การแปรผันของไมโตคอนเดรียลดีเอ็นเอของผึ้งมิ้ม *Apis florea* Fabricius, 1787 ในประเทศไทย จากการตรวจสอบโดยใช้เทคนิค PCR-RFLP

นางสาวปิยมาศ นานอก

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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MITOCHONDRIAL DNA VARIABILITY OF DWARF HONEY BEE *Apis florea* Fabricius, 1787 IN THAILAND USING PCR-RFLP TECHNIQUE

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ปิยมาศ นานอก : การแปรผันของไมโตคอนเดรียลดีเอ็นเอของผึ้งมิ้ม *Apis florea* Fabricius, 1787 ในประเทศไทย จากการตรวจสอบโดยใช้เทคนิค PCR-RFLP. (MITOCHONDRIAL DNA VARIABILITY OF DWARF HONEY BEE *Apis florea* Fabricius, 1787 IN THAILAND USING PCR-RFLP TECHNIQUE) อ. ที่ปรึกษา : ศ. ดร. สิริวัฒน์ วงษ์ศิริ, อ. ที่ปรึกษาร่วม : อ. ดร. สุรีรัตน์ เดี่ยววาณิชย์ จำนวนหน้า 91 หน้า. ISBN 974-03-0309-9

ได้มีการตรวจสอบความแปรผันทางพันธุกรรมของผึ้งมิ้ม (*Apis florea* Fabricius, 1787) ซึ่งเป็นผึ้งพื้นเมืองขนาดเล็กที่พบได้ทั่วไปในประเทศไทย โดยใช้เทคนิค PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) ของไมโตคอนเดรียลดีเอ็นเอ 3 บริเวณ คือบริเวณระหว่างยีน CO I และ CO II, ยีน L-rRNA, และ ยีน Cytb I-tRNA^{Ser} ซึ่งตัวอย่างผึ้งที่ใช้ในการศึกษาครั้งนี้มีจำนวนทั้งหมด 180 รัง โดยสุ่มเก็บตัวอย่างจากทั่วประเทศไทย รวมทั้งเกาะสมุยและเกาะพะงัน

หลังจากเพิ่มปริมาณไมโตคอนเดรียลดีเอ็นเอในทั้ง 3 บริเวณดังกล่าวแล้วผลิตภัณฑ์ดีเอ็นเอที่เพิ่มปริมาณได้มีขนาด 1590, 760 และ 870 คู่เบส ตามลำดับ จากนั้นนำดีเอ็นเอที่ได้มาตัดด้วยเอ็นไซม์ตัดจำเพาะ โดยเอ็นไซม์ที่ใช้ทั้งหมดคือ *Acl* I, *Afl* I, *Ase* I, *Bam*H I, *Bcl* I, *Dra* I, *Eco*R I, *Hin*d III, *Hin*f I, *Rsa* I, *Ssp* I และ *Swa* I แต่มีเพียง 6 เอ็นไซม์ คือ *Ase* I, *Bcl* I, *Dra* I, *Hin*d III, *Hin*f I, และ *Ssp* I ที่สามารถตัดผลิตภัณฑ์ดีเอ็นเอระหว่างบริเวณ CO I และ CO II ได้ และมีเพียง 3 เอ็นไซม์ที่สามารถตัดผลิตภัณฑ์ดีเอ็นเอในบริเวณยีน L-rRNA และ Cytb I-tRNA^{Ser} ได้ คือ *Ase* I, *Dra* I, และ *Ssp* I

จากการวิเคราะห์ความแปรผันของไมโตคอนเดรียลดีเอ็นเอภายในกลุ่มประชากรของผึ้งมิ้มในประเทศไทยโดยใช้เทคนิค PCR-RFLP พบว่าเมื่อตัดผลิตภัณฑ์ดีเอ็นเอระหว่างบริเวณยีน CO I และ CO II ด้วยเอ็นไซม์ *Ase* I สามารถจำแนกความแตกต่างออกเป็น 2 รูปแบบ ซึ่งรูปแบบที่แตกต่างจากประชากรทั้งหมดนั้นพบเพียง 1 รังเท่านั้นคือผึ้งมิ้มที่มาจากจังหวัดประจวบคีรีขันธ์ สำหรับผลิตภัณฑ์ดีเอ็นเอในบริเวณยีน L-rRNA และ ยีน Cytb I-tRNA^{Ser} นั้น เมื่อวิเคราะห์ด้วยการใช้เอ็นไซม์ตัดจำเพาะตัดในบริเวณที่ต้องการแล้ว ไม่สามารถตรวจสอบความแปรผันของไมโตคอนเดรียลดีเอ็นเอภายในสปีชีส์ของผึ้งมิ้มในประเทศไทยได้

เนื่องจากบริเวณระหว่าง CO I และ CO II ของไมโตคอนเดรียลดีเอ็นเอของผึ้งมิ้ม มีขนาดเล็กกว่าบริเวณเดียวกันในไมโตคอนเดรียลดีเอ็นเอของผึ้งพันธุ์ *A. mellifera* และ ผึ้งโพรง *A. cerana* ดังนั้นจึงอาจเป็นสาเหตุที่พบความแตกต่างในผึ้งมิ้มได้น้อยกว่าในทั้งสองสปีชีส์ดังกล่าว

นอกจากนี้ พฤติกรรมการอพยพตามฤดูกาล การแยกรัง การหนีรัง รวมทั้งความสามารถในการปรับตัวให้เข้ากับสิ่งแวดล้อมได้ดีของผึ้งมิ้ม ทำให้ผึ้งชนิดนี้สามารถดำรงชีวิตและแพร่กระจายในธรรมชาติได้อย่างรวดเร็ว ซึ่งอาจจะเป็นสาเหตุที่ทำให้ไมโตคอนเดรียลดีเอ็นเอในกลุ่มประชากรของผึ้งมิ้มในประเทศไทยไม่มีความแตกต่างกันมากนัก

ภาควิชา ชีววิทยา ลายมือชื่อนิสิต .....

สาขาวิชา สัตววิทยา ลายมือชื่ออาจารย์ที่ปรึกษา .....

ปีการศึกษา 2544 ลายมือชื่ออาจารย์ที่ปรึกษาร่วม .....

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KEY WORD: honey bee / *Apis florea* Fabricius, 1787 / mtDNA / PCR-RFLP / Thailand

PIYAMAS NANORK : MITOCHONDRIAL DNA VARIABILITY OF DWARF HONEY BEE *Apis florea* Fabricius, 1787 IN THAILAND USING PCR-RFLP TECHNIQUE. THESIS ADVISOR : PROF. SIRIWAT WONGSIRI, Ph.D., THESIS CO-ADVISOR : SUREERAT DEOWANISH, D. Agr., 91 pp. ISBN 974-03-0309-9.

Dwarf honey bee (*Apis florea* Fabricius, 1787) is a native species of honey bee in Thailand. Genetic variation within dwarf honey bees sampled from various parts (the North, the North-East, the East, the central, and the South including Samui Island and Pha-Ngan Island) of Thailand were determined by PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) technique.

Three mtDNA regions (inter CO I-CO II region, L-rRNA gene, and Cytb I-tRNA^{Ser} gene) were amplified. The size of inter CO I-CO II region, L-rRNA gene, and Cytb I-tRNA^{Ser} gene are 1590, 760, and 870 bp, respectively. A set of restriction enzymes (*Acl* I, *Afl* I, *Ase* I, *Bam*H I, *Bcl* I, *Dra* I, *Eco*R I, *Hin*d III, *Hin*f I, *Rsa* I, *Ssp* I, and *Swa* I) were used to analyse the variation. Six restriction enzymes; *Ase* I, *Bcl* I, *Dra* I, *Hin*d III, *Hin*f I, and *Ssp* I could digest the PCR products of inter CO I-CO II region, and only three restriction enzymes; *Ase* I, *Dra* I, and *Ssp* I could be digested the PCR products of L-rRNA gene and Cytb I-tRNA^{Ser} gene.

No variation was found from PCR- RFLP analysis in L-rRNA gene and Cytb I-tRNA^{Ser} gene among *A. florea* populations in Thailand (180 colonies). Two different haplotypes were found in the inter CO I-CO II region when digested the PCR products with *Ase* I. The different haplotype was deteced from a colony of *A. florea* in Prachub Kiri Khan (lower part of central Thailand).

The size of PCR product in the inter CO I-CO II region of *A. florea* was smaller than *A. mellifera* and *A. cerana*. Thus the variation in this region of mtDNA of *A. florea* may be less with the deleted sequences.

Furthermore, the basic biology of *A. florea* such as the seasonal migration, swarming, and absconding including the well adaptation of this species might be caused they can be survived in the environment and distributed very widely and rapidly. Thus low of mtDNA variation among them was detected.

Department Biology Student's signature .....

Field of study Zoology Advisor's signature .....

Academic year 2001 Co-advisor's signature .....

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LIST OF ABBREVIATIONS

A, T, C, G	=	nucleotide containing the base Adenine, Thymine, Cytosine, and Guanine, respectively.
ATPase	=	adenosine triphosphatase
bp	=	base pair
^{o}C	=	degree celcius
cm	=	centrimetre
CO I	=	cytochrome oxidaase I
CO II	=	cytochrome oxidaase II
Cytb I	=	cytochrome b I
DNA	=	deoxyribonucleic acid
dNTPs	=	deoxyribonucleotide triphosphates(dATP, dTTP, dGTP, dCTP)
ddNTPs	=	dideoxyribonucleotide triphosphates(ddATP, ddTTP, ddGTP, ddCTP)
EDTA	=	ethylenediamine tetraacetic acid
HCl	=	hydrochloric acid
Kb	=	kilobase
KCl	=	potassium chloride
L-rRNA	=	large subunit ribosomal RNA
$MgCl_2$	=	magnesium chloride
ml	=	mililitre
mM	=	millimolar
MtDNA	=	mitochondrial DNA
mg	=	milligram
ng	=	nanogram

PCR	=	polymerase chain reaction
RFLP	=	restriction fragment length polymorphism
RNA	=	ribonucleic acid
rpm	=	revolution per minute
SDS	=	sodium dodecyl sulfate
Tris	=	tris (hydroxy methyl) aminomethane
tRNA	=	transfer RNA
UV	=	ultraviolet
V	=	volt
W	=	watt
μg	=	microgram
μl	=	microlitre
μM	=	micromolar

CHAPTER I

INTRODUCTION

Economically, honey bees are the most important beneficial insect, giving us honey, bee's wax, royal jelly, pollen, and bee venom. Honey bee products are playing an increasing role as health foods and in apitherapy (Crane, 1990; 1993). Bee venom is routinely used for treatment of arthritis and other autoimmune diseases. However, the most important contribution of honey bees is the pollination of many economic crops and wild plants (Appanah and Kevan, 1995).

Honey bees are eusocial insects. Three types of individuals or castes, can be found in a honey bee colony including the queen (a fertile female), workers (subfertile female) and drones (male). Both queen and workers are produced from fertilized eggs (diploid individuals), but drones develop by parthenogenesis (haploid individuals) from unfertilized eggs (Wongsiri, 1988).

Apis species can be grouped, on the basis of morphology and behavior, into three lineages. Representatives from each of three lineages occur over a wide range of habitats and climates (Smith, 1991). Maa (1953) divided honey bees into three genera: *Micrapis*, the dwarf honey bees, with two species (*Micrapis florea* and *M. andreniformis*); *Megapis*, the giant bees, with four species (*Megapis breviligula*, *M. binghami*, *M. dorsata* and *M. laboriosa*); and *Apis*, the cavity-nesting bees. *Apis* was further subdivided into two genera, 1) *Apis*, The European and African cavity-nesting bees, with seven species and several subspecies; and 2) *Sigmatapis*, the Asian cavity-nesting bees,

with 11 species and several subspecies. Subsequently biologists tended to ignore Maa's classification and recognized one extant genus, *Apis*, and four species corresponding to each of Maa's genera or subgenera: *Apis florea*, the dwarf bee, *A. dorsata*, the giant bee, *A. cerana*, the cavity-nesting bee, and *A. mellifera*, the western cavity-nesting bee (Smith, 1991). Recently the number of species has been expanded, so that 9 species are currently recognized: *A. dorsata* Fabricius, 1793, *A. laboriosa* F. Smith, 1871, *A. florea* Fabricius, 1787, *A. andreniformis* (Smith, 1858), *A. mellifera* Linnaeus, 1758, *A. cerana* Fabricius, 1798, *A. nuluensis*, *A. kochevnikovi* Buttel-Reepen, 1906 and *A. nigrocincta* Smith, 1861 (Koeniger and Koeniger, 2000).

The species that construct a simple exposed comb are *A. dorsata*, *A. laboriosa*, *A. florea* and *A. andreniformis*. The species *A. cerana*, *A. nuluensis*, *A. kochevnikovi* and *A. nicrocincta*, construct their nest in sheltered cavities, These nests comprise multiple parallel combs (Koeniger and Koeniger, 2000; Otis, 1991; Ruttner, 1988).

Biodiversity is the total sum of life's variety in a region. There are three levels of biological organization : species diversity, genetic diversity and ecological diversity (Wilson, 1988). Thailand is probably the center of diversity for honey bees with five species of honey bees; *A. dorsata*, *A. florea*, *A. andreniformis*, *A. cerana* and *A. mellifera* are found (Wongsiri *et al.*, 1987).

A. dorsata, *A. florea*, *A. andreniformis* and *A. cerana* are native to Thailand but *A. mellifera* is an "exotic" species. Only *A. cerana* and *A. mellifera* can be kept and managed in hives for commercial

beekeeping (Ruttner, 1988; Wongsiri *et al.*, 1987, 1990, 1996), but all wild species are hunted.

In order to evaluate and more precisely describe the genetic diversity of *Apis*, different methods have been used. The first was morphometrics (Ruttner, 1988; Smith, 1999). Morphometric characters are quantitative characters with generally high heritability (Cornuet and Garnery, 1991). In Thailand, *A. cerana* was studied morphometrically by Limbipichai (1990) and Sylvester *et al.* (1998). They concluded that this species could be separated into four ecotypes; 1) northern to central Thailand, 2) Southern Thailand to the end of Malaysian Peninsula, 3) Samui Island and, 4) less distinctly, Phuket Island. Nonetheless, this method, in which genotypes cannot be established through phenotypes, gives only an indirect and possibly biased measure of genetic diversity. The second approach is the study of allozymic variation which allows the determination of genotypes (Smith and Brown, 1987; Sylvester, 1986; Yik-Yuen *et al.*, 1991). Finally, studies of the genetic variation of *Apis* at the DNA level have been developed (Cornuet and Ganery, 1991). Various molecular biology techniques, such as RAPD (random amplified polymorphic DNA) (Hunt and Page, 1995; Suazo *et al.*, 1998), RFLP (restriction fragment length polymorphism) (Deowanish *et al.*, 1996; Garnery *et al.*, 1993; Meusel and Moritz, 1992; Sheppard and McPheron, 1993; Smith and Brown, 1990) , Microsatellite (Frank *et al.*, 1998; Estoup *et al.*, 1995; Oldroyd *et al.*,1996, 1998; Rowe *et al.*, 1997), and DNA sequencing (Cameron, 1993; Crozier and Crozier, 1993; Flook *et al.*, 1995; Garnery *et al.*, 1991; Sheppard and McPheron, 1993; Willis *et al.*, 1992), have been used to study the diversity of *Apis* species.

One of the general techniques is to look for restriction fragment length polymorphisms (RFLPs). This procedure can be applied to nuclear (Hall, 1986) as well as to mitochondrial DNA (Moritz *et al.*, 1986; Smith, 1991; Smith and Brown, 1988). Mitochondrial DNA (mtDNA) has been used as a marker to distinguish between African and European honey bee matrilines (Smith and Brown, 1988; Hall and Smith, 1991).

Besides its potential discreminating power, mtDNA can provide valuable information on the phylogenetic links between subspecies (Cornuet and Garnery, 1991).

Genetic diversity of *A. cerana* in Thailand was studied by Pramual (1994), Bugharuang (1996), Deowanish (1997), Sihanuntavong (1997), Laoaroon (1998) and Sittipraneed *et al*. (2001) by using various molecular biology techniques. In summary, *A. cerana* could be separated into three groups: northern to central Thailand group, Southern Thailand group and Samui Island group. This conclusion fits well with results from morphometric analyses.

The dwarf honey bee, *A. florea*, is widely distributed over the different geograpical areas of Thailand (Akaratanakul, 1990; Wongsiri *et al.*, 1990). *A. florea* is an excellent pollinator and easy to maintain in orchards (Free, 1981; Wongsiri *et al.*, 1990). Their honey and nest products have increased the income of villagers in Thailand (Lekprayoon and Wongsiri, 1989; Wongsiri *et al.*, 1996). "Bee hunter" is well known profession and hundreds of *A. florea* nests may be "harvested" per week by one man and sold as a whole at the Bangkok

market (Dziadyk, 1993; Wongsiri *et al.*, 2000). As honey is harvested by destroying the brood, *A. florea* populations in Thailand were rapidly reduced (Wongsiri *et al.*, 1996).

The study of *A. florea* in Thailand has been confined to investigations into behavior, ecology, distribution and morphology (Wongsiri *et al.*, 1996). The study of genetic diversity and intraspecific variation within this species has not been done. *A. florea* populations are expected to show considerable genetic structure, because when this species swarms and abscond, the flight range of the swarm is relatively short (only a few up to several 100 m, Ruttner, 1988; Tirgari, 1971). In general, the nesting site of *A. florea* is the plain up to 500 m from sea level. However, seasonal migrations occur up to 1,500 m from sea level and even higher in the north of Thailand and in the south of China (Free, 1981; Wongsiri *et al.*, 1996). The migrations may be an adaptation to survival in response to seasonal changes and lack of food (Ruttner, 1986).

The objectives of this study are to determine diversity of *A. florea* from different regions of Thailand by using PCR-RFLP techniques on various mtDNA regions, and to develop the appropriate techniques and PCR conditions for study genetic variation in *A. florea*. The results will provide information on basic biology; biodiversity, geographic variation and genetic relationship among *A. florea* populations in Thailand. These may be applied for conservation biology and breeding improvement of *A. florea* strain in the future.

CHAPTER II

LITERATURE REVIEW

2.1 Biology of *Apis florea* Fabricius, 1787

The dwarf honey bee, *A. florea* is a native wild species and the most common of honey bee in Thailand (Figure 2.1, 2.2). Its preferred nesting sites are in the substratum of dense bushes and small trees of the tropics (Free, 1981; Ruttner, 1988), but nests are also found, for example, on high trees and roofs of building on the campus of Chulalongkorn University (Lekprayoon and Wongsiri, 1989; Wongsiri *et al.*, 1996). It usually inhibits on the plains, but also occurs up to about 1,500 m. from sea level (Free, 1981; Ruttner, 1986). *A. florea* is an excellent pollinator and easy to maintain in the tropical fruit orchards. In India, up to 75 % of the insect visitors in a *Brassica* field are likely to be *A. florea* (Free, 1981; Ruttner, 1986; Wongsiri *et al.*, 1996).

A. florea is able to survive in very hot climates with maximum temperatures near 40^0C, and dry climates. It occurs from Oman and Iran in the west through Pakistan, India, Sri Lanka, Indochina, Malaysia, to parts of Indonesia in the east (Free, 1981; Ruttner, 1988). It is frequently found in tropical forests, in woods and even in farming areas. In southeast Asia it is usual to find a nest of *A. florea* in almost any village (Akratanakul, 1990). Wongsiri *et al.* (1996, 2000) reported that, *A. florea* is distributed throughout Thailand, but has not been found in the southern Malaysian Peninsula or Indonesia, Borneo, the Philippines or in the surrounding islands where it is replaced by *A. andreniformis*.

However, it has been recently reported in Kaula Lampur (Malaysia) and Palawan (the Philippines) (Wongsiri *et al.*, 2001). Whether Malaysia and the Philippines is part of its natural range or represents a recent range expansion is not yet known.

Figure 2.1 Dwarf honey bee, *A. florea*

Figure 2.2 Nest of dwarf honey bee, *A. florea.*

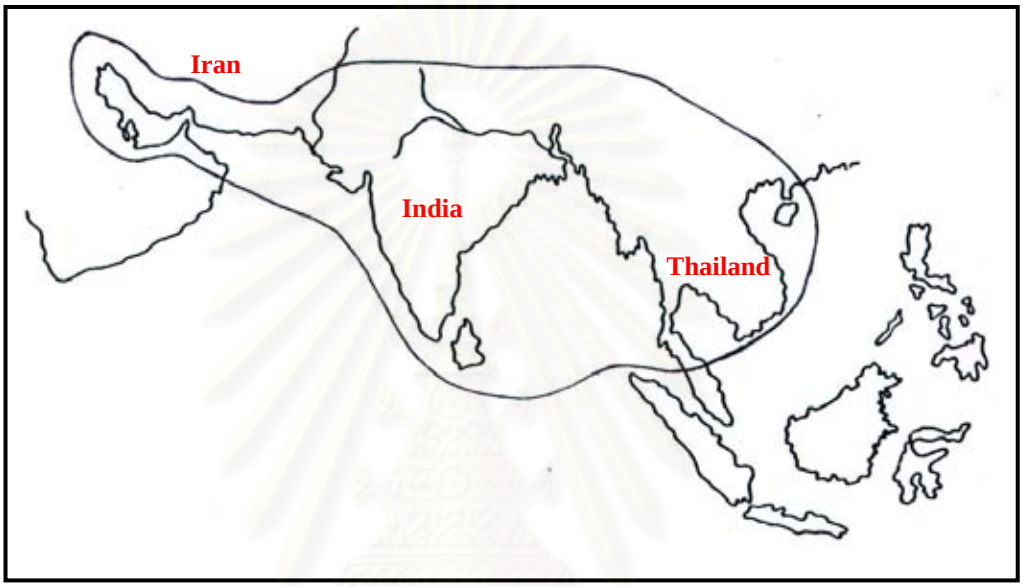

Figure 2.3 Distribution map of *A. florea*. (adapted from Ruttner, 1988; Wongsiri *et al.*, 2001)

2.2 Genetic variation in honey bees

The taxonomy of *A. florea* has been recognized as follows (Ruttner, 1986; Wongsiri, 1989)

Kingdom Animalia
Phylum Arthropoda
Class Insecta
Order Hymenoptera
Super-family Apoidea
Family Apidae
Genus *Apis*
Species *Apis florea* Fabricius, 1787

In order to quantify the genetic diversity of honey bee in genus *Apis*, different methods have been used. The first approach was characterized by morphological appearance (Ruttner, 1986, 1988; Smith, 1999). Morphometrics measurement, which deals with quantitative characters (Cornuet and Garnery, 1991), was a classical and reliable method for investigating biodiversity (Hepburn and Radloff, 1998; Ruttner, 1986, 1988; Smith, 1999; Smith and Brown, 1988). But it also has some shortcomings. For example some important characters, particularly those related to size, are environmentally influenced (Smith and Brown, 1988), and this method does not directly relate between genotypes and phenotypes. It gives only an indirect and possibly biased measure of genetic diversity (Cornuet and Garnery, 1991).

Allozyme or isozyme analysis is another approach to determine genetic diversity (Cornuet and Ganery, 1991, Sheppard and McPheron, 1993; Sylvester, 1986). Allozyme variation, detected by electrophoresis and histochemical staining, is a good way to access genetic variability in natural populations, but is not effective in honey bees. This technique can be used to determine the interspecific variation in honey bees, but have insufficient variability for detecting differentiation between subspecies (Cornuet, 1986), because honey bee and most other haplo-diploid hymenopteran are very uniform in their allozymes (Sylvester, 1986).

DNA analysis is the direct approach to determine genetic variation and relatedness among honey bee populations.

Within eukaryotic cells, DNA which found inside nucleus is nuclear DNA and the extranuclear DNA can be found in mitochondria and chloroplast are called mtDNA and chloroplast DNA, respectively (Hoy, 1994). Nuclear DNA, the combination of paternal genotypes can be used to study genetic divergence in population, and within populations structure (Cornuet and Garnery, 1991; Moritz *et al.*, 1986, Hepburn and Radloff, 1998).

Animal mitochondrial DNA (mtDNA) has proven extremely useful in population genetic and systematic studies (Hall and Smith, 1991). It has proved to be a powerful tool in discriminating various subspecies of honey bees (Cornuet and Garnery, 1991; Hall and Smith, 1991; Moritz *et al.*, 1986; Smith and Brown, 1988).

In most animal, including honey bees, mtDNA is maternally inherited without recombination (Singh *et al.*, 1995; Smith and Brown, 1988). It is a small, circular, double stranded DNA molecule, generally about 16,000 bp long. The mitochondrial genome contains 13 protein-coding genes, the genes for 22 tRNAs, 2 ribosomal RNAs and noncoding region, containing the origin of replication (Crozier and Crozier, 1993). The gene content is highly conserved (Moritz, 1994). There are many identical copies in each cell, while the nuclear genome is usually present in just two copies per cell (one from each parent in a diploid organism) (Moritz, 1986; Smith, 1991).

The complete sequence of the honey bee (*Apis mellifera ligustica*) mtDNA reported by Crozier and Crozier (1993), is 16,343 bp long. The gene order for this molecule is shown in Figure 2.4. Relative to their positions in the *Drosophila yakuba* map, 11 of 22 tRNA genes are in different positions, but the gene-coding protiens, control region and rRNAs are in the same relative position. The base composition is 84.9% A-T, whereas in *D. yakuba* is 78.6% A-T.

One of the useful properties of mtDNA, is its exclusively maternal inheritance, meaning that all offspring of a single queen will have an identical mtDNA molecule (Hepburn and Radloff, 1998), and mtDNA is not sensitive to environmental selection pressure (Avise, 1994; Franck *et al.*, 2000). These make mtDNA an excellent way to use in the analysis of phylogeographic variation and relationships among populations of bees (Avise, 1994, Franck *et al.*, 1998, 2000; Garnery *et al.*, 1991, 1993; Smith, 1991, 1999). Moreover, the high mutation rate of mtDNA is another useful feature for determination of population

structure at intraspecific level. Different portions of mitochondrial genome evolve at different rates. The non-coding region of mtDNA evolves rapidly, while genes (coding regions) evolve very slowly (Smith, 1991). Thus different regions can provide the level of genetic variation appropriate to the question of hand.

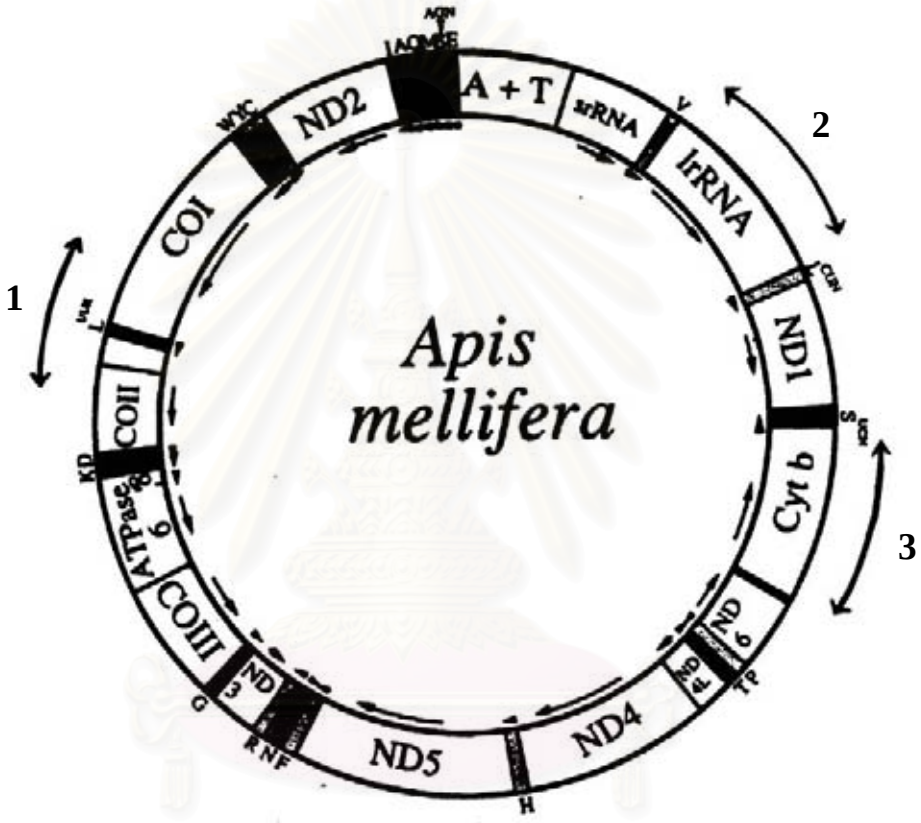

Figure 2.4 Map of circular mitochondrial genome of honey bee *A. mellifera* (Crozier and Crozier, 1993).
Numbers show the regions which were amplified in this study; 1 = inter CO I-CO II, 2 = LrRNA, and 3 = Cytb I-tRNA^{Ser}.

2.3 Mitochondrial DNA polymorphism in honey bees

Various techniques of molecular biology, such as Restriction Fragment Length Polymorphism (RFLP), Polymerase Chain Reaction (PCR), microsatellite DNA and DNA sequencing, have been used to investigate variation among and between honey bee populations (Hepburn and Radloff, 1998).

RFLPs are variations in the length of DNA fragments generated by specific restriction endonuclease. Cleavage variation is caused by mutations that create or eliminate recognition sites for the restriction enzymes (Hoy, 1994). MtDNA-RFLP is a popular technique for evaluating levels of genetic variation among and between various species. RFLP analysis provided information about the differences between sequences in mtDNA. It can be used effectively and economically, to analyze variation in populations.

The Polymerase Chain Reaction (PCR) is a powerful technique that can amplify large amounts of DNA from the small amounts of DNA template (Cornuet and Ganery, 1991). The PCR involves combining a DNA sample (the template) with oligonucleotide primers, deoxynucleotide triphosphases (dNTPs), and a DNA polymerase, usually *Taq* polymerase, in buffer. PCR reactions contain three important steps: denaturation of double stranded DNA template at high temperature, annealing to allow primers to form hybrid molecules at optimal temperature, and extension at working temperature of DNA polymerase (Hoy, 1994). PCR-amplified DNA can be used for

analysing variation in length (as in microsatellites), restriction (Hall and Smith, 1991) or sequence (Cornuet *et al.*, 1991; Garnery *et al.*, 1991).

RFLP and PCR-RFLP methods have been used to investigate interspecific, and intraspecific variation in honey bees. Sylvester and Wongsiri (1993) used nuclear DNA probes from European honey bee, *Apis mellifera*, and identified genetic variation in Asian species of *Apis* (*A. cerana, A. dorsata, A. florea* and *A. andreniformis*) and suggest that there may be interspecies differences. They found that it should be possible to obtain single probes which will simultaneously be useful for detecting variation within colonies, among populations or races and among species.

The intergenic region between the CO I and CO II genes in mtDNA of *A. mellifera* has been used to investigate the relationship among the species *A. cerana, A. florea* and *A. dorsata* (Cornuet *et al.*, 1991). They found that the length of this region differed among the species. These *Apis* species have an intergenic sequence of 89 bp in *A. cerana*, 32 bp in *A. florea*, and 24 bp in *A. dorsata*. In *A. koschevnikovi* and *A. mellifera*, the length of this region are 94 and ~ 200-900 bp respectively (Hepburn *et al.*, 2001). This sequence is free to evolve rapidly because it is non-coding protein gene. It can be provide information analysable at the intraspecific level (Hepburn *et al.*, 2001).

MtDNA polymorphism has potential to provide information on the history of Asian *Apis*. Smith (1991, 1993) determined genetic variation in 5 Asian honey bees; *A. cerana, A. koschevnikovi, A. dorsata, A. florea* and *A. andreniformis* compared with *A. mellifera*.

It was found that *Apis* mtDNA shows both restriction site polymorphisms and length polymorphims. But genomes of *Apis* species were too divergent for the construction of a phylogeny by this means: few or no fragments were shared among species. Thus, similar pairwise comparisons based on shared restriction fragments were not possible among them.

Garnery *et al.* (1991) sequenced the 5' end of mitochondrial CO II gene in *A. mellifera*, *A. cerana*, *A. dorsata*, and *A. florea*. Phylogenetic trees were built using neighbor-joining and parsimony methods. Three branches, 1) *A. dorsata*, 2) *A. florea* and 3) *A. cerana-A. mellifera* were found. The divergence almost simultaneously, *A. cerana* and *A. mellifera* were closely related and separated after the divergence of *A. dorsata* and *A. florea.*

The complete sequence nucleotide sequence of mitochondrial CO II gene of 5 species of honey bee; *A. andreniformis*, *A. cerana*, *A. florea* and *A. koschevnikovi* were determined by Willis *et al.* (1992). The results suggest that *A. dorsata* is the least derived species, followed by the branch of *A. florea* / *A. andreniformis* and *A. koschevnicovi*, and then *A. mellifera* and *A. cerana*.

Smith *et al.* (2000) sequenced a non-coding region between the $tRNA^{leu}$ and the CO II gene of mitochondrial genome of *A. cerana* and *A. nigrocincta* from Korea and the Philippines. Both neighbor-joining and parsimony analyses were carried out. They found 5 five major groups of haplotypes; 1) Asian mainland group, 2) Sundaland group, 3) Palawan group, 4) Luzon-Mindanao group, and 5) *A. nigrocincta*.

The changes in sea-level during Pleistocene glaciations is the cause of geograhpic distribution of these mtDNA haplotypes (Smith *et al.*, 2000).

Other interesting sources of phylogenetic information in mitochondrial genome are genes of large subunit (16S) ribosomal RNA (Cameron, 1993), Cytochrome b, ATPase 6, and ATPase 8 (Crozier and Crozier, 1992). These genes are in the same relative positions as in *Drosophila* mtDNA. These genes are appropriate for examining deep levels of relationship because they contain highly conserved regions. Interestingly, they have evolved at a significantly greater overall rate than those of *Drosophila* (Cameron, 1993; Crozier and Crozier, 1992).

Both interspecific and intraspecific variation of honey bee can be analysed using PCR-RFLP. Restriction mapping and restriction fragment length polymorphisms of *A. mellifera* mtDNA have been reported. Restriction endonuclease (*Bcl* I, *Bgl* II, *Eco*R I, *Hin*f I, *Nde* I and *Xba I*) were used to investigate the polymorphisms (Meixner *et al.*, 1993; Moritz *et al.*, 1986; Schiff and Sheppard, 1993; Sheppard *et al.*, 1996; Smith and Brown, 1988).

Moritz *et al.* (1986) studied on mtDNA polymorphism in *A. mellifera*. They isolated mtDNA from worker honey bee larvae, and digested mtDNA by restriction enzymes (*Bgl* II) showed a mtDNA different between *A. mellifera carnica*, *A. mellifera ligustica* and *A. mellifera caucasica.*

Ruttner (1988) classified the *A. mellifera* based on morphometric studies, recognizing four groups. There are 1) M-group, which includes *A. mellifera mellifera, A. mellifera iberica, A. mellifera intermissa,* and several other north African subspecies, 2) A-group, which includes *A. mellifera larmarckii, A. mellifera scutellata, A. mellifera capensis, A. mellifera unicolor* and other sub-Saharan subspecies, 3) C-group, which includes *A. mellifera carnica, A. mellifera ligustica*, and 4) O-group, which includes *A. mellifera caucasica* and subspecies of the Middle East. But the difference between M and O groups is not cleared.

The intergenic CO I-CO II region has been shown to be useful for the biogeographic classification of *A. mellifera* species (Cornuet *et al.*, 1991; Moritz *et al.*, 1994). Moritz *et al.* (1994) amplified a region between the CO I and CO II genes in samples of 102 colonies of *A. mellifera* covering the south of the 27th parallel of latitude in Africa. A total of nine different mitotypes were detected from size and restriction site of *Dra* I digestion in this mtDNA region.

Cornuet and Garnery (1991) divided the mtDNA variability of *A. mellifera* into 3 major lineages. 1) African colonies (lineage A) including *A. mellifera intermissa, A. mellifera adansonii, A. mellifera scutellata, A. mellifera capensis* and *A. mellifera monticola* subspecies, 2) *mellifera* colonies (lineage M); *A. mellifera ligustica, A. mellifera carnica* and 3) *caucasica* colonies (lineage C).

Franck *et al.* (2000) used mtDNA and microsatellite to confirm the existence of lineage O of *A. mellifera* from Lebanon (subspecies *syriaca*) compared with other lineages (lineage A,C and M). They found that the genetic differentiation at mtDNA and microsatellite loci between Lebanese bees and populations from other branches also support the hypothesis of branch O.

The variation in 12 subspecies of *A. mellifera* ; *A. mellifera mellifera, A. mellifera iberica, A. mellifera sicula, A. mellifera intermissa, A. mellifera scutellata, A. mellifera monticola, A. mellifera adansonii, A. mellifera caucasica, A. mellifera ligustica, A. mellifera meda, A. mellifera carnica, A. mellifera anatoliaca*, were determined by amplified the intergenic CO I-CO II region using PCR technique and followed by restriction with *Dra* I. Twenty one different haplotypes have been found. This approach is a useful way to study on the genetic structure and the evolution of the large fraction of the species (Garnery *et al.*, 1993).

Meixner, Sheppard and Poklukar (1993) used a set of restriction enzymes; *Acc* I, *Bcl* I, *Bgl* II, *Eco*R I, and *Xba* I to determine the variation in mtDNA of *A. mellifera carnica* and *A. mellifera ligustica*. They found only *Xba* I revealed a polymorphism with an asymmetrical distribution between the subspecies.

These techniques have also been used to distinguish the relationship of European and African honey bees. Studying the influence of "killer bee" or Africanized bees in widely separated regions such as the U.S.A., the Yucatan peninsula and Argentina, this technique can be

identified their populations (Hall and Muralidharn, 1989; Hall and Smith, 1991; Sheppard *et al.*, 1991; Smith *et al.*, 1989).

Genetic variation of *A. cerana* in Thailand was studied by Pramual (1994), Bugharuang (1996), Deowanish (1997), Sihanuntavong (1997), and Laoaroon (1998) using various molecular techniques.

Pramual (1994) showed restriction polymorphisms after digesting mtDNA with *Eco*R I. Later Bugharuang (1996) amplified intergenic CO I-CO II region and determined the variations by nucleotide sequence. Laoaroon (1998) used microsatellite variation to investigate the intraspecific variation within *A. cerana*. PCR-RFLP technique was used by Deowanish (1997) and Sihanuntavong (1997). They used restriction endonuclease; *Bcl* I, *Bgl* II, *Eco*R I, *Hin*d III, *Dra* I, *Hin*f I, *Hae* III, *Nde* I, *Spe* I, *Taq* I, and *Rsa* I to determine the polymorphism in the inter CO I-CO II region, sRNA gene, L-rRNA gene, and Cytb I-tRNA^{Ser} among *A. cerana* populations. They separated the populations of *A. cerana* into three groups; 1) northern to central Thailand group, 2) southern Thailand, and 3) Samui Island group.

MtDNA variation in *A. florea* in Thailand was studied by Smith (1991). She compared *A. andreniformis* collected from Malaysia with Thailand (3 colonies) and India (2 colonies), and found polymorphism between species, but no differences within each species. However Smith had only 5 colonies of *A. florea* . Much more sampling is needed for a study of the biogeography of the dwarf honey bees.

A. florea is an important insect pollinator in the ecosytem (Free, 1981; Ruttner, 1986). But there have been a limited number of publications concerning the biogeography and genetic variation of this species which are necessary and useful for understanding its evolution and basic biology.

CHAPTER III

MATERIALS AND METHODS

3.1 Instruments

- Autoclave Ken Clave max-26DX (Ikiken Co., Ltd., Japan)
- Automatic micropipette P10, P20, P100, P200 and P1000 (Gilson-Medical Electronics S.A., France)
- Balance 180A (Precisa Balances, Switzerland)
- Boekel dry bath incubator (Boekel Scientific, U.S.A.)
- Camera Nikon FM2 (Nikon Co., Japan)
- -20^0C Freezer WCF (Whirlpool Ltd., Thailand)
- Horizontal gel electrophoresis apparatus
  : MGU-402T (C.B.S. Scientific company, Inc., U.S.A.)
  : Taitec Pico-2 (Taitec Co.,Japan)
- Incubator BM 400 (Memmert GambH, Germany)
- Magnetic stirrer PC-320 (Corning, Inc., U.S.A.)
- Microcentrifuge Kubota 1120 (Kubota Co., Japan)
- Personal microcentrifuge C9314-3 (National Labnet Co., U.S.A.)
- Polaroid DS-34 camera (Polaroid Co., U.S.A.)
- Shaker VRN-360 (Gemmy Industrial Co., Taiwan)
- Thermal cycler : The MiniCycler Model PTC-150 (M.J. Research, Inc., U.S.A.)
- Utraviolet Transilluminator model TVC-312R/F (Spectronics Corporation, U.S.A.)

- Vortex Genie-2 Model G560E (Scientific Industries, Inc., U.S.A.)
- Water bath model SH28L (Polyscience, U.S.A.)

3.2 Inventory supplies

- Black and white paint film TriX-pan400 (Eastman Kodak Company, U.S.A.)
- Filter paper whatman 3 MM (Whatman Internation Ltd., England)
- Microcentrifuge tubes 0.5, 1.5 ml (Treff lab, Switzerland)
- Pipette tips (Treff Lab, Switzerland)
- Whatman laboratory sealing film (Whatmat Internation Ltd., England)

3.3 Chemicals

- Absolute ethanol (Merck, Germany)
- Agarose, Seakem LE (FMC Bio products, U.S.A.)
- Boric acid (Merck, Germany)
- Bromophenol blue (Merck, Germany)
- Chloroform (Merck, Germany)
- Ethylene diaminetetraacetic acid disodium salt dihydrate (Fluka, Switzerland)
- Ethidium bromide (Fluka, Switzerland)
- GeneAmp PCR core reagent (GibcoBRL, U.S.A.)
  - : *Taq* DNA polymerase
  - : 10X PCR buffer
  - : 50 mM $MgCl_2$ solution

- 100 mM dNTPs : dATP, dCTP, dGTP and dTTP (Boehringer Mannheim, Germany)
- Isoamyl alcohol (Merck, Germany)
- Phenol saturated solution (Amresco, U.S.A.)
- Sodium acetate (Merck, Germany)
- Sodium chloride (Merck, Germany)
- Sodium dodecyl sulfate (SDS) (Sigma, U.S.A.)
- Tris-(hydroxy methyl)-aminomethane (Fluka, Switzerland)
- Xylene cyanol (Sigma, switzerland)

3.4 Oligonucleotide primers

- Bio Service Unit, Thailand
- Biosynthesis Inc., U.S.A.

3.5 Enzymes

- Proteinase K (GibcoBRL Life Technologies Inc., U.S.A.)
- Restriction enzymes (New England Biolabs Inc., U.S.A.)
- *Taq* DNA polymerase (GibcoBRL Life Technologies Inc., U.S.A.)

3.6 Sample collections

Adult *Apis florea* workers were collected from natural colonies or from flowers in different locations. From a colony, 10-20 bees were collected; 180 samples of *A. florea* were from 37 provinces in various parts of Thailand including Samui Island and Pa-Ngan Island. Details of the samples collections are shown in Figure 3.1 and Appendix 2.

Samples were preserved in 95% ethanol and then stored at 4^0C until DNA extraction.

3.7 DNA extraction

Total DNA was extracted from individual thoraxes of adult workers, generally following the procedure of Hall and Smith (1991). Samples were homogenized and placed in a 1.5 ml microcentrifuge tube containing 500 μl of STE buffer (100 mM NaCl, 50 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0). The cells were lysed by adding 20% SDS, after which 10 mg/ml proteinase K solution was added to a final concentration of 450 μg/ml and the homogenate incubated at 60^0C for 2 hours. The suspension was then centrifuged at 8,000 rpm for 10 minutes. After centrifugation, the supernatant was removed to new tube and extracted twice with equal volume of phenol : chloroform : isoamyl alcohol (25:24:1), mixed gently for 15 minutes and spun at 8,000 rpm for 10 minutes at room temperature. The aqueous phase (upper layer) was transfered to a new tube and extracted once with an equal volume of chloroform : isoamyl alcohol (24:1). Subsequently, 3 M sodium acetate were added to a final concentration of 0.3 M. DNA was precipitated with double volume of cold absolute ethanol, and overnight incubation at -20^0C. DNA was pelleted by centrifugation at 12,000 rpm for 10 minutes. The pellet was briefly washed with 70 % ethanol, air-dried and resuspended in TE buffer (10 Tris-HCl pH 8.0 and 1 mM EDTA pH 8.0) and kept at 4^0C for the next step.

3.8 PCR amplification

Three different regions of *A. florea* mtDNA were amplified by PCR, using primers which has been designed from the *A. mellifera* sequence. Amplifications were carried out in a 25 μl volume and 0.024 U/μl (0.6 U/ reaction) of *Taq* DNA polymerase (Gibco) were provided. Primer sequences, PCR conditions and appropriate concentrations of each reagent are shown in Table 1.

3.9 Agarose gel electrophoresis

Agarose gel electrophoresis was performed in order to separate DNA fragments on the basis of their moecular sizes, 0.7 % agarose gel was used to check the result of DNA extraction and 1.5 % agarose gel was used to check the result of PCR amplification. The appropriate amount of agarose (Seakem LE or MetaPhor) was weighed out and mixed with TBE buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA pH 8.0) and heated until complete solubilisation in a microwave oven, then poured into an electrophoretic mould. After the gel was completely set, 3 μl of the loading dye (0.25 % bromphenol blue, 40 % ficoll 400 and 0.25 % xylene cyanol) was mixed with 3 μl of each DNA sample before loaded into each well. Digested products were electrophoresed at 110V for 1.5 hours or until bromphenol blue (lowest dye) migrated to approximately 2 cm from the bottom of the gel. The gel was stained with a 2.5 μg/ml ethidium bromide solution for 5 minutes and destained in distilled water for 30 minutes. DNA was visualized under UV light and photographed. λ DNA-*Hin*d III digested marker, 1 kb DNA

ladder and 100 bp DNA ladder were used as the standard molecular size.

3.10 Restriction enzyme digestion

PCR products of CO I-CO II, L-rRNA and Cytb I-tRNA^{Ser} were singly digested with 12 restriction enzymes (*Acl* I, *Afl* I, *Ase* I, *Bam*H I, *Bcl* I, *Dra* I, *Eco*R I, *Hin*d III, *Hin*f I, *Rsa* I, *Ssp* I, and *Swa* I) according to the manufacturer's instructions.

Digestion was carried out in a 10 μl volume containing approximately 50 ng of DNA, appropriate concentration of restriction enzymes, 1 μl of 10X reaction enzyme buffer and appropriate amount of sterile deionised water. The reaction mixture was incubated at 37^0C for 5-6 hours or overnight.

The restriction fragments were separated on 2% agarose gel at 110V for about 2 hours, stained with ethidium bromide and detected on UV light.

Figure 3.1 Sampling area of *A. florea* in Thailand.

Table 1. PCR conditions of each region.

MtDNA regions	Primers	References	Primer conc. (μM)	$MgCl_2$ conc. (mM)	Running program	No. of cycles
Inter CO I-CO II	Primer CO I : 5'-TTGATTTTTTGGTCATCCAGAAGT-3' Primer CO II : 5'-CCACAAATTTCTGAACATTGACC–3'	Roehrdanz, R.L. (1993)	0.12	3	92^0C 1 min 55^0C 1 min 72^0C 2 min extend 72^0C10 min	35
L-rRNA	Primer 1 : 5'-TTTTGTACCTTTTGTATCAGGGTT–3' Primer 2 : 5'-CTATAGGGTCTTATCGTCCC–3'	Hall & Smith (1991)	0.12	4	92^0C 1 min 55^0C 1 min 72^0C 2 min extend 72^0C10 min	35
Cytb I-$tRNA^{Ser}$	Primer 1 : 5'–TATGTACTACCATGAGGACAAATATC-3' Primer 2 : 5'-GAAAATTTTATTTCTATATTAT(AorG)--TTTTCA-3'	Oldroyd, B.P. (cited in Deowanish, 1997)	0.12	4	92^0C 1 min 55^0C 1 min 72^0C 2 min extend 72^0C10 min	35

Table 2. List of restriction enzymes used for analyse mtDNA variation of *A. florea*

Restriction enzymes	Recognition site	Restriction enzymes	Recognition site
Acl I	AA / CGTT TTGC / AA	*Eco*R I	G / AATTC CTTAA / G
Afl II	C / TTAAG GAATT / C	*Hin*d III	A / AGCTT TTCGA / A
Ase I	AT / TAAT TAAT / TA	*Hin*f I	G / ANTC CTNA / G
*Bam*H I	G / GATCC CCTAG / G	*Rsa* I	GT / AC CA / TG
Bcl I	T / GATCA ACTAG / T	*Ssp* I	AAT / AAT TTA / TTA
Dra I	TTT / AAA AAA / TTT	*Swa* I	ATTT / AAAT TAAA / TTTA

3.11 Data analysis

The data of restriction fragments were organized into 1-0 matrix (Appendix 3); 1 indicate the presence of fragment, and 0 indicate the absence. The genetic distance was estimated using Euclidean distance. The statistical analysis was carried out in NTSYS pc 2.01 D (Rohlf, 1997) and the phylogenetic reconstruction using UPGMA (unweighted pair group method with arithmetic mean).

CHAPTER IV

RESULTS

4.1 Total DNA extraction

Total DNA was extracted from the thorax of each worker. The extracted DNA was dissolved in 30 μl of TE buffer. High molecular weight DNA of *A. florea* is larger than 23.1 kb in length, slightly sheared DNA were observed (Figure 4.1). The amount of DNA extracted was estimated by agarose gel electrophoresis compared with the λ/*Hin*d III standard DNA marker. Usually, about 25 ng/μl was obtained per individual specimen. That result was suitable for subsequent experiments.

4.2 PCR amplification and optimization of PCR conditions

PCR technique was used to amplify the amount of DNA. The PCR is a technique for the *in vitro* amplification of specific DNA sequences by the simultaneous primer extension of complimentary strands of DNA. Many amounts of copies can be amplified from a few amount of DNA template. The mitochondrial analysis in this study was based on the inter CO I-CO II region, L-rRNA gene and Cytb I-tRNA^{Ser} gene.

Optimization of the inter CO I-CO II region was attempted by varying $MgCl_2$ concentration ranging from 0-5.0 mM at a constant concentration of 0.12 μM primers. PCR-amplified DNA was firstly

appeared at 2.0 mM $MgCl_2$. Non specific band was not found. Primer dimer was not observed. The increasing of $MgCl_2$ concentration to be higher than 3.0 mM did not yield significantly higher amount of amplified product, therefore an optimal $MgCl_2$ concentration was chosen at this concentration. The primer concentration was used at 0.12 μM, because it was originally developed from closely related species (*A. cerana*) (Sihanuntavong, 1997).

For amplification of L-rRNA gene, 0-5.0 mM $MgCl_2$ concentration was optimized at a concentration of 0.12 μM primer. The amplified product was observed at 1.0 mM $MgCl_2$ and increased with higher $MgCl_2$ concentration until 3.0 mM. Non specific product was found below PCR product but its amount was not high enough to interfere an interpretation of the restriction enzyme digestion results. Thus 3.0 mM $MgCl_2$ concentration was selected to use for amplification of L-rRNA gene in this study.

The optimal amplification conditions of Cytb I-$tRNA^{Ser}$ gene, 0-5.0 mM $MgCl_2$ concentration was determined at 0.12 μM primer. The PCR product was first observed at 2.0 mM $MgCl_2$ concentration. Non specific product was not observed. The optimal concentration of $MgCl_2$ for this gene was 4.0 mM.

The approximated sizes of PCR products of inter CO I-CO II region, L-rRNA gene, and Cytb I-$tRNA^{Ser}$ are shown in Table 3, Figure 4.2, 4.3 and 4.4. No difference in length of PCR products was found in all regions.

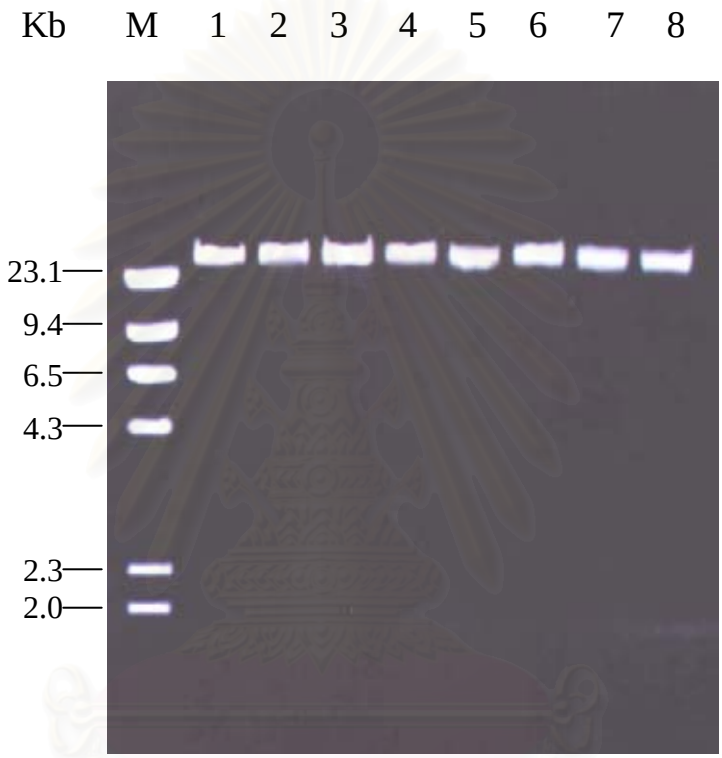

Figure 4.1 High molecular weight DNA extracted from thorax of *A. florea*.

Lane M : λ/*Hin*d III standard DNA marker

Lane 1-8 : Total DNA from eight *A. florea* individuals

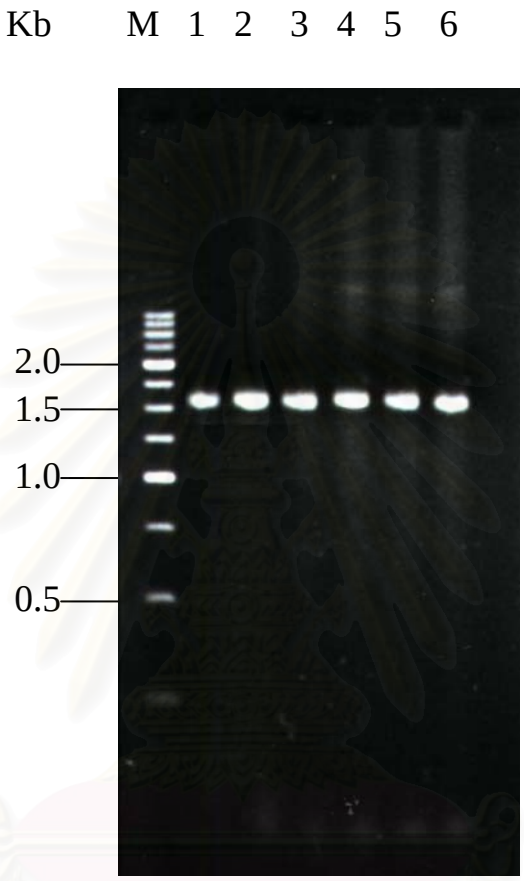

Figure 4.2 PCR amplified products of inter CO I-CO II region of mtDNA of *A.florea*.

Lane M : 250 bp DNA ladder

Lane 1-6 : PCR amplified products from 6 *A. florea* individuals

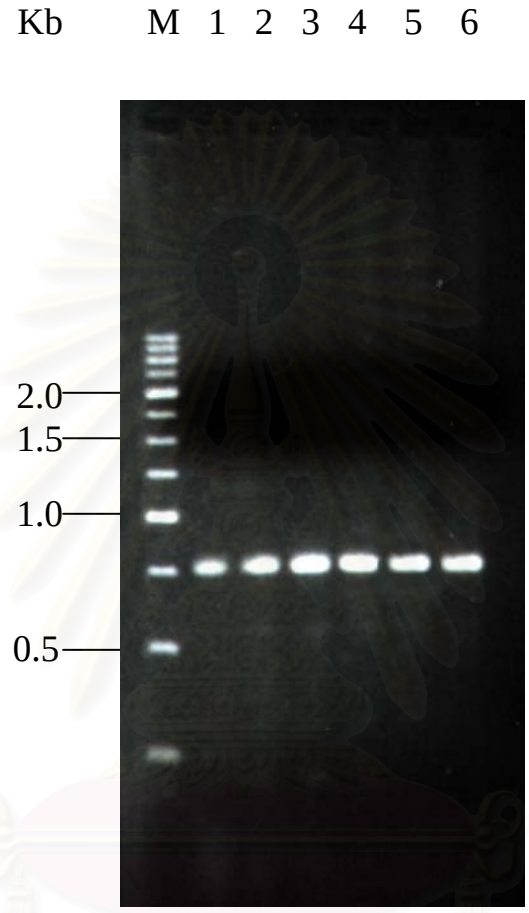

Figure 4.3 PCR amplified products of L-rRNA gene of mtDNA of *A.florea*.

Lane M : 250 bp DNA ladder

Lane 1-6 : PCR amplified products from 6 *A. florea* individuals

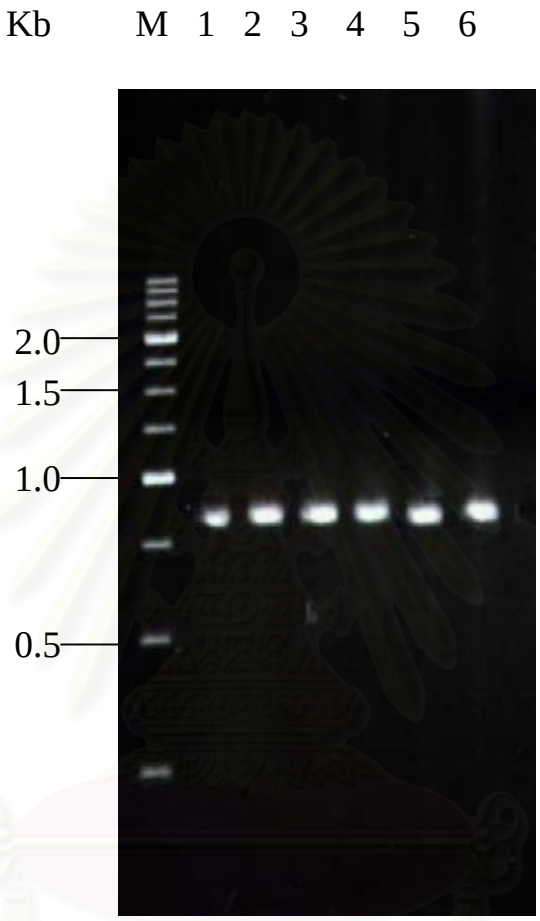

Figure 4.4 PCR amplified products of Cytb I-tRNA^{Ser} gene of mtDNA of *A.florea*.

Lane M : 250 bp DNA ladder

Lane 1-6 : PCR amplified products from 6 *A. florea* individuals

Table 3 Approximated size of PCR products of *A. florea* from this study

MtDNA regions	Size (bp)
Inter CO I-CO II	1590
L-rRNA	760
Cytb I- $tRNA^{Ser}$	870

4.3 The PCR-RFLP in inter CO I-CO II region of mtDNA of *A. florea*

PCR product of inter CO I-CO II region of mtDNA was preliminary investigated with 12 restriction enzymes; *Acl* I, *Afl* I, *Ase* I, *Bam*H I, *Bcl* I, *Dra* I, *Eco*R I, *Hin*d III, *Hin*f I, *Rsa* I, *Ssp* I, and *Swa* I. Only 6 restriction enzymes; *Ase* I, *Bcl* I, *Dra* I, *Hin*d III, *Hin*f I, and *Ssp* I could be digested the PCR products.

The approximately 50 μg of PCR products of *A. florea* were digested with 8 units of *Ase* I, 10 units of *Bcl* I, 10 units of *Dra* I, 10 units of *Hind* III, 10 units of *Hin*f I, and 8 units of *Ssp* I. The incubation time was 12 hours.

The number of restriction fragments and recognition patterns of PCR products after digested with each restriction enzymes are shown in Table 4.

The restriction fragments from *Bcl* I digestion (Figure 4.6) presented 3 bands which are 980, 320, and 290 bp. All samples of *A. florea* in Thailand have the same pattern.

The *Dra* I digestion showed 5 fragments; 790, 420, 215, 100 and 65 bp (Figure 4.7). No variation among populations was detected.

*Hin*d III fragments (Figure 4.8) showed 2 bands; 900 and 690 bp. The digestion of *Hin*d III could not be investigated the intraspecific variation among all samples.

The 4 restriction fragments (640, 380, 340 and 230 bp) were detected after cut with *Hin*f I (Figure 4.9). Only one pattern was found.

Figure 4.10 showed the number and pattern of restriction fragments when digested with *Ssp* I. A pattern of 7 bands; 425, 325, 280, 230, 180, 80 and 70 bp were presented among populations.

Ase I is only one restriction enzyme which showed polymorphism (Figure 4.5). Two patterns from the total 11 bands were found. One colony from Prachub Kiri Khan (lower part of southern Thailand) which has different pattern from the others.

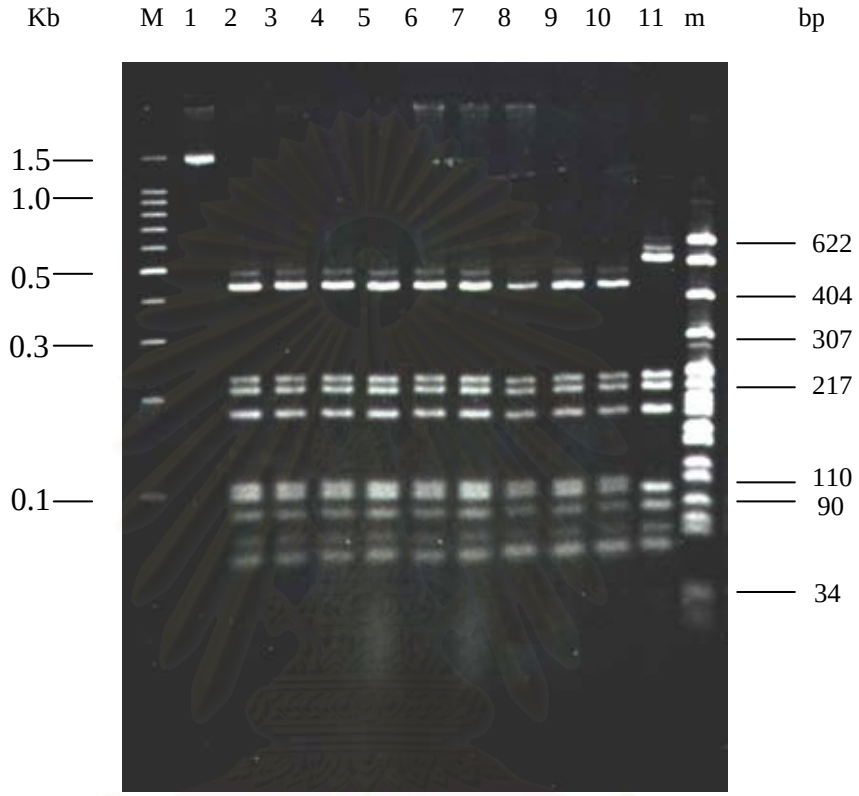

Figure 4.5 Two different restriction patterns in inter CO I-CO II region of mtDNA of *A. florea* after digested with *Ase* I.

Lane M : 100 bp DNA ladder

Lane m : pBR322/*Msp* I marker

Lane 1 : Undigested PCR product

Lane 2-10 : Haplotype A

Lane 11 : Haplotype B (sample of *A. florea* from a colony in Prachub Kiri Khan)

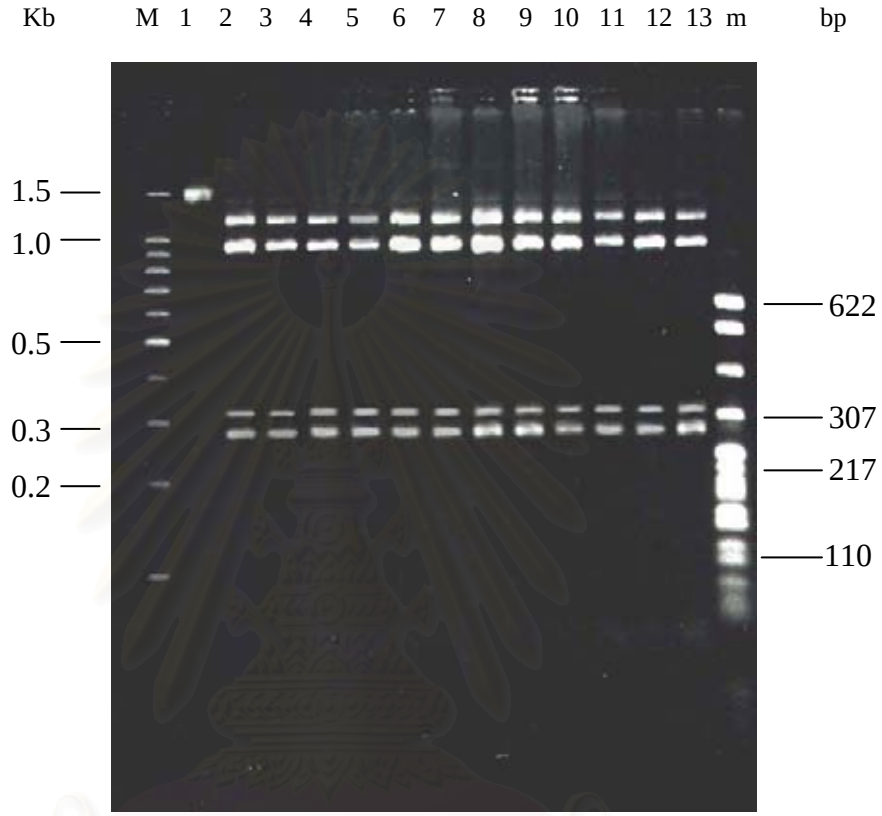

Figure 4.6 Restriction pattern in inter CO I-CO II region of mtDNA of *A. florea* after digested with *Bcl* I.

Lane M : 100 bp DNA ladder

Lane m : pBR322/*Msp* I marker

Lane 1 : Undigested PCR product

Lane 2-13 : Samples of *A. florea* after digested with *Bcl* I

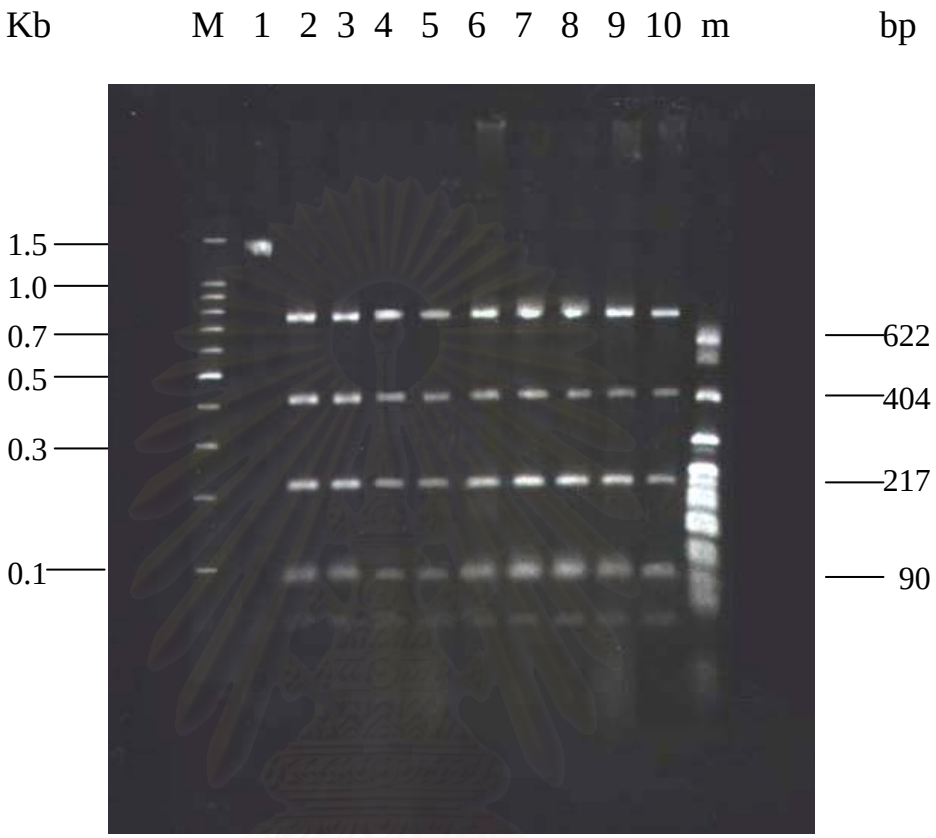

Figure 4.7 Restriction pattern in inter CO I-CO II region of mtDNA of *A. florea* after digested with *Dra* I.

Lane M : 100 bp DNA ladder

Lane m : pBR322/*Msp* I marker

Lane 1 : Undigested PCR product

Lane 2-10 : Samples of *A. florea* after digested with *Dra* I

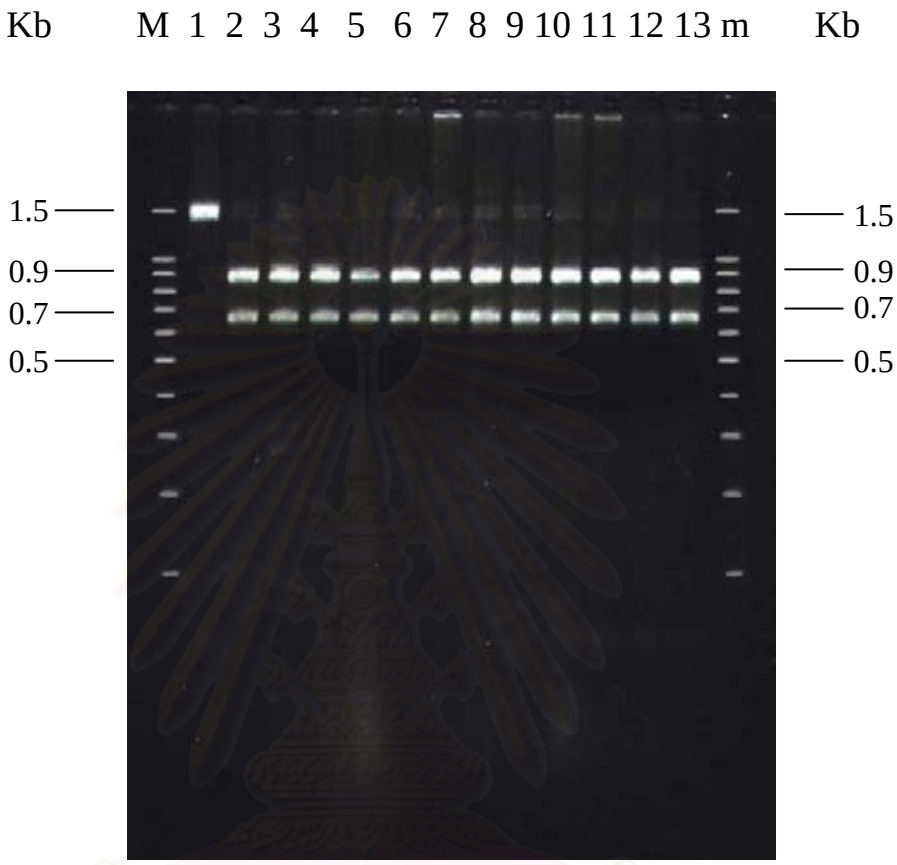

Figure 4.8 Restriction pattern in inter CO I-CO II region of mtDNA of *A. florea* after digested with *Hin*d III.

Lane M : 100 bp DNA ladder

Lane m : pBR322/*Msp* I marker

Lane 1 : Undigested PCR product

Lane 2-13 : Samples of *A. florea* after digested with *Hin*d III

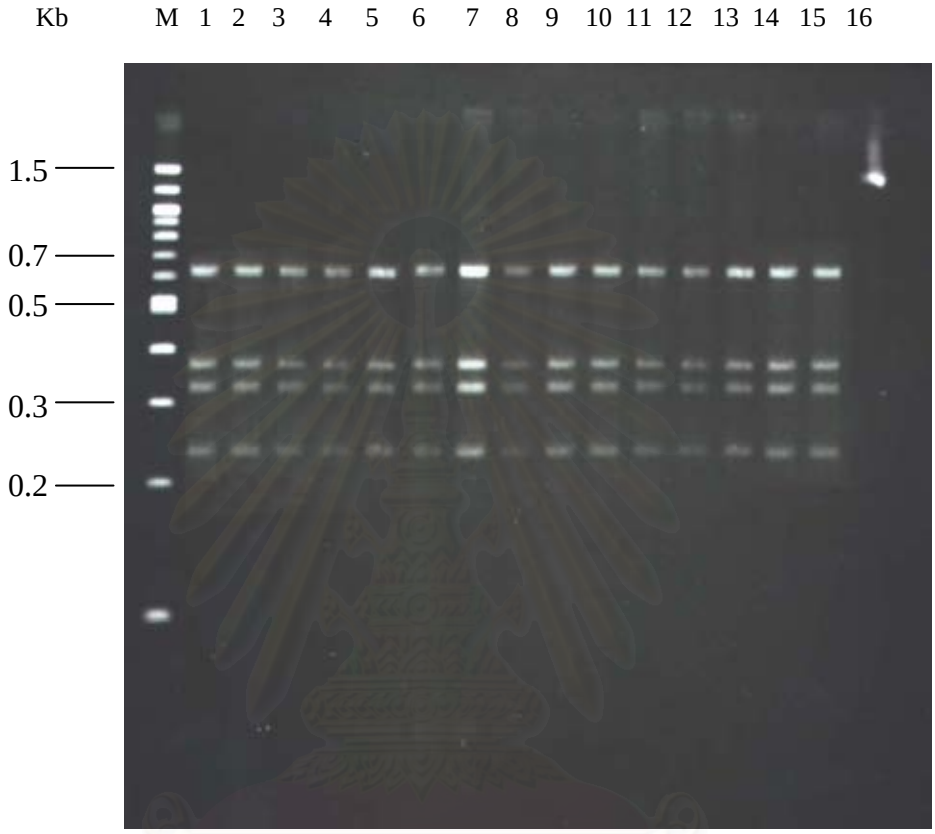

Figure 4.9 Restriction pattern in inter CO I-CO II region of mtDNA of *A. florea* after digested with *Hin*f I.
Lane M : 100 bp DNA ladder
Lane 1-15 : Samples of *A. florea* after digested with *Hin*f I
Lane 16 : Undigested PCR product

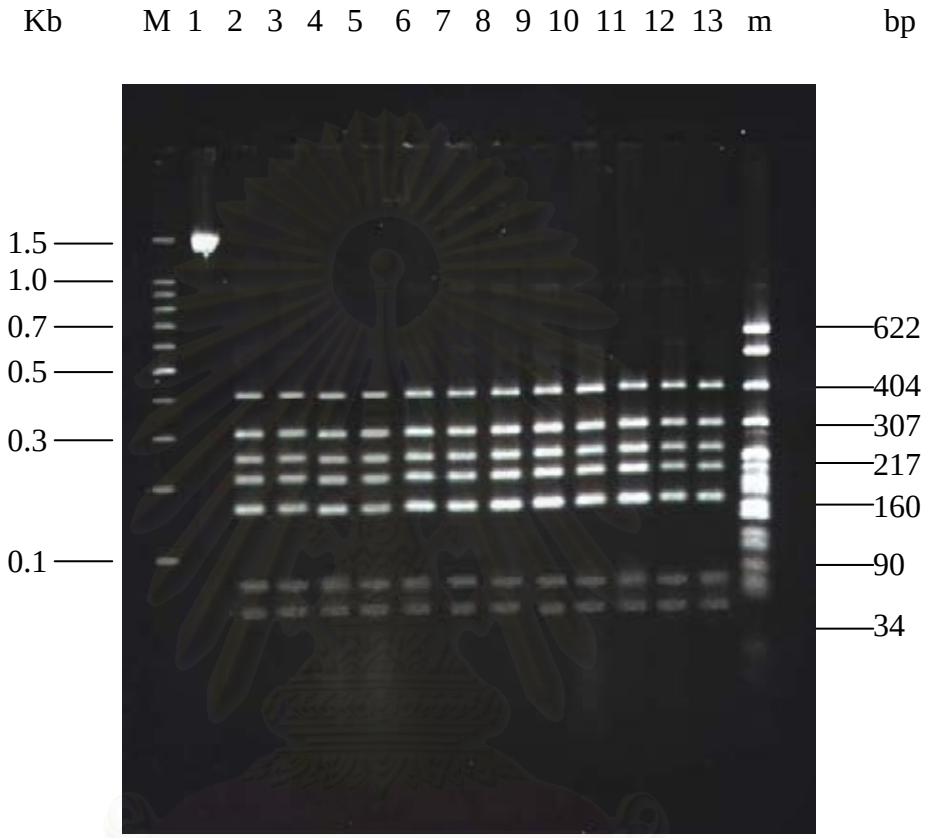

Figure 4.10 Restriction pattern in inter CO I-CO II region of mtDNA of *A. florea* after digested with *Ssp* I.

Lane M : 100 bp DNA ladder

Lane m : pBR322/*Msp* I marker

Lane 1 : Undigested PCR product

Lane 2-13 : Samples of *A. florea* after digested with *Ssp* I

4.4 The PCR-RFLP in L-rRNA gene of mtDNA of *A. florea*

Twelve restriction enzymes; *Acl* I, *Afl* I, *Ase* I, *Bam*H I, *Bcl* I, *Dra* I, *Eco*R I, *Hin*d III, *Hin*f I, *Rsa* I, *Ssp* I, and *Swa* I were used for preliminary investigation. However, *Ase* I, *Dra* I, and *Ssp* I were selected to digest PCR products of *A. florea*.

The size and number of restriction fragments after digested with 8 units of *Ase* I, 10 units of *Dra* I and 8 units of *Ssp* I are shown in Table 4.

The pattern and number of restriction fragments after treated with *Ase* I is shown in Figure 4.11. Three bands (360, 300 and 100 bp) were detected. No intraspecific variation was found.

The fragments; 300, 120, 100, 80, 60, 60 and 40 bp were found when analyzed with *Dra* I (Figure 4.12). The variation among the samples could not be investigated.

Ssp I restriction fragments (Figure 4.13) showed a pattern of 2 bands; 460 and 300 bp among the populations.

4.5 The PCR-RFLP in CytbI-tRNA^{Ser} gene of mtDNA of *A. florea*

A set of 12 restriction enzymes were used to determine the variation in *A. florea* population in Thailand. There were *Acl* I, *Afl* I, *Ase* I, *Bam*H I, *Bcl* I, *Dra* I, *Eco*R I, *Hin*d III, *Hin*f I, *Rsa* I, *Ssp* I, and *Swa* I, but only 3 restriction enzymes; *Ase* I, *Dra* I and *Ssp* I could be digested the PCR products of this gene.

The digestion of *Ase* I, *Dra* I and *Ssp* I showed 5 bands (225, 225, 210, 130 and 80 bp), 3 bands (400, 270 and 200 bp) and 3 bands (590, 190 and 90 bp), respectively.

Figure 4.14, 4.15 and 4.16 showed the restriction pattern, the size and number of restriction fragments when cut the PCR product with *Ase* I, *Dra* I, and *Ssp* I , respectively. The details of the number and size of restriction fragments are shown in Table 4.

The variation could not be detected from the results of *Ase* I, *Dra* I, and *Ssp* I digestion.

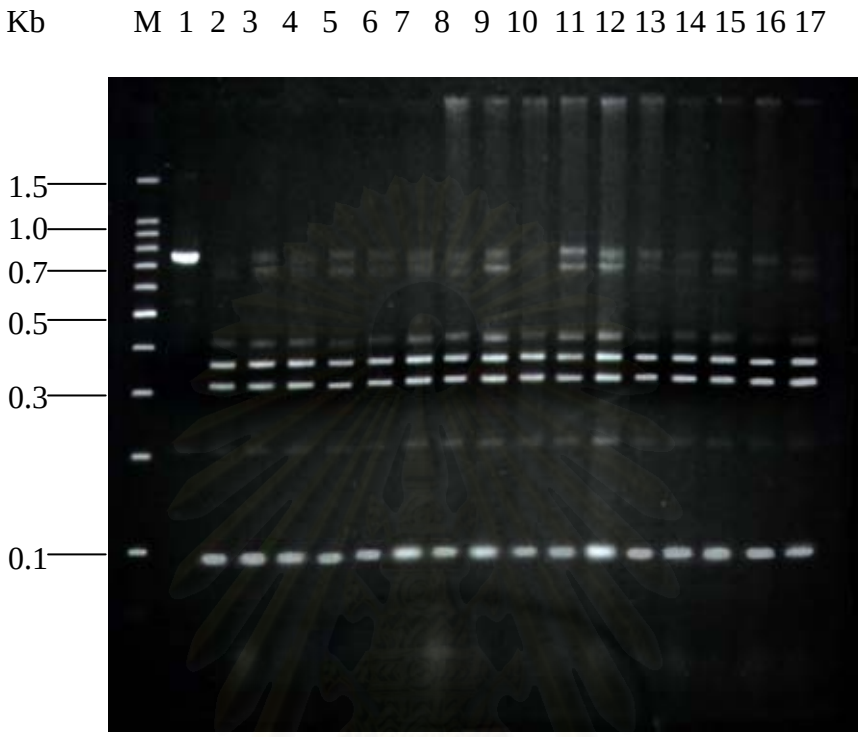

Figure 4.11 Restriction pattern in L-rRNA gene of mtDNA of *A. florea* after digested with *Ase* I.

Lane M : 100 bp DNA ladder

Lane 1 : Undigested PCR product

Lane 2-17 : Samples of *A. florea* after digested with *Ase* I

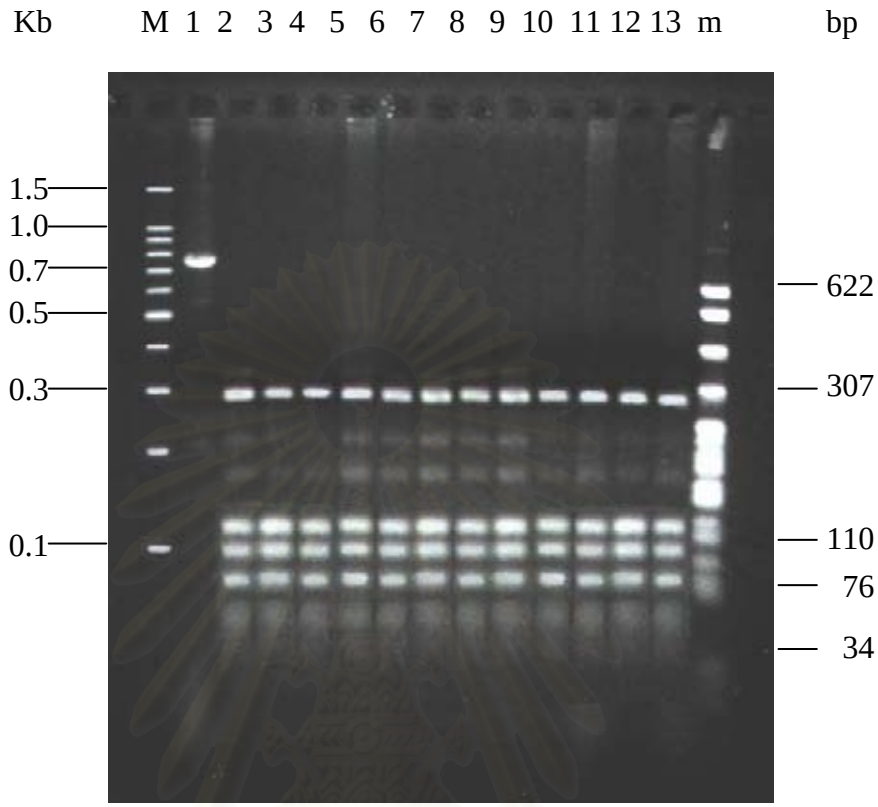

Figure 4.12 Restriction pattern in L-rRNA gene of mtDNA of *A. florea* after digested with *Dra* I.

Lane M : 100 bp DNA ladder

Lane m : pBR322/*Msp* I marker

Lane 1 : Undigested PCR product

Lane 2-13 : Samples of *A. florea* after digested with *Dra* I

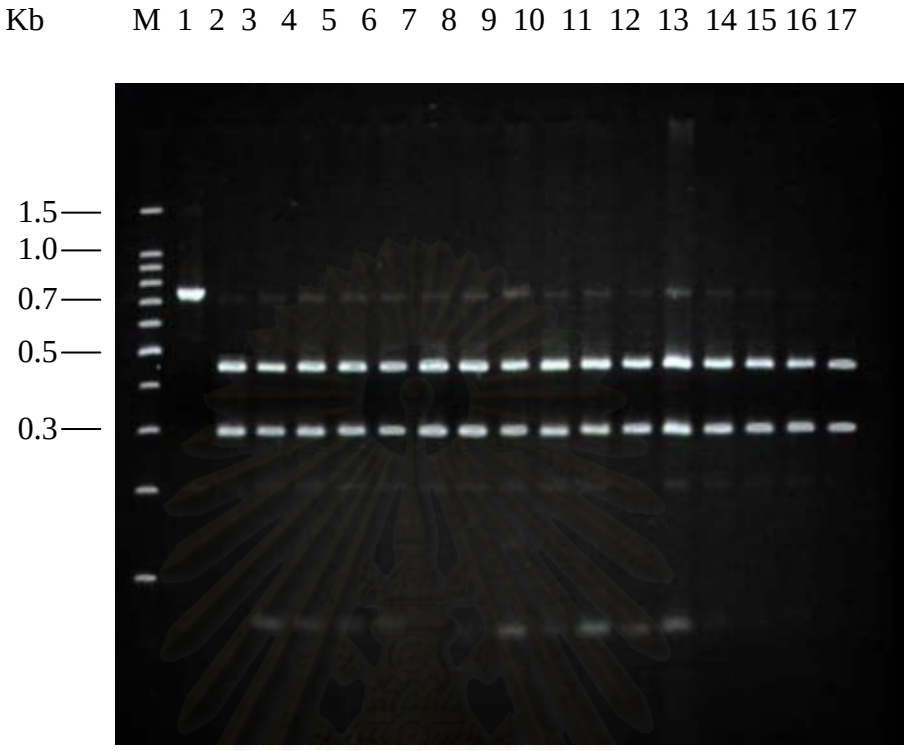

Figure 4.13 Restriction pattern in L-rRNA gene of mtDNA of *A. florea* after digested with *Ssp* I.

Lane M : 100 bp DNA ladder

Lane 1 : Undigested PCR product

Lane 2-17 : Samples of *A. florea* after digested with *Ssp* I

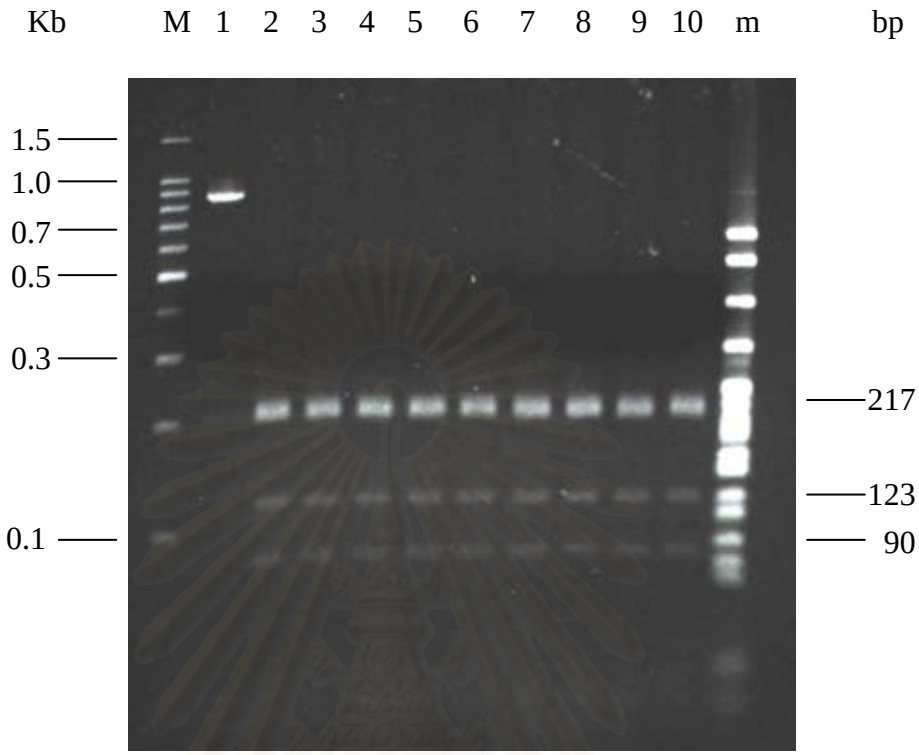

Figure 4.14 Restriction pattern in Cytb I-tRNA^{Ser} gene of mtDNA of *A. florea* after digested with *Ase* I.

Lane M : 100 bp DNA ladder

Lane m : pBR322/*Msp* I marker

Lane 1 : Undigested PCR product

Lane 2-10 : Samples of *A. florea* after digested with *Ase* I

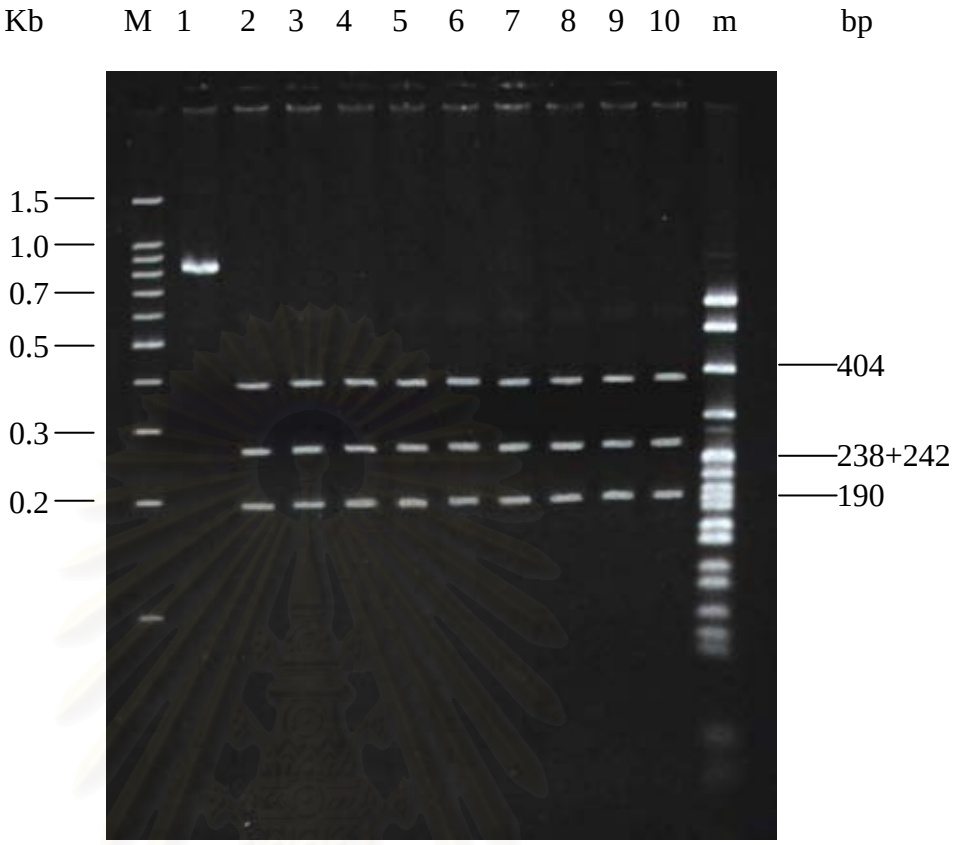

Figure 4.15 Restriction pattern in Cytb I-tRNA^{Ser} gene of mtDNA of *A. florea* after digested with *Dra* I.

Lane M : 100 bp DNA ladder

Lane m : pBR322/*Msp* I marker

Lane 1 : Undigested PCR product

Lane 2-10 : Samples of *A. florea* after digested with *Dra* I

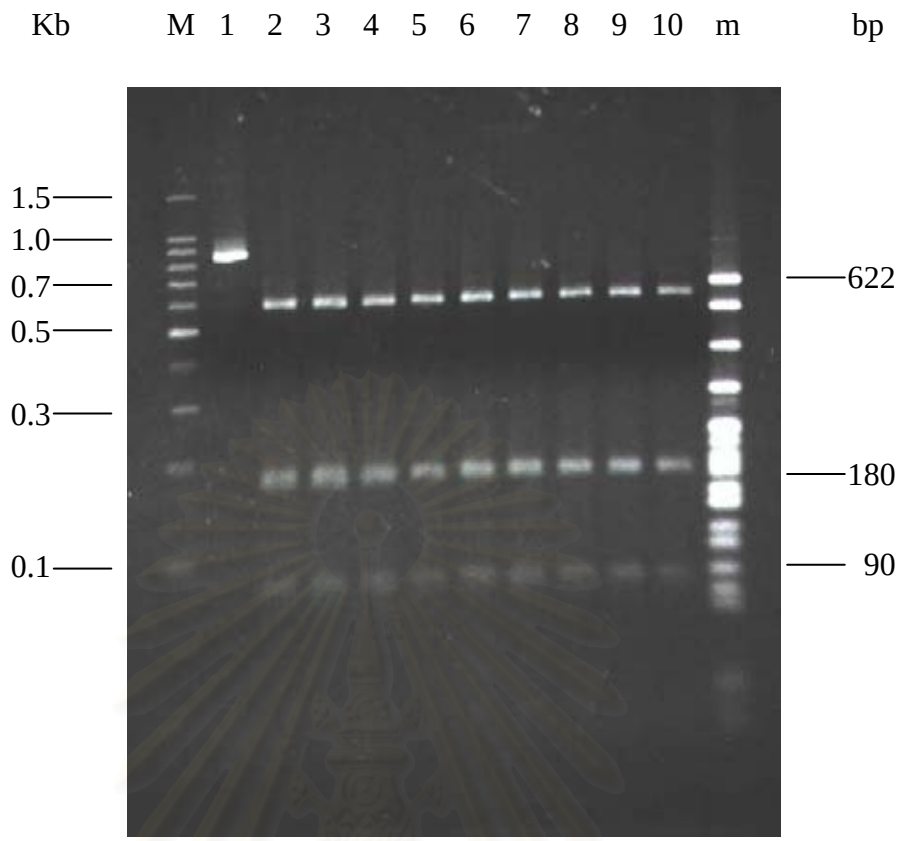

Figure 4.16 Restriction pattern in Cytb I-tRNA^{Ser} gene of mtDNA of *A. florea* after digested with *Ssp* I.

Lane M : 100 bp DNA ladder

Lane m : pBR322/*Msp* I marker

Lane 1 : Undigested PCR product

Lane 2-10 : Samples of *A. florea* after digested with *Ssp* I

Table 4 Numbers and approximated size of restriction fragments and number of patterns recognized by PCR-RFLP analysis on inter COI-COII region, L-rRNA and CytbI-tRNA^{Ser} of *A. florea* in Thailand.

MtDNA gene	Restriction enzymes	No. of bands	No. of patterns	Approximated fragment sizes (bp)	Locations
Inter CO I-CO II	*Ase* I	11	2	455, 230, 215, 190, 110, 100, 85, 75, 65, 65	All samples
				565, 230, 215, 190, 100, 85, 75, 65, 65	Prachub Kiri Khan
	Bcl I	3	1	980, 320, 290	All samples
	Dra I	5	1	790, 420, 215, 100, 65	All samples
	*Hin*d III	2	1	900, 690	All samples
	*Hin*f I	4	1	640, 380, 340, 230	All samples
	Ssp I	7	1	425, 325, 280, 230, 180, 80, 70	All samples
L-rRNA	*Ase* I	3	1	360, 300, 100	All samples
	Dra I	7	1	300, 120, 100, 80, 60, 60, 40	All samples
	Ssp I	2	1	460, 300	All samples
Cytb I-tRNA^{Ser}	*Ase* I	5	1	225, 225, 210, 130, 80	All samples
	Dra I	3	1	400, 270, 200	All samples
	Ssp I	3	1	590, 190, 90	All samples

4.6 Analysis of mtDNA polymorphisms of *A. florea* in Thailand by PCR-RFLP

The variation in L-rRNA and Cytb I-tRNA^{Ser} after digested with restriction enzymes (*Ase* I, *Dra* I and *Ssp* I) was not found. Whereas, two haplotypes were found when digested the PCR product of inter CO I-CO II region with *Ase* I. The different pattern was found in only one colony from Prachub Kiri Khan (lower part of central Thailand).

The genetic distance among the populations of *A. florea* in Thailand after investigated from PCR-RFLP in the inter CO I-CO II of mtDNA is 1.7321. Cluster analysis of *A. florea* populations by UPGMA is shown in Figure 4.14. A colony (C56) from Prachub Kiri Khan was separated from the others.

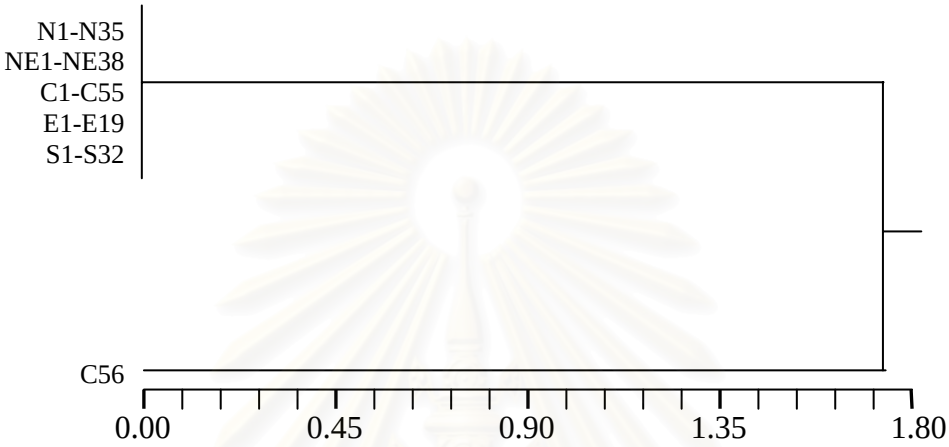

Figure 4.17 A UPGMA dendogram showing the relationship among populations of *A. florea* in Thailand using data of PCR-RFLP of inter CO I-CO II region of mtDNA.

CHAPTER V

DISCUSSION

After digested with a number of restriction enzymes; *Acl* I, *Afl* I, *Ase* I, *Bam*H I, *Bcl* I, *Dra* I, *Eco*R I, *Hin*d III, *Hin*f I, *Rsa* I, *Ssp* I, and *Swa* I. Six restriction enzymes; *Ase* I, *Bcl* I, *Dra* I, *Hin*d III, *Hin*f I and *Ssp* I could be digested the PCR product in the inter CO I- CO II region of mtDNA in *A. florea* populations. Whereas only 3 restriction enzymes; *Ase* I, *Dra* I, and *Ssp* I could be digested the PCR products of L-rRNA gene and Cytb I-$tRNA^{Ser}$ gene.

No variation was found from PCR-RFLP analysis in L-rRNA gene and Cytb I-$tRNA^{Ser}$ gene among *A. florea* populations in Thailand. Two different haplotypes were found in the inter CO I-CO II region when digested the PCR product with *Ase* I. The different haplotype was detected from only one colony of *A. florea* in Prachub Kiri Khan (lower part of central Thailand). Ten restriction fragments (haplotype A); 455, 230, 215, 190, 110, 100, 85, 75, 65, and 65 bp were investigated from the analysis. The sample of *A. florea* from all parts of Thailand showed haplotype A except the colony from Prachub Kiri Khan Province which found the different haplotype (haplotype B). Haplotype B showed 9 restriction fragments; 565, 230, 215, 190, 100, 85, 75, 65, and 65 bp. The interconnection between haplotype A and B can be explained by a loss of the restriction site generating two fragments; 455 and 110 bp in haplotype A resulted in an appearance of a 565 bp fragment in haplotype B. On the other hand, gaining of restriction site within a 565 bp fragment in haplotype B resulted in an occurance of 455 and 110 bp

fragments in haplotype A. The loss and gain of restriction site was resulted from the difference of nucleotide. The variation which occurred in this sample of *A. florea* probably resulted from a mutation in the inter CO I-CO II region of mtDNA. DNA sequencing could be utilised to confirm this conjecture.

The inter CO I-CO II region is a popular region for investigate the difference both within and between species. Cornuet and Garnery,(1991) found that the total length of *A. mellifera* mtDNA is variable, approximately ranging between 16,500 and 17,00 bp. This range results from length variability in several regions of the molecule.

L-rRNA gene and Cytb I-tRNA^{Ser} gene are coding regions which the evolution rate is slower than the non-coding region (Cornuet and Garnery, 1991; Hepburn *et al.*, 2001). Thus the lack of variation among populations of *A. florea* in Thailand in these genes is not surprising. Sihanuntavong *et al.* (1999) has been used L-rRNA gene to detect genetic variation in *A. cerana*. They separated *A. cerana* populations into 2 groups; Northern and Southern Thailand. While results from the study of Deowanish (1996) found that L-rRNA gene of mtDNA of *A. cerana* could not give enough variation for study intraspecific variation in *A. cerana*. Comparison of the results from PCR-RFLP of inter CO I-CO II region and L-rRNA gene indicated the inter CO I-CO II region is more suitable than L-rRNA gene to study genetic variation within *A. cerana* populations. However the inter CO I-CO II region is non-coding region which evolves very rapidly. The difference in this mtDNA region of *A. florea* in Thailand could be detected in only one colony. The lack of variation in the CO I-CO II intergenic region may be

because the size of non-coding region between CO I and CO II. The lack of variation in the CO I-CO II intergenic region may be because gene of *A. florea* is smaller than *A. mellifera* and *A. cerana* because the sequence between $tRNA^{Leu}$ gene and CO II gene has been deleted (Cornuet and Garnery, 1991). Cornuet and Garnery (1991) found the size between $tRNA^{Leu}$ and CO II gene of *A. mellifera*, *A. cerana*, *A. dorsata*, and *A. florea* were 250, 89, 24, and 32 bp, respectively. Thus the variation in the inter CO I-CO II region of mtDNA of *A. florea* may be less with the deleted sequences.

An *A. florea* colony can be move in a short distance (only a few up to several 100 m) (Ruttner, 1988; Tirgari, 1971). However swarm colonies up to 8 swarms a year (Free, 1981). If a colony moves no more than about 100 m, the honey bees will revisit the old site to collect the honey and the wax from the old comb for build the new comb (Free, 1981; Ruttner, 1988). The long-distance movement of *A. florea* colony also occurs when a colony absconds, which do two or three times a year (Free, 1981). The lacking of food is probably the seasonal migration (Free, 1981; Ruttner, 1988). In Thailand, seasonal migration behavior of *A. florea* is occurred during dearth periods (Deowanish *et al.*, 2001). Temperature changes and the invasion by enemies such as wax moths, bee mites, ants or human are also important in inducing changes of its nesting site (Free, 1981). Another cause of widely distributed of *A. florea* is human by bee hunters (Crane, 1993; Wongsiri *et al.*, 1996).

More over, *A. florea* can be nest in various types of place. It can build the comb on the branch of tree or shrub, and also nest in small caves with wide aperture or in sheltered areas of building, or even in wells far below ground surface (Ruttner, 1988; Wongsiri *et al.*, 1990; 1996).

From this study, One type across the whole population of *A. florea* in Thailand might be caused of the new invasion, either of a species or of a mitochondrial DNA. Because of the rapid distribution of this species, it makes their mtDNA among them are not different. In case that, *A. florea* in Thailand have different type of mtDNA, but some type is good to survive in the environment better than other types of mtDNA in the same species. It can make mtDNA within this species are relatively identical (Oldroyd, personal communication).

The genetic variation in the inter CO I-CO II region, L-rRNA gene, and Cytb I-tRNA^{Ser} gene in mtDNA of *A. florea* could not be determined the significant difference by the PCR-RFLP approach. To investigate the intraspecific variation in this species in Thailand, may be different technique such as microsatellite DNA could be detected the variation within this species.

The honey bee DNA in this study was extracted from thorax of worker bee. Thorax is an active organ which full of muscles and believed to contain of high quantity of mitochondrial DNA (Hoy, 1994). Only one band of high molecular weight of total DNA was presented after agarose gel electrophoresis. The size of this band is larger than 23.1 kb (Figure 4.1). The result of extraction method in this

study gave small amount of extracted DNA, but enough to amplify in subsequence step.

In this study, the approximated size of PCR products after amplification the mtDNA in the inter CO I-CO II region, L-rRNA gene and Cytb I-tRNA^{Ser} gene were 1590, 760, and 870 bp, respectively. Comparison of amplified products of *A. florea* with the amplified product of *A. cerana* which study by Deowanish (1996) indicated that the PCR product on inter CO I-CO II region was shorter than *A. cerana* (1,710 bp).

CHAPTER VI

CONCLUSIONS

1. The PCR products of inter CO I-CO II region, L-rRNA gene, and Cytb I-tRNA^{Ser} gene of *A. florea* are 1590, 760, and 860 bp, respectively.

2. The restriction fragment patterns of *A. florea* in Thailand could be divided into two haplotypes, haplotype A and haplotype B, when PCR product of inter CO I-CO II region in mtDNA were digested with *Ase* I. But there were low genetic variation level. Only one colony from Prachub Kiri Khan was separated from the others after clustering by UPGMA (Figure 4.17).

3. No variation was found in L-rRNA gene and Cytb I-tRNA^{Ser} in this species. The restriction pattern among populations are identical after analyze by *Ase* I, *Dra* I, and *Ssp* I in each gene.

4. The seasonal migration, swarming, and absconding including the well adaptation of *A. florea* might be caused this species can be survived in the environment and distributed very rapidly.

5. More sensitivity of different technique and different DNA marker might be detected the genetic variation in this species.

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APPENDICES

APPENDIX 1

PCR-RFLP analysis

1. DNA extraction

Bee thorax

↓

Homoginize on ice with 500 µl TE buffer in 1.5 microcentrifuge tube.

↓

Add 25 µl of 20% SDS

↓

25 µl of 10 mg/ml proteinase K

Vortex and incubate in water bath at 60^{o}C of 2 hr. or at 37^{o}C over night

↓

Centrifuge at 8,000 rpm, 10 min

↓

Move supernatant to new tube

↓

Add 1 volume of phenol/chloroform/isoamyl alcohol (25:24:1)

↓

And mix gently by hand

Centrifuge at 8,000 rpm, 10 min

↓

Move supernatant to new tube and repeat the previous step

Centrifuge at 8,000 rpm, 10 min

↓

Move supernatant to new tube and add 1 volume

of chloroform/ isoamyl alcohol(24:1)

and mix gently by hand

↓

Centrifuge at 8,000 rpm, 10 min

↓

Move supernatant to new tube and add 1/10 volume

of 3 M sodium acetate(NaOAc)

Add 2 volume of cold EtOH, Keep at -20 ^{o}C overnight

↓

Centrifuge at 12,000 rpm, 10 min

Remove the solution and add 500 µl of cold 70% EtOH to wash the pellet

↓

Centrifuge at 12,000 rpm, 10 min

↓

Remove the solution and air dry the pellet

↓

Add 30 µl TE buffer mix gently, incubate at 37 ^{o}C, 30 min

↓

Keep at 4 ^{o}C

2. PCR amplification

Step 1. Prepare reaction mixture as follow in 0.5 ml microcentrifuge tube.

	Stock conc.	Final conc.	Volume (μl)
1. PCR buffer	10x	1x	2.5
2. dNTPs			
- dATP	2.5 mM	100 μM	1.0
- dTTP	2.5 mM	100 μM	1.0
- dCTP	2.5 mM	100 μM	1.0
- dGTP	2.5 mM	100 μM	1.0
3. Primer I	10 μM	0.12 μM	0.3
4. Primer II	10 μM	0.12 μM	0.3
5. Taq polymerase	5 U	0.6 U/ reaction	0.12
6. Autoclaved D.W	-	-	12.78
7. Template DNA	25 ng/μl	50 ng	2.0
8. Mg^{2+}	25 mM	3 mM	3.0
Total			25 μl

Step 2. Spin the mixture.

Step 3. Run PCR under themocycle as show in Table1.

Step 4. Check PCR product (3 μl) on 1.5% agarose gel electrophoresis.

3. Restriction enzyme digestion (Total volume = 10 μl)

PCR product (5 μl)

Add 1 μl RE's buffer

RE (8-10 units)

↓

Autoclaved D.W.

Incubate at 37 ^{o}C overnight (or at least 5 hr.)

↓

Separate the restriction fragment on 2% agarose gel

Remarks : The volume of RE depends on stock concentration and final concentration of each RE.

: The volume of autoclaved D.W. will add to make the total volume = 10 μl.

APPENDIX 2

Details of sample collections.

Sample number	Sampling area	Date	Sample number	Sampling area	Date
N1	Chiang Rai	6/6/00	N29	Phitsanulok	17/2/00
N2	Chiang Rai	6/6/00	N30	Lam Phun	18/2/00
N3	Chiang Rai	6/6/00	N31	Phetchaboon	20/2/99
N4	Chiang Rai	7/6/00	N32	Phetchaboon	20/2/99
N5	Chiang Rai	7/6/00	N33	Uttaradit	21/2/00
N6	Chiang Rai	7/6/00	N34	Uttaradit	21/2/00
N7	Chiang Rai	7/6/00	N35	Kampangpetch	29/4/00
N8	Chiang Rai	8/6/00	NE1	Loei	8/4/96
N9	Chiang Rai	8/6/00	NE2	Nong Kai	8/12/96
N10	Chiang Rai	8/6/00	NE3	Nong Kai	25/5/00
N11	Chiang Mai	18/2/00	NE4	Nong Kai	25/5/00
N12	Chiang Mai	18/2/00	NE5	Nong Kai	30/5/00
N13	Chiang Mai	19/2/00	NE6	Nong Kai	30/5/00
N14	Chiang Mai	19/2/00	NE7	Udontani	24/5/00
N15	Chiang Mai	20/2/00	NE8	Udontani	24/5/00
N16	Chiang Mai	22/3/00	NE9	Udonthani	24/5/00
N17	Chiang Mai	22/3/00	NE10	Udonthani	24/5/00
N18	Chiang Mai	22/3/00	NE11	Sakon Nakorn	25/5/00
N19	Chiang Mai	22/3/00	NE12	Nakorn Phanom	27/5/00
N20	Pha Yao	6/6/00	NE13	Nakorn Phanom	27/5/00
N21	Pha Yao	6/6/00	NE14	Nakorn Phanom	27/5/00
N22	Pha Yao	6/6/00	NE15	Nakorn Phanom	27/5/00
N23	Pha Yao	6/6/00	NE16	Ubon Ratchathani	28/5/00
N24	Pha Yao	6/6/00	NE17	Ubon Ratchathani	28/5/00
N25	Tak	5/6/00	NE18	Ubon Ratchathani	28/5/00
N26	Phitsanulok	17/2/00	NE19	Ubon Ratchathani	28/5/00
N27	Phitsanulok	17/2/00	NE20	Roi Ed	28/5/00
N28	Phitsanulok	17/2/00	NE21	Roi Ed	28/5/00

Sample number	Sampling area	Date	Sample number	Sampling area	Date
NE22	Khon Kaen	28/3/00	C15	Lopburi	6/12/99
NE23	Khon Kaen	28/3/00	C16	Suphanburi	23/5/99
NE24	Khon Kaen	28/3/00	C17	Suphanburi	23/5/99
NE25	Khon Kaen	28/3/00	C18	Suphanburi	23/5/99
NE26	Khon Kaen	28/3/00	C19	Suphanburi	23/5/99
NE27	Khon Kaen	6/12/99	C20	Suphanburi	23/5/99
NE28	Khon Kaen	6/12/99	C21	Suphanburi	23/5/99
NE29	Khon Kaen	6/12/99	C22	Suphanburi	3/1/99
NE30	Chaiyaphum	23/5/00	C23	Angthong	17/5/00
NE31	Chaiyaphum	23/5/00	C24	Angthong	17/5/00
NE32	Chaiyaphum	23/5/00	C25	Samutprakarn	10/3/96
NE33	Chaiyaphum	23/5/00	C26	Samutprakarn	10/3/96
NE34	Nakorn Ratchaseema	26/6/99	C27	Nakornpathom	14/4/96
NE35	Nakorn Ratchaseema	26/6/99	C28	Nakornpathom	15/4/96
NE36	Nakorn Ratchaseema	26/6/99	C29	Samutsongkram	12/7/99
NE37	Nakorn Ratchaseema	26/6/99	C30	Samutsongkram	12/7/99
NE38	Nakorn Ratchaseema	16/4/00	C31	Samutsongkram	24/2/99
C1	Nakornsawan	21/2/00	C32	Samutsongkram	24/2/99
C2	Nakornsawan	15/5/99	C33	Samutsongkram	24/2/99
C3	Nakornsawan	15/5/99	C34	Ratchaburi	22/7/00
C4	Lopburi	13/5/98	C35	Ratchaburi	22/7/00
C5	Lopburi	19/6/99	C36	Phetchaburi	15/3/96
C6	Lopburi	19/6/99	C37	Phetchaburi	15/3/96
C7	Lopburi	19/6/99	C38	Phetchaburi	11/7/99
C8	Lopburi	19/6/99	C39	Phetchaburi	11/7/99
C9	Lopburi	19/6/99	C40	Kanchanaburi	13/4/96
C10	Lopburi	20/6/99	C41	Kanchanaburi	13/6/99
C11	Lopburi	20/6/99	C42	Kanchanaburi	13/6/99
C12	Lopburi	20/6/99	C43	Prachaub Kirikan	23/3/96
C13	Lopburi	20/6/99	C44	Prachaub Kirikan	23/3/96
C14	Lopburi	20/6/99	C45	Prachaub Kirikan	24/3/96

Sample number	Sampling area	Date	Sample number	Sampling area	Date
C46	Prachaub Kirikan	15/5/99	E19	Chacheng sao	13/4/00
C47	Prachaub Kirikan	15/5/99	S1	Chumporn	15/5/96
C48	Prachaub Kirikan	1/6/99	S2	Chumporn	15/5/96
C49	Prachaub Kirikan	1/6/99	S3	Chumporn	16/5/96
C50	Prachaub Kirikan	1/6/99	S4	Chumporn	1/6/99
C51	Prachaub Kirikan	1/6/99	S5	Chumporn	2/6/99
C52	Prachaub Kirikan	1/6/99	S6	Chumporn	2/6/99
C53	Prachaub Kirikan	1/6/99	S7	Chumporn	5/6/99
C54	Prachaub Kirikan	12/5/00	S8	Chumporn	5/6/99
C55	Prachaub Kirikan	12/5/00	S9	Suratthani	12/5/96
C56	Prachaub Kirikan	12/5/00	S10	Suratthani	11/5/00
E1	Chantaburi	17/3/96	S11	Suratthani	11/5/00
E2	Chantaburi	18/3/96	S12	Kho Samui, Suratthani	3/6/99
E3	Chantaburi	18/3/96	S13	Kho Samui, Suratthani	3/6/99
E4	Chantaburi	31/3/00	S14	Kho Samui, Suratthani	4/6/99
E5	Chantaburi	31/3/00	S15	Kho Samui, Suratthani	4/5/00
E6	Chantaburi	31/3/00	S16	Kho Samui, Suratthani	4/5/00
E7	Chantaburi	1/4/00	S17	Kho Samui, Suratthani	4/5/00
E8	Chantaburi	12/4/00	S18	Kho Samui, Suratthani	4/5/00
E9	Trad	7/3/97	S19	Kho Samui, Suratthani	4/5/00
E10	Trad	6/3/97	S20	Kho Samui, Suratthani	4/5/00
E11	Trad	31/3/00	S21	Kho Samui, Suratthani	4/5/00
E12	Trad	31/3/00	S22	Kho Samui, Suratthani	4/5/00
E13	Trad	31/3/00	S23	Kho Samui, Suratthani	4/5/00
E14	Trad	31/3/00	S24	Kho Samui, Suratthani	4/5/00
E15	Rayong	12/4/00	S25	Kho Samui, Suratthani	4/5/00
E16	Rayong	12/4/00	S26	Kho Samui, Suratthani	4/5/00
E17	Rayong	12/4/00	S27	Kho Pa-ngan, Suratthani	10/3/00
E18	Rayong	12/4/00	S28	Kho Pa-ngan, Suratthani	10/3/00

Sample number	Sampling area	Date
S29	Kho Pa-ngan, Suratthani	10/3/00
S30	Kho Pa-ngan, Suratthani	10/3/00
S31	Kho Pa-ngan, Suratthani	10/3/00
S32	Kho Pa-ngan, Suratthani	11/3/00

Appendix 3

Matrix data of RFLP analysis (Inter CO I-CO II region).

	N1	N2	N3	N4	N5	N6	N7	N8	N9	N10	N11	N12	N13	N14	N15	N16	N17	N18
*Ase*I 1 (565 bp)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
*Ase*I 2 (455 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 3 (230 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 4 (215 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 5 (190 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 6 (110 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 7 (100 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 8 (85 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 9 (75 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 10 (65 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ase I 11 (65 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Bcl I 1 (980 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Bcl I 2 (320 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Bcl I 3 (290 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 1 (790 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 2 (420 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 3 (215 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 4 (100 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 5 (65 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Hin*d III 1 (900 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Hin*d III 2 (690 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Hinf I 1 (640 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Hinf I 2 (380 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Hinf I 3 (340 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Hinf I 4 (230 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 1 (425 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 2 (325 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 3 (280 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 4 (230 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 5 (180 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 6 (80 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 7 (70 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

	N19	N20	N21	N22	N23	N24	N25	N26	N27	N28	N29	N30	N31	N32	N33	N34
*Ase*l 1 (565 bp)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
*Ase*l 2 (455 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*l 3 (230 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*l 4 (215 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*l 5 (190 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*l 6 (110 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*l 7 (100 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*l 8 (85 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*l 9 (75 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*l 10 (65 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ase I 11 (65 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Bcl I 1 (980 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Bcl I 2 (320 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Bcl I 3 (290 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 1 (790 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 2 (420 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 3 (215 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 4 (100 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 5 (65 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Hin*d III 1 (900 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Hin*d III 2 (690 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Hin*f I 1 (640 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Hin*f I 2 (380 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Hin*f I 3 (340 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Hin*f I 4 (230 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 1 (425 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 2 (325 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 3 (280 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 4 (230 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 5 (180 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 6 (80 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 7 (70 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

	N35	NE1	NE2	NE3	NE4	NE5	NE6	NE7	NE8	NE9	NE10	NE11	NE12	NE13
*Ase*l 1 (565 bp)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
*Ase*l 2 (455 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*l 3 (230 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*l 4 (215 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*l 5 (190 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*l 6 (110 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*l 7 (100 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*l 8 (85 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*l 9 (75 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*l 10 (65 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ase I 11 (65 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Bcl I 1 (980 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Bcl I 2 (320 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Bcl I 3 (290 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 1 (790 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 2 (420 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 3 (215 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 4 (100 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 5 (65 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Hin*d III 1 (900 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Hin*d III 2 (690 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Hinf I 1 (640 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Hinf I 2 (380 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Hinf I 3 (340 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Hinf I 4 (230 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 1 (425 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 2 (325 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 3 (280 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 4 (230 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 5 (180 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 6 (80 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 7 (70 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1

	NE14	NE15	NE16	NE17	NE18	NE19	NE20	NE21	NE22	NE23	NE24	NE25
*Ase*I 1 (565 bp)	0	0	0	0	0	0	0	0	0	0	0	0
*Ase*I 2 (455 bp)	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 3 (230 bp)	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 4 (215 bp)	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 5 (190 bp)	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 6 (110 bp)	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 7 (100 bp)	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 8 (85 bp)	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 9 (75 bp)	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 10 (65 bp)	1	1	1	1	1	1	1	1	1	1	1	1
Ase I 11 (65 bp)	1	1	1	1	1	1	1	1	1	1	1	1
Bcl I 1 (980 bp)	1	1	1	1	1	1	1	1	1	1	1	1
Bcl I 2 (320 bp)	1	1	1	1	1	1	1	1	1	1	1	1
Bcl I 3 (290 bp)	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 1 (790 bp)	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 2 (420 bp)	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 3 (215 bp)	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 4 (100 bp)	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 5 (65 bp)	1	1	1	1	1	1	1	1	1	1	1	1
*Hin*d III 1 (900 bp)	1	1	1	1	1	1	1	1	1	1	1	1
*Hin*d III 2 (690 bp)	1	1	1	1	1	1	1	1	1	1	1	1
Hinf I 1 (640 bp)	1	1	1	1	1	1	1	1	1	1	1	1
Hinf I 2 (380 bp)	1	1	1	1	1	1	1	1	1	1	1	1
Hinf I 3 (340 bp)	1	1	1	1	1	1	1	1	1	1	1	1
Hinf I 4 (230 bp)	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 1 (425 bp)	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 2 (325 bp)	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 3 (280 bp)	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 4 (230 bp)	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 5 (180 bp)	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 6 (80 bp)	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 7 (70 bp)	1	1	1	1	1	1	1	1	1	1	1	1

	NE26	NE27	NE28	NE29	NE30	NE31	NE32	NE33	NE34	NE35	NE36	NE37
*Ase*I 1 (565 bp)	0	0	0	0	0	0	0	0	0	0	0	0
*Ase*I 2 (455 bp)	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 3 (230 bp)	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 4 (215 bp)	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 5 (190 bp)	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 6 (110 bp)	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 7 (100 bp)	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 8 (85 bp)	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 9 (75 bp)	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 10 (65 bp)	1	1	1	1	1	1	1	1	1	1	1	1
Ase I 11 (65 bp)	1	1	1	1	1	1	1	1	1	1	1	1
Bcl I 1 (980 bp)	1	1	1	1	1	1	1	1	1	1	1	1
Bcl I 2 (320 bp)	1	1	1	1	1	1	1	1	1	1	1	1
Bcl I 3 (290 bp)	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 1 (790 bp)	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 2 (420 bp)	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 3 (215 bp)	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 4 (100 bp)	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 5 (65 bp)	1	1	1	1	1	1	1	1	1	1	1	1
*Hin*d III 1 (900 bp)	1	1	1	1	1	1	1	1	1	1	1	1
*Hin*d III 2 (690 bp)	1	1	1	1	1	1	1	1	1	1	1	1
Hinf I 1 (640 bp)	1	1	1	1	1	1	1	1	1	1	1	1
Hinf I 2 (380 bp)	1	1	1	1	1	1	1	1	1	1	1	1
Hinf I 3 (340 bp)	1	1	1	1	1	1	1	1	1	1	1	1
Hinf I 4 (230 bp)	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 1 (425 bp)	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 2 (325 bp)	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 3 (280 bp)	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 4 (230 bp)	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 5 (180 bp)	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 6 (80 bp)	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 7 (70 bp)	1	1	1	1	1	1	1	1	1	1	1	1

	NE38	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15
*Ase*I 1 (565 bp)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
*Ase*I 2 (455 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 3 (230 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 4 (215 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 5 (190 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 6 (110 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 7 (100 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 8 (85 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 9 (75 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 10 (65 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ase I 11 (65 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Bcl I 1 (980 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Bcl I 2 (320 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Bcl I 3 (290 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 1 (790 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 2 (420 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 3 (215 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 4 (100 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 5 (65 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Hin*d III 1 (900 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Hin*d III 2 (690 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Hinf I 1 (640 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Hinf I 2 (380 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Hinf I 3 (340 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Hinf I 4 (230 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 1 (425 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 2 (325 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 3 (280 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 4 (230 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 5 (180 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 6 (80 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 7 (70 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

	C16	C17	C18	C19	C20	C21	C22	C23	C24	C25	C26	C27	C28	C29	C30	C31
*Ase*I 1 (565 bp)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
*Ase*I 2 (455 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 3 (230 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 4 (215 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 5 (190 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 6 (110 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 7 (100 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 8 (85 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 9 (75 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 10 (65 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ase I 11 (65 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Bcl I 1 (980 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Bcl I 2 (320 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Bcl I 3 (290 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 1 (790 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 2 (420 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 3 (215 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 4 (100 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 5 (65 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Hin*d III 1 (900 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Hin*d III 2 (690 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Hinf I 1 (640 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Hinf I 2 (380 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Hinf I 3 (340 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Hinf I 4 (230 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 1 (425 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 2 (325 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 3 (280 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 4 (230 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 5 (180 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 6 (80 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 7 (70 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

	C32	C33	C34	C35	C36	C37	C38	C39	C40	C41	C42	C43	C44	C45	C46	C47
*Ase*I 1 (565 bp)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
*Ase*I 2 (455 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 3 (230 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 4 (215 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 5 (190 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 6 (110 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 7 (100 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 8 (85 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 9 (75 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 10 (65 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ase I 11 (65 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Bcl I 1 (980 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Bcl I 2 (320 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Bcl I 3 (290 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 1 (790 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 2 (420 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 3 (215 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 4 (100 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 5 (65 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Hin*d III 1 (900 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Hin*d III 2 (690 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Hinf I 1 (640 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Hinf I 2 (380 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Hinf I 3 (340 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Hinf I 4 (230 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 1 (425 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 2 (325 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 3 (280 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 4 (230 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 5 (180 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 6 (80 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 7 (70 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

	C48	C49	C50	C51	C52	C53	C54	C55	C56	E1	E2	E3	E4	E5	E6	E7
*Ase*I 1 (565 bp)	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
*Ase*I 2 (455 bp)	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
*Ase*I 3 (230 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 4 (215 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 5 (190 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 6 (110 bp)	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
*Ase*I 7 (100 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 8 (85 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 9 (75 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 10 (65 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ase I 11 (65 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Bcl I 1 (980 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Bcl I 2 (320 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Bcl I 3 (290 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 1 (790 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 2 (420 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 3 (215 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 4 (100 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 5 (65 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Hin*d III 1 (900 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Hin*d III 2 (690 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Hinf I 1 (640 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Hinf I 2 (380 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Hinf I 3 (340 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Hinf I 4 (230 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 1 (425 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 2 (325 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 3 (280 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 4 (230 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 5 (180 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 6 (80 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 7 (70 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

	E8	E9	E10	E11	E12	E13	E14	E15	E16	E17	E18	E19	S1	S2	S3	S4	S5	S6
*Ase*I 1 (565 bp)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
*Ase*I 2 (455 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 3 (230 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 4 (215 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 5 (190 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 6 (110 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 7 (100 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 8 (85 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 9 (75 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*I 10 (65 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ase I 11 (65 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Bcl I 1 (980 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Bcl I 2 (320 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Bcl I 3 (290 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 1 (790 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 2 (420 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 3 (215 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 4 (100 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 5 (65 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Hin*d III 1 (900 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Hin*d III 2 (690 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Hin*f I 1 (640 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Hin*f I 2 (380 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Hin*f I 3 (340 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Hin*f I 4 (230 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 1 (425 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 2 (325 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 3 (280 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 4 (230 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 5 (180 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 6 (80 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 7 (70 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24
*Ase*l 1 (565 bp)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
*Ase*l 2 (455 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*l 3 (230 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*l 4 (215 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*l 5 (190 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*l 6 (110 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*l 7 (100 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*l 8 (85 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*l 9 (75 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Ase*l 10 (65 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ase I 11 (65 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Bcl I 1 (980 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Bcl I 2 (320 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Bcl I 3 (290 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 1 (790 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 2 (420 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 3 (215 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 4 (100 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dra I 5 (65 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Hin*d III 1 (900 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
*Hin*d III 2 (690 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Hinf I 1 (640 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Hinf I 2 (380 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Hinf I 3 (340 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Hinf I 4 (230 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 1 (425 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 2 (325 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 3 (280 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 4 (230 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 5 (180 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 6 (80 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ssp I 7 (70 bp)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

	S25	S26	S27	S28	S29	S30	S31	S32
*Ase*l 1 (565 bp)	0	0	0	0	0	0	0	0
*Ase*l 2 (455 bp)	1	1	1	1	1	1	1	1
*Ase*l 3 (230 bp)	1	1	1	1	1	1	1	1
*Ase*l 4 (215 bp)	1	1	1	1	1	1	1	1
*Ase*l 5 (190 bp)	1	1	1	1	1	1	1	1
*Ase*l 6 (110 bp)	1	1	1	1	1	1	1	1
*Ase*l 7 (100 bp)	1	1	1	1	1	1	1	1
*Ase*l 8 (85 bp)	1	1	1	1	1	1	1	1
*Ase*l 9 (75 bp)	1	1	1	1	1	1	1	1
*Ase*l 10 (65 bp)	1	1	1	1	1	1	1	1
Ase I 11 (65 bp)	1	1	1	1	1	1	1	1
Bcl I 1 (980 bp)	1	1	1	1	1	1	1	1
Bcl I 2 (320 bp)	1	1	1	1	1	1	1	1
Bcl I 3 (290 bp)	1	1	1	1	1	1	1	1
Dra I 1 (790 bp)	1	1	1	1	1	1	1	1
Dra I 2 (420 bp)	1	1	1	1	1	1	1	1
Dra I 3 (215 bp)	1	1	1	1	1	1	1	1
Dra I 4 (100 bp)	1	1	1	1	1	1	1	1
Dra I 5 (65 bp)	1	1	1	1	1	1	1	1
*Hin*d III 1 (900 bp)	1	1	1	1	1	1	1	1
*Hin*d III 2 (690 bp)	1	1	1	1	1	1	1	1
*Hin*f I 1 (640 bp)	1	1	1	1	1	1	1	1
*Hin*f I 2 (380 bp)	1	1	1	1	1	1	1	1
*Hin*f I 3 (340 bp)	1	1	1	1	1	1	1	1
*Hin*f I 4 (230 bp)	1	1	1	1	1	1	1	1
Ssp I 1 (425 bp)	1	1	1	1	1	1	1	1
Ssp I 2 (325 bp)	1	1	1	1	1	1	1	1
Ssp I 3 (280 bp)	1	1	1	1	1	1	1	1
Ssp I 4 (230 bp)	1	1	1	1	1	1	1	1
Ssp I 5 (180 bp)	1	1	1	1	1	1	1	1
Ssp I 6 (80 bp)	1	1	1	1	1	1	1	1
Ssp I 7 (70 bp)	1	1	1	1	1	1	1	1

BIOGRAPHY

Miss Piyamas Nanork was born on November 12, 1976 in Khon Kaen, Thailand. She graduated with Bachelor degree of Science in Biology from Mahasarakam University in 1997 and has studied for Master degree of Science at Department of Biology, Chulalongkorn University since 1998. She works in Department of Biology, Faculty of Science, Mahasarakam University.