree of nucleotide and amino acid sequences from both methods are different.

From these results, it may be suggest that the PTH gene is able to classify the primates only into Infraorder level (Platyrrhini and Catarrhini) in Suborder Anthropoidea. To classify into Genus level, it should be used the blood-protein allele (Fooden and Lanyon, 1989) or 27 e-globin gene (Schneider *et al*., cited in Martins, 1996), and to classify into species or population level, it should be used the mitochondrial DNA (mtDNA), cytochrome b gene of the mtDNA (Martins, 1996), or displacement loop (D-loop).

Chapter VI

Conclusions and Recommendations

Conclusions

1) The primer designed from human PTH gene can amplify whole protein coding region of the PTH gene in 8 species of primates range from 500-553 bp, consisted of one intron and two exons. Intron is located between exon II and exon III. Exon II encodes the 25 amino acids of pre-sequence and first four amino acids of pro-sequence, and exon III encodes the remaining 2 amino acids of pro-sequence and 84 amino acids of mature PTH.

2) The intron contains 102 bp in *Cebus apella* and *Callithrix jacchus* (New world monkeys), but the other 6 species (Old world monkeys) contain 103 bp.

3) The nucleotide and amino acid sequences of PTH gene in 8 species of primates show a high homology with human sequences, especially between *Pan paniscus* and human.

4) Phylogenetic trees based on nucleotide sequence from both programs, PHYLIP and PAUP, classify 8 species of primates into 2 groups in Infraorder level; Infraorder Platyrrhini (New world monkeys) including *Cebus apella* and *Callithrix jacchus*, and Infraorder Catarrhini (Old world monkeys) including *Macaca fascicularis, Papio hamadryas, Presbytes obscura, Hylobates lar, Gorilla gorilla,* and *Pan paniscus*.

Recommendations

1) In Thailand, the study of primate does not widely carry out, then the researcher in this field has to cooperate with the foreign researchers. If there are more Thai researchers, or grant to support the study in this field, it would be very profitable, because we can get our own basic knowledge of primate in Thailand.

2) From the results showing the different number in nucleotide of intron between new world and old world monkeys, it may suggest that this difference may be used to discriminate the new world from the old world monkeys. However, only 2 species of new world monkeys (*Cebus apella* and *Callithrix jacchus*) have been determined the PTH sequences in this study. To be a reliable data, nucleotide sequences of PTH gene of more animals in this group (new world monkeys) should be determined.

3) To get more useful information, the further study of 3-dimension structure and receptor binding site of PTH in primate should be performed. This result may support the use of primate as an animal model for studying the bone metabolism related to changes of PTH.

4) To clarify the result, the PTH gene should be reconfirmed by determining the other strand of dsDNA or by cloning method.

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Appendices

Appendix I

The taxonomy of Order Primates

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The taxonomy of Order Primates

Order Primates

- Suborder Prosimii
  - Infraorder Lemuriformes
    - Superfamily Lemuroidea
      - Family Lemuridae (6 genera)
      - Family Indriidae (3 genera)
      - Family Daubentiniidae (1 genus)
  - Infraorder Lorisiformes
    - Family Lorisidae (5 genera)
  - Infraorder Tarsiiformes
    - Family Tarsiidae (1 genus)
- Suborder Anthropoidea
  - Infraorder Platyrrhini
    - Superfamily Cebidea
      - Family Ceboidae (12 genera)
      - Family Callithricidae (2 genera)
  - Infraorder Catrhhini
    - Superfamily Cercopithesoides
      - Family Cercopithecidae (15 genera)
    - Superfamily Hominoidea
      - Family Hylobatidae (3 genera)
      - Family Pongidae (3 genera)
      - Family Hominidae (1 genus)

(Lekagul and McNeely, 1977)

Appendix II

Two Worlds of Monkeys: New world and Old world

Appendix II

Two Worlds of Monkeys: New World and Old World

Monkeys, the most numerous and diversified primates, are grouped into great divisions: New World and Old World species. Although they apparently evolved from different ancestors, they resemble each other, both physically and behaviourally- a remarkable example of parallel evolution. Fossils show they once ranged far into subpolar latitudes, but they are today almost entirely tropical.

Monkeys are traditionally tropical animals. Almost all of them are found living in a broad belt of hot rain forest or savannah running right round the earth roughly between the Tropics of Cancer and Capricorn. The key identifies the principal kinds of living Old World and New World monkeys (Eimerl and De Vore, 1972).

Monkeys of the New World (Family Cebidae): in the vast forests which range from the western mountain ranges of the South America to the Atlantic, and from the southern edge of the Argentina, there are is an immense population of Monkeys, divided into 26 species. They differ markedly from the Old World Monkeys. Their faces are bare and tend to be flatter; they have a relatively large cranial capacity, their eyes are small, and their fur is thick and woolly. They have long and usually prehensile tails and most species have nails on all figures and toes. The average South American species is less intelligent than those of the Old World and their movements are less jerky. Only one species is nocturnal (Harlow and Parsons, 1974). A few new

World Monkeys have also evolved arms and shoulders that are suitable for swinging hand over hand through the trees like the Asiatic gibbons (Eimerl and De Vore, 1972).

Classification of Family Cebidae - New World Monkey:

1) The nostrils are well separated and face laterally (platyrrhine).
2) There are no cheek pouches.
3) There is no tubular, bony, external auditory meatus.
4) The dental formula is usually I 2/2, C 1/1, Pm 3/3, M 3/3.
5) The thumb when present is slightly opposable.
6) The tail is long and prehensile or short.
7) There are no ischial callosities on the buttocks.
8) The gait is quadrupedal.
9) They are found in South America.

(Webb, Wallwork, and Elgood, 1979)

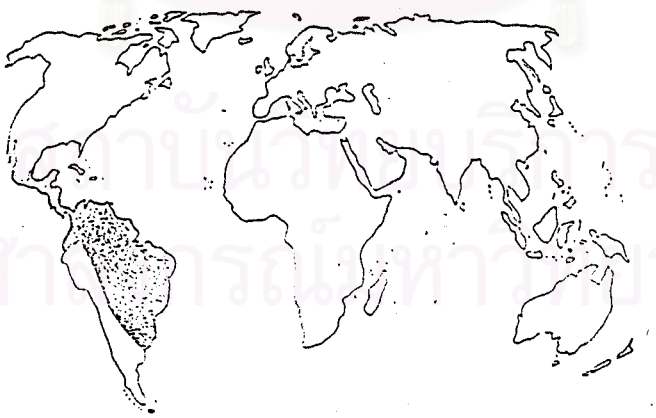


Figure A1.1 Distribution of New world monkeys, shading area.(Kando *et al.*, 1968)

Old World Monkeys (Family Cercopithecidae): the tailed Monkeys of the Old World, of which there are 60 species, are in many respects higher in the relatives of the New World. They are found in all the warmer zones of the eastern hemisphere except Madagascar and Austrasia. Old World Monkeys have the same number of teeth as Man, while their close-set nostrils are more human looking than those of New World Monkeys. They also appear to have some degree of color vision. Although the tails of most species are long, they are never prehensile. Old World monkeys have a well-developed thumb and they are in general more intelligent than are their New World relation (Harlow and Parsons, 1974).

Classification of Family Cercopithecidae - Old World monkeys

1) The nostrils are close together and face downward (catarrhine).
2) Cheek pouches are present.
3) The tympanic forms a tubular external auditory meatus.
4) The dental formula is I 2/2, C 1/1, Pm 2/2, M 3/3.
5) The thumb when present is opposable
6) The tail when present is not prehensile.
7) There are ischial callosities on the buttocks.
8) The gait is quadrupedal.
9) They are found in Africa, Arabia and from Afghanistan to Japan.

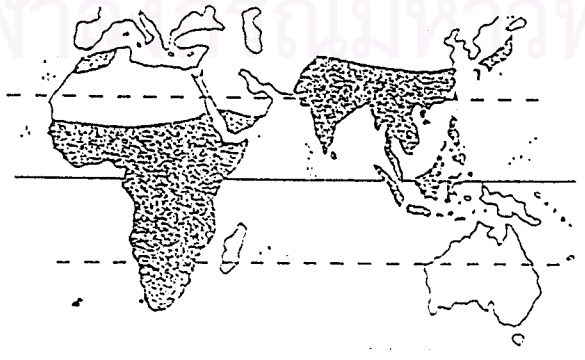


Figure A1.2 Distribution of Cercopithecidae (Webb et al., 1979)

Appendix III

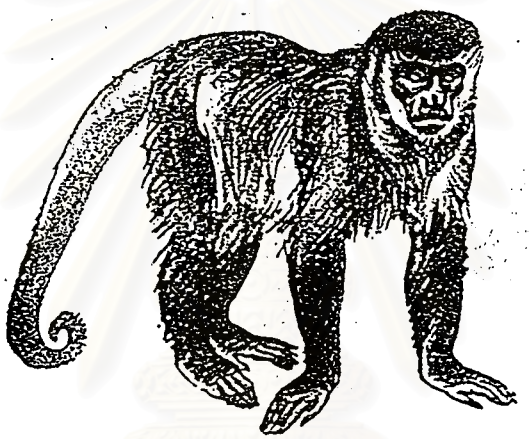
The natural history of 8 species of primates in this study

Appendix III

The natural history of 8 species of primates in this study

1) ***Cebus apella*** (Linneaus, 1758)

Common name: Black-capped capuchin or brown capuchin



Features:

The coat is light to dark brown and lighter on the underside. The hands and feet are always black. The face has distinctive black sideburns extending to the chin. The tufts on each side of the head and forming a triangle over the forehead are formed by dark brown hairs. These medium-sized monkeys are robust. Males are larger than females. Tails are semi-prehensile and used as temporary anchors to support the weight.

Life cycle:

Age at maturity is not certain, but females are probably sexually at between four and seven years and males one year later. The menstrual cycle lasts 15 to 20 days, with a slight show of bleeding. A single birth after a gestation period of about two years, if the infant survives. There is no breeding season. Copulation is by female choice: she follows the dominant male in the middle of her four to six days of receptivity. This considered to be her ovulatory period. In the final two days of her cycle, she copulates with up to six subordinates males in a day.

Habitat:

A highly adaptable and widespread species, this species is found in all types of humid forest, usually below 500 m, although some have been found as high as 2700 m in Colombia.

2) ***Callithrix jacchus*** (Linneaus, 1758)

Common name: Common marmoset or tufted-ear marmoset

Features:

The common marmoset is a small New World monkeys. The tail is longer than the body. All digits have curved, sharp claws except for the big toe. Body fur is mixed grey and black. Black and grey rings are found on the tail and there are bushy, white ear tufts completely covering the ears. Common marmoset have a specialized dentition with enlarged incisors that have only a thin layer of enamel. These teeth are chisel-like in shape and are used for biting holes in trees to get the resin, gums and saps. Callithrix have a cylindrical spiny penis; the females'labia majora are unpigmented and scrotum-like in size and form.

Life cycle:

The female's age at sexual maturity is around two years. The gestation period is nearly five months. In captivity, the female comes into estrus shortly after delivery (post-partum estrus), and copulations are observed 10 to 20 days after birth. The estrus

cycle lasts about 13 to 16 days. Twins are typical and births usually occur at six-month intervals. The interbirth intervals observed in captivity ranged from four months to five months.

Habitat:

The common marmoset is mainly found in the dry forests, coastal and upland scrub forests, gallery forests, edge habitats and secondary forest environments of Brazil. They easily adapt to humane habitation, occupying cultivated areas and gardens even in cities such as Sao Paulo

3) ***Macaca fascicularis*** (Raffles, 1821)

Common name: Crab-eating macaque or longtailed macaque or cynomolgus macaque

Features:

The long-tailed macaques is lithe and gracile, with a body weight around 4 kg in females, and close to 6 kg in males, making them among the lightest of macaques. Many subspecies have a crest down the center of the head, alternatively, there is a 'cap' of hair at the crown which is darker than elsewhere in the head. The tail is quite long, reaching to the ground. Fur is grey to brownish-grey, with white facial whiskers, and tufts above and beside the eyes that are lighter-colored, although in some there are dark patches below each eye. Mature females have longer hair along the side of the face and beneath the jaw , rather like Victorian "muttonchop whiskers". Lifespan is over 20 years, and animals have been known to live up to 27 years.

Life cycle:

Age at first reproduction for females is between four and five years, although the first menstrual cycle is apparent at between 2.5 and 3 years. Bleeding averages 3

days, with the cycle lasting 28 days. Sexual swelling and reddening of the genitalia are not pronounced and vary greatly between individuals. Males are sexually mature beginning at 4.5 years of age. Gestation lasts approximately 5.5 months. In Malaysia, there is a birth peak between May and July. The newborn weighs about 345 g, with a single birth being common. The interbirth interval is approximately one year. Weaning occurs at around 14 months.

Habitat:

The long-tailed macaque is able to live in a variety of habitats-primary, riverine, or coastal forest- provided they are close to water. As an 'edge' species, it can live in disturbed habitats along the edge, rather than in the interior of forest. This species is found at altitudes ranging from as high as 2000 m to sea level, although it is more frequently found at lower elevations. It species most of its time in the canopy, but can range on the ground, which it readily does in human-disturbed areas such as fields, temple precincts and botanical gardens.

4) ***Papio hamadryas*** (Linneaus, 1758)

Common name: Hamadryas baboon

Features:

Body weight for males is between 20 and 30 kg and for females, around 10 to 15 kg. These large baboons have distinctive characteristics: males have a large ruff or mantle and are considerably larger than females.

Life cycle:

Some authorities find there is no birth seasonality, while others report that in Ethiopia birth peaks were found between May and July and again in November to December. Gestation is thought to be around five to six months. Females reach sexual maturity at between four and five years, and males between five and seven. Females show clear swelling as a sign of estrus. The interbirth interval is approximately two years.

Habitat:

Home range is not known exactly. Published source, however, give ranges of between 10 and 20 km. Population density has been observed at 1.9/ km2 and as high as nearly 4/km2. *P. hamadryas* prefer subdesert steppe. This species is also found in short-grass plains and alpine grass meadows. It can range up to 2300 m in elevation in Ethiopia. Sleeping rocks reach heights of 15 to 25 m and are a limiting factor in this species' distribution

5) ***Presbytes (Trachypithecus) obscura*** (Reid, 1837)

Common name: Ducky leaf monkey or spectacled leaf monkey

Features:

The species is distinguished by its black face with a wide ring of white around each eye, as well as white patch over the center of the mouth including both lips. The back and limbs are grey, and sometimes have an orange sheen, while the extremities are black. The belly is creamy yellow to almost orange or brown. Adult females weigh 6.5 kg, and males 8.30 kg.

Life cycle:

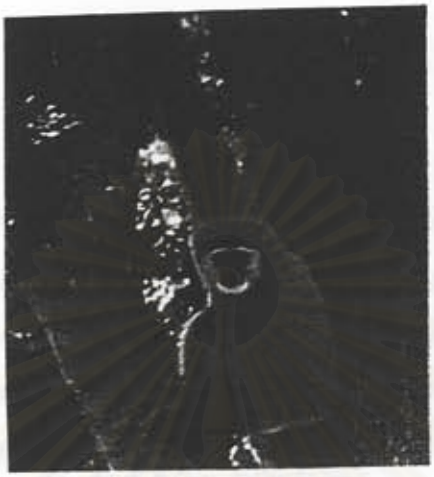
The neonate weighs 480 g. The single birth follows a gestation of five months. The female has an estrus swelling. Details of the life cycle are unknown, but females generally mature at between three to four years, and males a year later. The interbirth interval in not known, but may be one to two years.

Habitat:

The spectacled-leaf monkey inhabits both primary and old-growth secondary forest, and also invades human settlements.

6) ***Hylobates lar*** (Linneaus, 1771)

Common name: White-handed gibbon or common gibbon



Features:

Hylobatids are tailless, and extremely long forearms which make them excellent brachiators. *H. lar* is regionally distinct in pelage. In Sumatra and the Malay Peninsula, it is brown to red or beige; in Thailand, the darkest coats are present, dark brown to black or light beige, with a ring around the face that is white, as are the hands and feet. They have dense fur and bare, pigmented faces. The adult male has black, tufted fur in the pubic region. Males weigh 5.7 kg. Throat sacs beneath the chin are apparent in both males and females, from which a low-pitched boom and a high-pitched bark are emitted.

Life cycle:

Males may mature as young as six years of age, but females, between eight and ten. The estrus cycle is about 27 days. Female genitalia become turgid and change color during ovulation, and they alter shape and appearance during the ovarian cycle,

seven months' gestation, and the infant is weaned at approximately two years. The interbirth interval is 2.5 years. In captivity, this species has lived to 30 years.

Habitat:

The lar gibbon occupies a variety of types of semi-deciduous and tropical evergreen forest in both lowland and highland, and both primary and secondary, forest. It has been seen at elevations as high as 2400 m. Most observations are at or above sea level.

7) ***Pan paniscus*** (Schwarz, 1929)

Common name: Pygmy chimpanzee

Features:

As with all the Great Apes, *P. paniscus* are tailless. Body skin is white, but black on the face and hands. The cost is sparse and predominantly black in color, but aging turns the hair on the back to grey and darkens the skin. Infants have a tail tuft of white fur that remains into adulthood. They are slender and have long hindlimbs; their clavicles are short and their molars small. Arms are longer than hindlimbs, and the thumb is short. Females reach almost 86 cm in height, and males are slightly taller. Females weigh about 30 kg,, and male somewhat more (39 kg).

Life cycle:

The mean cycle length of adult females is about 46 days, with a swelling phase of 22 days in captive chimpanzee. In P. paniscus, the labia majora are retained into adulthood. There is no birth seasonality. Gestation is thought to last 7.7 to 8 months. A single birth is typical. In the female, swelling begin at adolescence and for some

years, the female will become pregnant. Females begin to swell again within less than one year after giving birth, but the interval until the next birth is 5 to 5.5 years. Weaning occurs at around four years of age, and it is thought that lactation prevents ovulation.

Habitat:

The pygmy chimpanzee utilized all vegetation types and forest strata, but is most often in the crowns and shrub levels of dry, primary forest.

8) ***Gorilla gorilla*** (Savage & Wyman, 1847)

Common name : Gorilla

Features:

The western lowland gorilla, *G. g. gorilla* is brown grey with reddish highlights. The adult male silver-back is distinguished by silver hairs extending down to his rump and thighs. Gorilla females weigh 82.3 kg, with a range from 67 to 97 kg. Male weigh 161 kg and range from 141 to 181 kg. Females are 150 cm in height, while males are nearly 170 cm tall.

Life cycle:

Gorilla females exhibit very little sexual swelling, and , although the group has a one-male unit structure, it is the female who initiates copulation. Age at sexual maturity for females is from 6.5 to 8 years, but breeding begins at around 10 years for females. Sexual competition between males is strong and restricts access, so that males breed only at around 15 to 20 years of age. There is no breeding season. Single births are usual, after gestation of between eight and nine months, and occur

throughout the year, peaking between July and August and again between September and December. The neonate weigh only around 2 kg, and its pinkish-grey color skin is nearly naked, with sparse fur greyish in color. It is weaned at about three years of age.

Habitat:

Gorillas live in lowland and montane forest, preferring open-canopy forests that are moist and lush but which allow light to reach the forest floor. Lowland gorillas inhabit a variety of habitats, from primary and secondary forest to the edges of clearing such as fields and roads (Burton, 1995).

Appendix IV

Chemicals and instruments

Chemicals

Acrylamide	BIO-RAD, USA
Tris	BIO-RAD, USA
EDTA (ethylenediamine tetraacetic acid)	BIO-RAD, USA
Urea	BIO-RAD, USA
Phenol	Wako, Japan
Chloroform	Wako, Japan
Ampli *Taq* Gold™	PERKIN ELMER, USA
SequiTherm EXCEL™II Long-Read sequencing Kit-LC	EPICENTRE Technologies
100-bp DNA Ladder	GibcoBRL, USA
Ketamine hydrochloride	Park Davis, Australia
Heparin	Park Davis, Australia
NaCl	Wako, Japan
Dialysis bag	Wako, Japan
Proteinase K	Promega corporation, USA
RNAase A	Promega corporation, USA
$AgNO_3$ (silver nitrate)	Wako, Japan
CTAB	Wako, Japan
NH_4OH (ammonium hydroxide)	Wako, Japan
Ammonia	Wako, Japan
Dyna bead	Dynal A.S. Osio, Norway
Formaldehyde	Wako, Japan

Instruments

PCR thermal cycler	PERKIN ELMER, USA
Shimadzufluorescent automatic sequencer DSQ-1	Shimdzu , Japan

Appendix V

Genetic distance analysis

The estimated nucleotide sequence divergence between pairs of species used in this study based on Kimura's two parameter method.

Species	*H. sapiens*	*P. paniscus*	*G. gorilla*	*H. lar*	*M.fascicularis*	*P. hamadryas*	*P. obscura*	*C. apella*	*C. jacchus*	Bovine
H. sapiens										
P. paniscus	0.0020									
G. gorilla	0.0101	0.0081								
H. lar	0.0121	0.0101	0.0162							
M.fascicularis	0.0286	0.0286	0.0349	0.0223						
P. hamadryas	0.0286	0.0286	0.0349	0.0223	0.0080					
P. obscura	0.0265	0.0265	0.0328	0.0203	0.0141	0.0141				
C. apella	0.0539	0.0539	0.0608	0.0472	0.0560	0.0560	0.0515			
C. jacchus	0.0476	0.0476	0.0542	0.0411	0.0495	0.0453	0.0452	0.0318		
Bovine	0.1650	0.1650	0.1649	0.1621	0.1619	0.1619	0.1643	0.1840	0.1771	

Pairwise comparisons indicating the number of nucleotide differences (below diagonal) and sequence divergence (above diagonal) of PTH. The human and bovine PTH were included as an ingroup and an outgroup references, respectively.

Species	*H. sapiens*	*P. paniscus*	*G. gorilla*	*H. lar*	*M.fascicularis*	*P. hamadryas*	*P. obscura*	*C. apella*	*C. jacchus*	Bovine
H. sapiens	-	0.002	0.008	0.010	0.026	0.026	0.024	0.050	0.044	0.143
P. paniscus	1	-	0.006	0.008	0.026	0.026	0.024	0.050	0.044	0.143
G. gorilla	4	3	-	0.012	0.030	0.030	0.028	0.054	0.048	0.142
H. lar	5	4	6	-	0.022	0.022	0.020	0.046	0.040	0.143
M.fascicularis	13	13	15	11	-	0.008	0.014	0.054	0.048	0.143
P. hamadryas	13	13	15	11	4	-	0.014	0.054	0.044	0.143
P. obscura	12	12	14	10	7	7	-	0.050	0.044	0.145
C. apella	24	24	26	22	26	26	24	-	0.031	0.163
C. jacchus	22	22	24	20	24	22	22	15	-	0.156
Bovine	71	71	70	71	71	71	72	78	77	-

The estimated genetic distances of PTH amino acid sequences between pairs of species.

Species	*H. sapiens*	*P. paniscus*	*G. gorilla*	*H. lar*	*M.fascicularis*	*P. hamadryas*	*P. obscura*	*C. apella*	*C. jacchus*	Bovine
H. sapiens										
P. paniscus	0.00000									
G. gorilla	0.01666	0.01666								
H. lar	0.00829	0.00829	0.02509							
M.fascicularis	0.02490	0.02490	0.04188	0.01575						
P. hamadryas	0.03337	0.03337	0.05051	0.02500	0.00828					
P. obscura	0.02498	0.02498	0.04202	0.01013	0.01012	0.02502				
C. apella	0.06839	0.06839	0.08627	0.05979	0.07698	0.08597	0.07724			
C. jacchus	0.04206	0.04206	0.05943	0.03361	0.05050	0.05922	0.05067	0.04251		
Bovine	0.15899	0.15899	0.15929	0.15000	0.16795	0.17779	0.16040	0.21874	0.18843	

Pairwise comparisons illustrating the number of PTH amino acid differences (below diagonal) and sequence divergence (above diagonal) of 8 primate species in this study. The human and bovine sequences were included as an ingroup and an outgroup references, respectively.

Species	*H. sapiens*	*P. paniscus*	*G. gorilla*	*H. lar*	*M.fascicularis*	*P. hamadryas*	*P. obscura*	*C. apella*	*C. jacchus*	Bovine
H. sapiens	-	0.000	0.017	0.009	0.026	0.035	0.026	0.070	0.043	0.148
P. paniscus	0	-	0.017	0.009	0.026	0.035	0.026	0.070	0.043	0.148
G. gorilla	2	2	-	0.026	0.043	0.052	0.043	0.087	0.061	0.148
H. lar	1	1	3	-	0.017	0.026	0.017	0.061	0.035	0.139
M.fascicularis	3	3	5	2	-	0.009	0.017	0.078	0.052	0.157
P. hamadryas	4	4	6	3	1	-	0.026	0.087	0.061	0.165
P. obscura	3	3	5	2	2	3	-	0.078	0.052	0.148
C. apella	8	8	10	7	9	10	9	-	0.043	0.200
C. jacchus	5	5	7	4	6	7	6	5	-	0.174
Bovine	17	17	17	16	18	19	17	23	20	-

Biography

Miss Kanya Anukulthanakorn was born on 12 September 1975 in Uttaradit province, Thailand. She graduated her bachelor's degree of Science in Biology in 1996 from the Department of Biology, Faculty of Science, Naresuan University, Phitsanulok. She continued her graduated study for a master's degree of Science in Zoology at Chulalongkorn University in 1997.