ความหลากหลายทางพันธุกรรมของประชากรผึ้งหลวง *Apis dorsata* Fabricius, 1793 ในประเทศไทย วิเคราะห์โดยใช้ดีเอ็นเอเครื่องหมาย

นางสาวสุชีรา อินทร์ศวร

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GENETIC VARIATION OF GIANT HONEYBEES *Apis dorsata* Fabricius, 1793 IN THAILAND ANALYZED BY DNA MARKERS

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A Thesis Submitted in Partial Fulfillment of the Requirements

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การศึกษาความผันแปรทางพันธุกรรมและโครงสร้างประชากรผึ้งหลวง *Apis dorsata* 154 ตัวอย่าง จากบริเวณ 5 พื้นที่ภูมิศาสตร์ของประเทศไทย คือ ภาคเหนือ, ภาคตะวันออกเฉียงเหนือ, ภาคกลาง, ภาคใต้ และเกาะสมุย โดยทำการตรวจสอบด้วยเทคนิค PCR-RFLP (Polymerase Chain Reaction–Restriction Fragment Length Polymorphism) ของไมโตคอนเดรียลดีเอ็นเอ 4 บริเวณ (บริเวณระหว่างยีน CO I- CO II, ยีน Cytb I - $tRNA^{Ser}$, ยีน ATPase6-8 และ ยีน lrRNA) ด้วยเทคนิค PCR สามารถทำการเพิ่มปริมาณชิ้นดีเอ็นเอ ของบริเวณบริเวณระหว่างยีน CO I- CO II, ยีน Cytb I - $tRNA^{Ser}$, ยีน ATPase6-8 และ ยีน lrRNA โดยใช้ไพรเมอร์คู่จำเพาะขนาบข้างดังกล่าว ได้ชิ้นดีเอ็นเอขนาด 1040, 845, 820 และ 750 คู่เบส ตามลำดับ เมื่อตัดชิ้นดีเอ็นเอทั้ง 4 บริเวณด้วยเอนไซม์ตัดจำเพาะ *Dra* I และ *Hin*f I พบว่าจะได้รูปแบบของแถบดีเอ็นเอเป็น 1, 1, 1 และ 2 รูปแบบ และ 3, 4 และ 1 รูปแบบ ตามลำดับ เมื่อรวมผลทั้ง 4 บริเวณที่ตัดด้วยเอนไซม์ตัดจำเพาะทั้ง 2 ตัว นี้ จะให้รูปแบบรวมของแถบดีเอ็นเอเป็น 6 รูปแบบ จากการวิเคราะห์ค่า geographic hetergeneity พบว่าการกระจายตัวของความถี่ของรูปแบบดีเอ็นเอของประชากรผึ้งหลวงไม่แตกต่างกันในทุกคู่ของกลุ่มประชากรที่นำมาเปรียบเทียบ ดังนั้นในการตรวจสอบไมโตคอนเดรียลดีเอ็นเอด้วย PCR-RFLP ไม่สามารถบอกความแตกต่างทางพันธุกรรมประชากรผึ้งหลวงในประเทศไทยได้

การตรวจสอบด้วยไมโครแซทเทลไลท์ดีเอ็นเอในผึ้งหลวง 155 ตัวอย่าง ครอบคลุมทั้ง 5 พื้นที่ในประเทศไทย โดยใช้ไมโครแซทเทลไลท์ไพรเมอร์ของผึ้งพันธุ์ *A. mellifera* 13 คู่ พบความหลากหลายของอัลลีลใน 3 ตำแหน่งของไมโครแซทเทลไลท์ (A14, A24 และ A88) โดยมีจำนวนอัลลีลต่อตำแหน่งเป็น 6, 8 และ 20 ตามลำดับ มีค่าเฮเทอโรไซโกซิตี้ เฉลี่ยอยู่ในช่วง 0.68-0.74 และให้ค่าเฉลี่ยของจำนวนอัลลีลในแต่ละตำแหน่งอยู่ในช่วง 6.0-9.0 หลังจากวิเคราะห์ geographic hetergeneity พบว่าที่ตำแหน่งของไมโครแซทเทลไลท์ A24 พบการกระจายตัวของความถี่อัลลีล ของประชากรผึ้งหลวงที่เกาะสมุยแตกต่างอย่างมีนัยสำคัญ กับกลุ่มประชากรผึ้งหลวงจากแผ่นดินใหญ่ (ภาคเหนือ, ภาคตะวันออกเฉียงเหนือ, ภาคกลาง และ ภาคใต้) ดังนั้นไมโครแซทเทลไลท์ที่ ตำแหน่ง A24 สามารถบอกความผันแปรทางพันธุกรรม ของประชากรผึ้งหลวงในประเทศไทยบนแผ่นดินใหญ่กับเกาะสมุยได้

ภาควิชา ชีวเคมี ลายมือชื่อนิสิต .....

สาขาวิชา ชีวเคมี ลายมือชื่ออาจารย์ที่ปรึกษา .....

ปีการศึกษา 2544 ลายมือชื่ออาจารย์ที่ปรึกษาร่วม .....

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PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) analysis is used to detect genetic variability and population structure in mitochondrial DNA of Thai giant honeybees (*A. dorsata*) from 5 geographic areas (north, north-east, central, south and Samui Island). PCR amplification using specific primers flanking to intergenic COI-COII region, CytbI-tRNA^{Ser} gene, ATPase6-8 gene and lrRNA gene yielded PCR products of 1040, 845, 820 and 750 bp, respectively. Digestion of these PCR products with *Dra* I and *Hin*f I revealed one, one, one and two haplotypes and three, four, and one haplotypes, respectively. Six composite haplotypes were generated from these study. Geographic heterogeneity in the distribution frequency was determined using χ^2 test. Six composite haplotypes did not show any significant differences in the distribution frequency in all pairwise comparisons. Therefore, determination of mtDNA by PCR-RFLP did not a sensitive marker for studying intraspecific variation *A. dorsata* in Thailand.

Three microsatellite loci (A14, A24 and A88) were used to study on population genetic of *A. dorsata* in 5 geographic areas by 13 *A. mellifera* microsatellite loci. These microsatellite were shown to be polymorphic with number of alleles at each locus of 6, 8 and 20 alleles, respectively. Population analyses based on 3 loci revealed heterozygosity between 0.68-0.74 and average number of alleles per locus were ranged 6.0-9.0. The analysis of geographic heterogeneity, the A24 locus indicated that the allele distribution frequencies of *A. dorsata* in Samui Island was significant difference from mainland (north, north-east, central and south). Microsatellite locus A24 can be used to investigate genetic differentiation of *A. dorsata* from mainland and Samui Island of Thailand.

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LIST OF ABBREVIATIONS

A, T, C, G	=	nucleotide containing the base adenosine, thymine, guanine and cytosine, respectively.
ATPase	=	adenosine triphosphatase
bp	=	base pair
^{o}C	=	degree celcius
cm	=	centrimetre
CO I	=	cytochrome oxidase I
CO II	=	cytochrome oxidase II
CytbI	=	cytochrome b I
DNA	=	deoxyribonucleic acid
dNTPs	=	deoxyribonucleotide triphosphate (dATP, dTTP, dGTP, dCTP)
ddNTPs	=	dideoxyribonucleotide triphosphate (ddATP, ddTTP, ddGTP, ddCTP)
EDTA	=	ethylenediamine tetraacetic acid
HCl	=	hydrochloric acid
Kb	=	kilobase
KCl	=	potassium chloride
lrRNA	=	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
TEMED	=	*N, N, N′, N′,* -tetramethyl ethylenediamine
Tris	=	tris(hydroxy methyl)aminomethane
tRNA	=	transfer RNA
UV	=	ultraviolet
V	=	volt
W	=	watt
μg	=	microgram
μl	=	microliter
μM	=	micromolar

CHAPTER I

INTRODUCTION

Honeybees, the highly eusocial Hymenopteran insects belonging to the genus *Apis*, are some of the most economically important insects. Their advantages are not only in the valuable hive products such as honey, pollen, royal jelly, beewax, propolis and bee venom they produce, but also from their ability to pollinate many crops and wild plants, which enhances plant biodiversity (Crane, 1990; 1993; Appanah and Kevan, 1995). A colony consists of one queen (female), a large number of workers (up to 50,000 worker bees, all female) and a few hundred of drones (all male). The females are heterozygotes (diploid 2n = 32), which come from fertilized eggs. The males are hemizygotes (haploid n = 16), which come from unfertilized eggs (Wongsiri, 1988).

On the basis of morphological structures, Ruttner (1988) classified honeybees into 4 species; the European honeybee, *A. mellifera* Linneaus, 1758; the Eastern honeybee, *A. cerana* Fabricius, 1798; the giant honeybee, *A. dorsata* Fabricius, 1793; and the dwarf honeybee, *A. florea* Fabricius, 1787.

Recently, 5 additional species of honeybees were recognized as the separated species by same investigators. These are the small dwarf honeybee, *A. andreniformis* Smith, 1858 (Wu and Kuang, 1987; Wongsiri *et al*., 1990); the mountain giant honeybee, *A. laboriosa* F. Smith, 1871 (Sakagami *et al*., 1980); the cavity-nesting honeybee, *A. koschevnikovi* Buttel-Reepen, 1906 (Koeniger *et al*., 1988; Rinderer *et al*., 1989); the cavity-nesting honeybee, *A. nigrocincta* Smith, 1861 (Otis, 1991; 1996); and the cavity-nesting honeybee, *A. nuluensis* (Otis, 1991; 1996).

In Thailand 5 of the 9 species of honeybees are found. *A. andreniformis*, *A. florea*, *A. cerana*, and *A. dorsata* are native bees and can be found from the Southern coastal flat to the high mountain and forest areas in the Northern part of Thailand. *A. mellifera* is the only exotic species, which was first introduced for research purpose at Chulalongkorn University in the early 1940s (Wongsiri *et al*., 2001). This species is used in apiculture with modern moveable comb hives. The numerical strength of *A. cerana* colonies is usually much less, and honey yields are smaller. It is therefore being rapidly supplanted by imported *A. mellifera* races, chiefly *A.m.ligustica* (Milner, 1996).

Giant honeybees, *A. dotsata* is distributed cover the area of Thailand (Wongsiri *et al*., 1989). It is an important pollinator of natural plants (Ruttner, 1988). Because of its high honey yield and the belief by people that honey from this bee is the best, several *A. dorsata* colonies are destroyed by bee hunters every year. However, the main impact of destroying *A. dorsata* is the destruction of the forests with its host plant.

Because of its high nesting and aggressive behavior, the study of *A. dorsata*'s biology is very different from other *Apis* species. Most reports are studies of behavior, morphology, distribution and ecology (Ruttner, 1988; Koeniger and Koeniger, 1980; Wongsiri *et al*., 2001). *A. dorsata* has different on nesting behavior in which some colonies live in aggregations, while others live as solitary colonies. Seasonal migration of *A. dorsata* is depended on food resources and predation. However, surprisingly *A. dorsata* of mainland to Borneo and Palawan has uniform morphological characters. Two subspecies of *A. dorsata: A. d. breviligula* and *A. d. binghami* differ from the species type in the length of tongue and the length of fore wing. Studies on variation of honeybees was firstly done using morphological character or morphometrics (Ruttner, 1988). This approach should be investigated with several characteristics from a large number of samples to reduce the bias from investigators and environmental effects on the measure of morphological characters. The second method, protein marker (allozyme) variation was used to study genetic variation in honeybees. However, the

limit of low polymorphism in haplo-diploid insects became a problem. After DNA markers have been developed, the genetic variation of honeybees was effectively investigated using several technique such as RFLP (Restriction Fragment Length Polymorphism), PCR-RFLP, microsatellites and DNA sequencing (Cameron, 1993; Cornuet *et al*., 1991; Estoup, 1994; Garnery *et al*., 1995; Meixner *et al*., 2000; Smith *et al*., 1991).

For Thailand, early studies on genetic variation and population differentiation of *A. cerana* were examined by restriction analysis of amplified mitochondrial genes and intergenic region (srRNA and lrRNA genes, and the intergenic CO I – CO II region) with *Dra* I (Sihanuntavong *et al*., 1999). Three distinct groups were found using the clustering algorithm UPGMA. These were: Northern Thailand (north, central and north-east) (A), peninsular Thailand and Phuket (B), and Samui Islands (C).

Moreover, Sittapraneed *et al.* (2001) studied level of genetic differentiation and population structure of Thai honeybees *A. cerana*, by using microsatellite DNA analysis. The analysis of geographic heterogeneity and phylogenetic reconstruction using the Neighbor-joining approach divided 5 geographic *A. cerana* samples to 3 different groups consisting of 1) Northern (north, central and north-east), 2) peninsular Thailand and 3) Samui Island.

Genetic diversity of giant honeybee (*A. dorsata*) has been one of interest in honeybee study since this species is wide spread throughout south-eastern Asia. Smith (1991) studied genetic diversity of this species in Thailand but with limited samples (from only 1 location). This species is distributed throughout Thailand and is one of the important pollinators. To understand the diversity of this species, it is useful to re-investigation the genetic variation of this species. In this thesis, I conducted the investigation of genetic polymorphism of mtDNA and nuclear DNA of *A. dorsata* sampled from five different geographic areas. Molecular markers using PCR-RFLP and microsatellite analysis were conducted to investigate genetic variation of *A. dorsata* in Thailand.

LITERATURE REVIEW

A. dorsata Fabricius, 1973, the giant honeybee, is the largest honeybee. Its distribution area covers most of the Indo-Malayan region. *A. dorsata* is found not farther than the Indus river to the west while to the east it is found all the Philippines islands (Figure 1.1). Its nest is an open-air, single colony which is usually found underneath a large branch of a tree, cliff or human-built-structure (Ruttner, 1988). Aggregation of many colonies in the same area (tree, building and cliff) is a unique character of this honeybee. However, a single or solitary colony of *A. dorsata* is common especially in the Philippines (Ruttner, 1988). Seasonal migration is also a common behavior in *A. dorsata*, which may be related to available food resources and predation.

The distance of *A. dorsata* seasonally migration is 100-200 km. The migration to the new nesting site is accomplished in several stops. The distance between stops is between 200 m to 5 km. Each stopover lasts approximately 1 to 3 days, within which time they refuel for the rest of their flight (Koeniger and Koeniger, 1980). Observation in northern Thailand indicates that if the nests are undisturbed, the colonies will abscond or migrate when their food reserves have been depleted, usually at the end of the summer months.

Morphometrics has long been the method used to study variation among honeybee populations. Maa (1953) divided the honeybees into three genera: *Micrapis*, the dwarf bees, with two species; *Megapis*, the giant bees, with four species; and *Apis*, the cavity-nesting bees. Furthermore, he recognized four giant honeybee species in the genus *Megapis*: *M. breviligula* from Luzon (and probably elsewhere in the Philippines); *M. binghami* from the Indonesian islands of Sulawesi and Sula; *M. laboriosa* from the Himalayan region: and *M. dorsata*, found over the India to the south-east Asia. Subsequent workers tended to ignore Maa's classification and recognized the four species of honeybee in the genus *Apis*, one species corresponding to each of Maa's genera or subgenera: *A. florea*, *A. dorsata*, *A. cerana* and *A. mellifera* (Ruttner,

Figure 1.1 Giant honeybees (*A. dorsata*)

1988). The genus *Apis* is morphologically rather distinct, and has well defined geographic boundaries. Consequently those of Maa classified giant honeybees have been much more readily accepted as valid taxa, at either the subspecific or specific level. Sakagami *et al*. (1980) clarified a taxonomic status of Himalayan giant honeybee as a separate species, *A. laboriosa* and showed distribution of *A. dorsata* as in Figure 1.2.

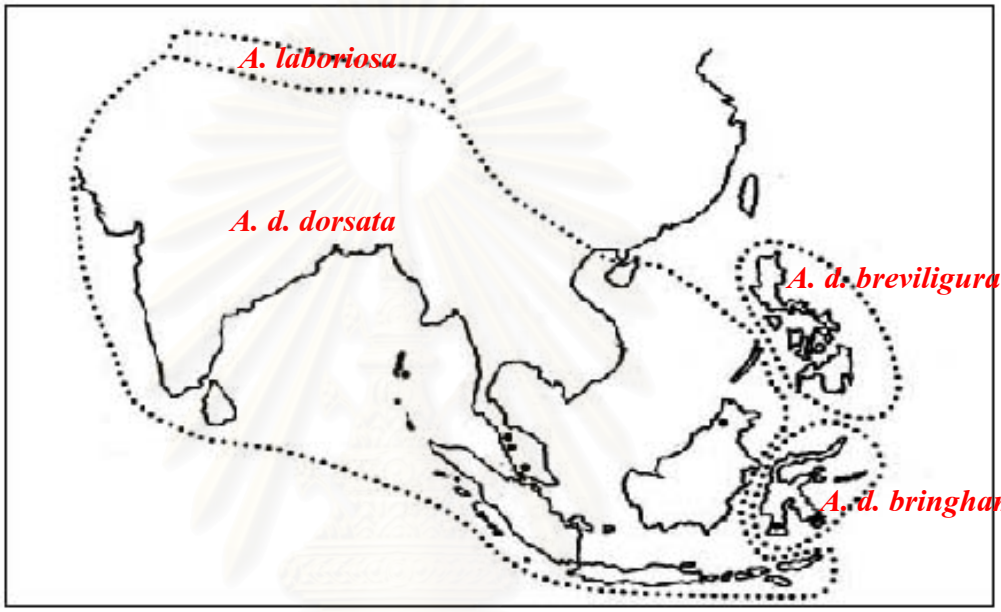

Figure 1.2 Distribution of *A. dorsata* in Asia with subspecies *binghami* and *breviligula* and the not yet definitely classified *laboriosa*. (Adapted from Ruttner, 1988)

The taxonomic categories of *A. dorsata* Fabricius, 1793 described from morphological characteristics by Ruttner (1988):

Kingdom: Animalia

Phylum: Arthropoda

Class: Insecta

Order: Hymenoptera

Family: Apidae

Genus: *Apis*

Species: *Apis dorsata*

Morphological characters have several drawbacks, such as their polygenic determinism, which hinders their use in population genetic studies. In recent years, various DNA markers have been developed. One has been mtDNA, which is particularly well suited for inferring phylogenetic relationships among the components of a species. In honeybees, the first studies confirmed the existence of three evolutionary branches, although slightly modifying their subspecies composition and their estimated time of divergence as inferred from morphometry (Garnery *et al*., 1992; Smith, 1991). More detailed studies are now exploring the evolutionary relationships among subspecies within the same branch.

Basic markers for population genetic analysis

Genetic analysis of natural populations has allowed biologists to ask a wide variety of questions which previously could only be answered by extensive observation of the group in question. A number of genetic markers have proven to be useful. These include protein and DNA markers.

1. Protein marker

Allozymes

An allozyme is a form of enzyme that has different alleles (forms) at the same genetic locus with different electrophoretic mobilities. The basic principle of allozyme electrophoresis is to run samples, using an electric current, through a medium (gel) that causes proteins to travel different distances through the gel, depending on size, shape and charge. These gels are then stained for a particular enzyme such that the location of the allele of the enzyme is indicated by coloured bands in the gel. The great advantage of allozyme electrophoresis is that heterozygous phenotypes can be identified because the alleles are codominant. Allozyme analysis is a technique of choice to begin with particularly when the species under investigation has not had any molecular analysis.

Although allozymes provide a straightforward genetic interpretation that is not susceptible to environmental effects, it is now well established that they exhibit a relatively low level of polymorphism in honeybees (Daly, 1991). This is thought to be a consequence of the haplodiploid genetic system in these species (Pamilo and Crozier, 1981). In the last decade techniques for the measurement of genetic variation in honeybees at the DNA level have been developed and are proving to be extremely powerful probes for the analysis of genetic variation. DNA analysis overcomes the limitations of morphometric characters and the relatively low polymorphism of allozymes. The main conclusion, however, is that honeybees have low variability for this set of traits (allozymes) and little differentiation between subspecies.

2. DNA markers

2.1 Mitochondrial DNA (MtDNA)

Mitochondria, the organelles responsible for aerobic metabolism in eukaryotic cells, possess their own small circular DNA molecules. Each cell may contain one to hundreds of mitochondria. Thus the first of the useful properties of mtDNA is its high copy number, while any nuclear gene may be present in just two copies per cell (one from each parent in diploid organisms). Some metabolically active tissues, such as eggs or insect flight muscle, are particularly rich in mitochondria and mtDNA. High copy number makes it easy to analyze PCR amplified directly from total DNA of animal tissues, without the necessity of isolating and cloning individual genes. The mtDNA of *A. mellifera* is showed in Figure 1.3.

Figure 1.3 Map of circular mitochondrial genome of the honeybee *A. mellifera* as described by Crozier and Crozier (1993).

Another useful characteristic of mtDNA is its small size. Most animal mitochondrial genomes consist of a single small, circular molecule, double-stranded DNA molecule of between 16,000 to 20,000 base pairs. The mtDNA sequence of *A. mellifera* was determined by Crozier and Crozier (1993). This organelle DNA contains 13 protein-encoding genes, the genes for 22 tRNAs and 2 rRNAs subunits, and a non-coding region containing the origin of replication. The transcribed regions are highly conserved. The non-coding A+T rich region of mtDNA evolves rapidly, while the genes such as those coding for the large and small subunits rRNA, or Cytochrome Oxidase subunits I and II (CO I and CO II) evolve at lower rates. Mutations in mtDNA are acquired faster than those in the nucleus, and thus provide a scale for measuring even recent population data. Most importantly, mtDNA is inherited maternally. The lack of recombination makes the history of its inheritance easier to trace and provides an

important tool for analysis recent population history. In addition, there has been significant study on the mtDNA genome, and much comparative information is available in the databases.

The mtDNA molecule of honeybees has been studied within and between populations. MtDNA variation can be identified from the DNA sequence: either some of the sequence from the large subunit ribosomal RNA gene (lrRNA) or non-coding intergenic cytochrome oxidase subunits I and II (inter CO I - CO II) (Cameron, 1993; Cornuet *et al*., 1991; Crozier *et al*., 1989; Crozier and Crozier, 1992; Koulianos and Crozier, 1996; Sittipraneed *et al*., 2001; Smith and Hagen, 1997; Smith *et al*., 2000). Besides this, mtDNA-RFLP is a technique used to evaluate levels of genetic variation in *A. mellifera* (Meixner *et al*., 1993; Meixner *et al*., 2000; Moritz *et al*., 1986; Sheppard *et al*., 1996; Smith and Brown, 1988, 1990; Smith *et al*., 1991). MtDNA-RFLP technique requires large amount of pure mtDNA. Recently, the Polymerase Chain Reaction (PCR) has provided a new technology for molecular population genetics. With this technique, millions of DNA molecules can be synthesised from the target DNA by the activity of thermostable DNA polymerase. The PCR-amplified fragments can be further employed for analysis of restriction site and fragment length polymorphisms in processes called PCR-RFLP. This technique has been used frequently to detect polymorphic mtDNA regions such as COI-COII region (Delarua *et al*., 1998; Garnery *et al*., 1993; Garnery *et al*., 1995; Meusel and Moritx, 1992). For honeybees in Thailand, mtDNA markers were used to study genetic variability of *A. cerana* populations using PCR-RFLP of various mtDNA regions, e.g. lrRNA gene, sRNA gene, intergenic CO I - CO II region and ATPase6-8 gene (Sihanuntavong, 1999; Songram, 1997).

2.2 Nuclear DNA

Nuclear genomes are much larger than mitochondrial genome. The size of nuclear genome range from $<10^6$ bp in some bacteria to $>10^{11}$ bp in some plants (Cavalier and Smith, 1985). The size of nuclear genome is about 3×10^9 bp in mammals. Diploid organisms have two copies of each genetic region (locus) on homologous pairs of chromosomes. These two

copies are called alleles, regardless whether they represent coding or non-coding regions of the genome. Coding regions (exons) are often interspersed with more variable non-coding regions (intron). Repetitive DNA consists of core sequences that are repeated in varying degree. They may be made up of coding segments such as the ribosomal RNA (rRNA) genes or non-coding tandemly repeated unit such as satellite, minisatellite and microsatellite DNA. Nuclear DNA has also been used as a genetic probe in honeybees, but most investigations have been directed to analyses of patriline structure.

Microsatellite

Microsatellites are short, 2-6 base pair, tandemly repeated sequences that occur randomly distributed through out the nuclear genome of all higher life forms. Microsatellite arrays are highly variable in length due to the change in the number of copies of the repeated sequence resulting from errors during replication of the DNA. Different microsatellites are defined for the purposes of assay and study by the unique sequences flanking them. Microsatellites generally occur in noncoding regions of the genome and their function if any is unknown. They are inherited in typically Mendelian fashion, that is each diploid individual has two copies (alleles) of the microsatellite one which has been passed on by the mother and one by the father. As there is a high degree of variability of sizes of microsatellite alleles it is not uncommon for an individual to have two different alleles at a locus and as the sizes of the alleles can be easily measured, and it is possible to determine which of the alleles was inherited from the mother and which from the father (Schlotterer, 1998).

Assay of microsatellites

The assay of microsatellites is carried out by polymerase chain reaction (PCR) amplification of a specific microsatellite region as defined by the unique primers for the microsatellite. Each microsatellite represents only a tiny portion of the whole genome and as such cannot practically be directly isolated and measured. These make it possible to amplify the target microsatellite millions of times and to produce sufficient copies of the microsatellite

to easily detect and measure the size of the PCR product on polyacrylamide gels. The use of very small amounts of DNA is very important as this allows noninvasive sampling which may be of critical importance in the case of rare or endangered species or in forensic applications where the available tissue sample may be very small. The assay of a very small portions of the genome also make it possible to study old partially-degraded DNA samples which can be recovered from bones or other biological material. Recently, microsatellites have been increasingly used as the marker of choice. There are some advantages to utilising microsatellites over the other markers, which make them desirable.

PCR analysis of small fragments also allows the analysis of degraded samples in which the mean fragment size of the genomic DNA has been severely reduced through environmental insult. Finally, microsatellites have been found to be variable even in populations which have low levels of allozyme and mitochondrial variation (Estoup *et al.* 1995, 1996). Microsatellites are useful for a number of analyses. They were originally utilised for genetic mapping and have been extensively used for linkage analyses in the association with disease susceptibility genes. In addition they have proven useful in the analysis of paternity and kinship and in the probability of sample identity at both the individual and population levels. In the study of entire populations microsatellites are also very useful. Microsatellite variation has been used to study the amount of hybridization between closely related species (Estoup, 1996). Comparison of levels of variation between species and populations have also proven useful in the assessment of overall genetic variation. The analysis of population substructure will be the focus of the following review.

Presently there are a wide variety of procedures which can be utilised to analyse microsatellite data. These include various estimates of genetic distance between populations. This illustrates the wide variety of treatments microsatellite data has received to estimate the same parameters, mainly genetic distance and population substructure (Murray, 1996).

Study on genetic variation of *A. dorsata* using DNA markers

A recent study on the mtDNA of *A. dorsata*, using RFLP techniques have indicated that there are three main lineages of *A. dorsata*, differentiated by their mitochondrial haplotypes. The first includes the samples from Pakistan, the Andamans, Thailand, Malaysia and Borneo (these bees are called the "mainland group"); a second group consists of the samples from Southern India; and a third consists of the samples from Sulawesi (Smith, 1991).

Several studies have reported genetic variation and distribution of population of *A. mellifera* using the polymorphism of mitochondrial intergenic CO I – CO II region as studied by the sequence technique (Cornuet *et al*., 1991; Crozier and Crozier, 1992; Willis *et al*., 1992) and RFLP technique (Garnery *et al*., 1993; Meusel and Moritz, 1992). Smith (1991) studied genetic variation of *A. cerana* in the south-east Asia using RFLP of mtDNA and the variation found in this region can be distinguished in 3 groups: 1) mainland (Japan, Thailand, Malaysia, Borneo and south of India), 2) Luzon in the Philippines and 3) the Andaman Islands.

Besides the above studies, Smith and Brown (1988) studied polymorphism in the restriction enzyme cleavage sites of honeybee mtDNA. These polymorphisms are potentially useful for the identification of Africanized bees (*A. mellifrea*) in the Western hemisphere and for study of honeybee phylogeny. In addition, Smith *et al*. (1991) studied restriction enzyme cleavage maps of mtDNA from the Spanish honeybees, *A. mellifera liberica* were compared with those from the other European subspecies (*A. m. mellifer*, *A. m. ligustica* and *A. m. carnica*) and the African subspecies (*A. m. intermissa* and *A. m. scutellata*). Two types of mtDNA were found in Spanish honeybees, one is a west European type like that of *A. m. mellifera*, and the other is an African type like that of *A. m. intermissa*.

MtDNA length variation in the cytochrome oxidase region of *A. mellifera* was studied by Meusel and Moritz (1992). They found that RFLP analysis with *Bcl* I of *A. m. capensis*

mtDNA showed a length variation in the CO I – CO II gene region. These can be used to distinct south African Cape (*A. m. capensis*) honeybees from European honeybees (*A. m. carnica*) honeybees.

Garnery *et al*. (1995) studied mtDNA variation in Morocco and Spanish honeybee populations by a rapid test involving the restriction by *Dra* I of a PCR-fragment of the CO I – CO II intergenic region. In Morocco, they found eight haplotypes, all of the African lineage, which were most probably colonized by two sublineages, one from the north-east and the other one from the south of the country. In Spain, they found eight haplotypes of the African lineage (six in common with Morocco) and four haplotypes of the west European lineage. The relative distribution of both lineages in Spain clearly demonstrates a south-north gradient extending from 10% of the west European lineage in Seville (south) and up to 100% in San Sebastain (north). Comparing Spanish and Moroccan frequencies of haplotypes of the African lineage, both countries have specific rare types.

Sheppard *et al*. (1996) examined mtDNA variation among endemic honeybees, *A. mellifera* subspecies using the restriction enzyme *Hin*f I. Twenty different mtDNA haplotypes were identified and their distribution among and within the subspecies improved resolution of relationships among some racial groups and provided unambiguous identification of some taxa.

Deowanish *et al*. (1996) studied mtDNA variation of *A. cerana* from Japan, Korea, Taiwan, Vietnam, Thailand, Nepal and the Philippines using RFLP analysis. Total DNA was digested using ten restriction enzymes. After digestion, DNA fragments on the gel were Southern-blot-transferred to nylon membrane and hybridized with labeled probe from $tRNA^{Leu}$ to CO II region. She could discriminate among different localities including groups from: 1) Japan, 2) Nepal, Vietnam and the north to central Thailand, 3) Korea-Tsushima, 4) Taiwan, 5) south of Thailand and 6) the Philippines.

The mtDNA of individuals from 79 colonies of *A. mellifera* from five Canary Islands was studied on the restriction of PCR products of the $tRNA^{leu}$ - CO II intergenic region with *Dra* I (Delarua *et al*., 1998). Fives haplotypes of the African lineage and one of the west European lineage were found. Besides this, two haplotypes (A14 and A15) are described for the first time. The wide distribution and high frequency of haplotype A15 suggest that it is characteristic of the Canarian Archipelago. The results of this study are in agreement that the colonization of the Canary Islands was carried out by successive waves of colonization from Africa rather than from Spanish population.

Thirty-nine samples of *A. mellifera monticola* and *A. m. scutellata* from three different regions of Kenya were analyzed for mtDNA variation using RFLP. Restriction digests with *Hpa* II and *Alu* I resulted in distinct patterns that together produced three different haplotypes. The haplotypes 2 and 3 were restricted to samples from the mountain forest, haplotype 1 was found in *A. m. scutellata* and in all samples from the Ngong Hills. These results support that *A. mellifera monticola* is a distinct subspecies and not an ecotype of *A. m. scutellata* (Meixner *et al*., 2000).

Smith *et al*. (2000) added new data from Korea and the Philippines to earlier mtDNA-based studies of the phylogeography of Asian cavity-nesting honeybees. A non-coding region that lies between the leucine tRNA gene and the cytochrome oxidase II gene of the mitochondrial genome of *A. cerana* and *A. nigrocincta* was sequenced. Fourty one different haplotypes were found and allocated *A. cerana* into 4 groups 1) an Asian mainland group, 2) a Sundaland group, 3) a Palawan group and 4) a Luzon-Mindanao group and *A. nigrocincta* into one group.

Microsatellite DNA is a codominant marker that can be very useful for population genetics, especially in species where allozyme variability is low. In *A. mellifera*, Estoup *et al*. (1993) have shown that microsatellites, which represent an abundant class of variable markers

in honeybee, enable highly precise dissection of the genetic structure of colonies. Furthermore, Estoup *et al*. (1994) present a precise assessment of the number of patriline and of genetic relatedness in honeybee colonies by screening their samples with a number of polymorphic microsatellite loci. They identified the 7-20 subfamilies (patrilines) present in five honeybee colonies belonging to three different subspecies (*A. m. mellifera*, *A. m. carnica* and *A. m. ligustica*). They showed that the genetic structure remained largely unchanged over time as long, as the colony is headed by the same queen.

In 1995, Estoup *et al*. used microsatellite technique to investigate microsatellite variation in *A. mellifera* subspecies from nine populations. A large amount of genetic variation was detected. Microsatellite analyses confirmed that *A. mellifera* evolved in three distinct and deeply differentiated lineages previously detected by morphological and mtDNA studies.

Moreover, genetic differentiation of European continental and island populations of *Bombus terrestris* (Hymenoptera: Apidae) has been studied using 10 microsatellite loci and a partial sequence of the COII mitochondrial gene (Estoup *et al*., 1996). No significant differentiation was found among populations of *B. terrestris* from the continent. In contrast, island populations were all significantly and most of them strongly differentiated from continental populations. Microsatellite and mtDNA data call for the protection of the island populations of *B. terrestris* against importation of bumble bees of foreign origin which are used as crop pollinators.

Furthermore, microsatellite have been used to study the levels of polyandry and intraconial genetic relationships in *A. florea* (Oldroyd *et al*., 1995), *A. dorsata* (Moritz *et al*., 1995; Oldroyd *et al*., 1996, Wattanachaiyingcharoen *submitted*), *A. andreniformis* (Oldroyd *et al*., 1997), and *A. cerana* (Oldroyd *et al*., 1998). Oldroyd et al. (1996) found *A. dorsata* queens mated with mean of 26.75± 5.42 drones, deduced from microsatellite genotypes at 3 loci (A14, A88, B124) for 42-194 workers from 5 colonies. However the most recent study has

shown *A*. *dorsata* queens can mate with more than 100 drones (Wattanachaiyingcharoen *submitted*). This apparent increase in mating frequency is probably due to the increased resolution of microsatellite markers and increased sample size in the latter study.

In 1998, Franck *et al*. examined the origin of west European subspecies of *A. mellifera* by analysing at 8 microsatellite loci and *Dra* I RFLP of the CO I – CO II mtDNA marker. When considering microsatellite markers for the seven loci (B124, A43, A88, A8, A113, A28, A24 and A7), Iberian populations do not present any trace of 'africanization' and are very similar to French populations. Therefore, the Iberian Peninsula is not a transition area.

CHAPTER II

MATERIALS AND METHODS

2.1 Instruments

- Autoclave HA-30 (Hirayama Manufacturing Co., Japan)
- Automatic micropipette P2, P10, P020, P100, P200, P1000 (Gilson Medical Electrical S.A., France)
- Electrophoresis apparatus
  - : Horizontal agarose gel electrophoresis apparatus
    - * Taitec Pico-2 (Taitec Corporation, Japan)
    - * MGU-402T (C.B.S. & Scientific Company, California)
  - : Power supply - EPS 250 (C.B.S. & Scientific Company, California)
  - : Vertical gel electroporesis apparatus for DNA sequencing - #DDH-400-33 (C.B.S. & Scientific Company, California)
  - : Power supply – ESP 3501 (Amersham Pharmacia Biotech)
- -20^{o}C freezer model WCF-657 (Whirlpool)
- Gel dryer model 583 (BIORAD Laboratories, U.S.A.)
- High speed microcentrifuge model 1120 (Kubota Corporation, Japan)
- Heating box (Boekel Scientific)
- Incubator BM-600 (Memmert Gamblt, Germany)
- Light box 2859 SHANDON (Shandon /scientific Co. Ltd., England)
- Microwave model TRX-249I (Turbora)
- PCR-Minicycler^{TM} model PTC-150 (MJ Research Inc., U.S.A.)
- Polaroid camera model CH-1314 (Spectronics Corporation, U.S.A.)
- Precisa-Balances model 180A (PAG OERLIKON AG, Switzerland)
- Shaker model VRN-360 (Gemmy Industrial Corp., Taiwan)
- Stirrer/Hotplate model PC-320 (Corning, U.S.A.)

- Ultraviolet Transilluminator model TVC-312R/F (Spectronics Corporation, U.S.A.)
- Vortex Genie-2 model G560E (Scientific Industries Inc., U.S.A.)
- Water Baths model SH28L (Polyscience, U.S.A.)

2.2 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)

2.3 Chemicals

- Absolute ethanol (Merck, Germany)
- Acetic acid (Merck, Germany)
- Acrylamide (Sigma, U.S.A.)
- Agarose, Seakem LE (FMC Bio products, U.S.A.)
- Ammonium persulfate (Promega, 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)
- Formaldehyde (BDH, England)
- GeneAmp PCR core reagent (GibcoBRL, U.S.A.) : research use only
  : *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)

- *N,N*-methylene-bis-acrylamide (Fluka, switzerland)
- *N, N,N',N'*-tetramethylenediamine (Fluka, Switzerland)
- OmniBase™ DNA cycle sequencing system kit (Promega, U.S.A.) : research use only
  : 5X DNA sequencing buffer
  : d/ddNTP nucleotide mixes
  : DNA sequencing stop solution
  : 5 U/µl Sequencing Grade *Taq* DNA Polymerase
  : 24 mer pUC/M13 forward primer
  : pGEM3Zf(+) control DNA
- Phenol saturated solution (Amresco, U.S.A.)
- Silver nitrate (BDH, England)
- Sodium acetate (Merck, Germany)
- Sodium carbonate (Merck, Germany)
- Sodium chloride (Merck, Germany)
- Sodium dodecyl sulfate (SDS) (Sigma, U.S.A.)
- Sodium thiosulfate (Sigma, U.S.A.)
- Tris-(hydroxy methyl)-aminomethane (Fluka, Switzerland)
- Urea (Merck, Germany)
- Xylene cyanol FF (Sigma, U.S.A.)

2.4 Oligonucleotide primers

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

2.5 Enzymes

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

2.6 Samples collections

A. dorsata samples were collected as pupae or adults. Shortly after collection in the field, samples were either frozen or equilibrated in several changes of ice cold 95% ethanol for 2-4 days. Alcohol preservation allowed shipping or transport at ambient temperatures. For storage thereafter, samples were kept at –20^{o}C. A total samples of 160 colonies were collected from a wide range of sites in the natural distribution of *A. dorsata*. The sampling areas were composed of 3 geographically different locations indicating 1) Northern (North, North-East and Central) 2) Southern and 3) Samui Island.

Fig. 2.1 Collection sites of *A. dorsata* in Thailand.

2.7 DNA extraction

In most cases, total DNA was prepared from individual bees using the modified method of Hall and Smith (1991). A thorax of each bee was cut with sterile scissors and homogenized in a microcentrifuge tube containing 500 μl STE buffer (100 mM NaCl, 50 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0). Then 25 μl 20% SDS solution and 25 μl 10 mg/ml Proteinase K were added. The mixture was incubated in 55°C water bath until the tissue was dissolved (2 hours) and the suspension was centrifuged at 4,000 xg for 10 minutes. The supernatant was extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), once with an equal volume of chloroform/isoamyl alcohol (24:1). After each extraction, phases were separated by centrifugation at 4,000 xg for 10 minutes. The upper phase was precipitated with one-fifth volume of 3 M sodium acetate pH 7.4 and 2 volumes of cold absolute athanol at –20°C overnight and then centrifuged at 6,000 xg for 10 minutes, repeated after rinsed the pellet with 70% ethanol. The DNA pellet was air-dried and resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0). The DNA solution was incubated at 37°C for 1-3 hours for complete redissolved and kept at 4°C for the next step.

2.8 Agarose gel electrophoresis / Concentration of DNA samples

Agarose gel electrophoresis is the standard method used to separate, identify and purify of DNA fragments under the influence of an applied electrical field on the basis of their molecular weight. The technique is simple, rapid to perform and capable of resolving fragments of DNA that cannot be separated adequately by other procedures, such as density gradient centrifugation. Furthermore, the location of DNA within the gel can be determined directly by staining with low concentrations of the fluorescent intercalating dye ethidium bromide; bands containing as little as 1-10 ng of DNA can be detected by direct examination of the gel in ultraviolet light.

Agarose gels were cast by melting the agarose in the presence of the desired buffer (1X TBE : 89 mM Tris-HCl, 89 mM boric acid, 2.5 mM Na_2EDTA pH 8.3) until a clear,

transparent solution was achieved, and cooled down to 60^{o}C in waterbath. The melt solution was then poured into a mold and allowed to harden. Upon hardening, the agarose forms a matrix, the density of which is determined by the concentration of the agarose. Sample was mixed with one-fifth volume of the loading dye buffer (15% Ficoll400, 0.25% bromphenol blue, 0.25% xylene cyanol FF). Then, an electric field was applied across the gel, DNA, which is negatively charged at natural pH, migrates toward the anode. When electrophoresis is completed, the gel was stained with ethidium bromide solution and destained in distilled water. In order to remove the unbound ethidium bromide from gel. DNA bands were visualized under the UV transilluminator and photographed through a red filter using Kodak Tri-X-pan 400.

Before further analysis, it is important to determine the concentration of the extracted DNA and quality of the extracted DNA. This can be done by comparison of fluorescent intensity with known concentration of DNA standards on agarose gels. Usually, 0.7% gel concentration gels are useful for assessing both the quantity and the quality of the genomic DNA. For 500 ng of standard DNA (λ/*Hin*d III), the orange-red fluorescent intensity of standard bands of 23.1, 9.4, 6.6, 4.4, 2.3, 2.1 and 0.6 Kb corresponding to 240, 98, 67, 45, 24, 21 and 6 ng DNA, respectively. The extracted DNA concentration of each individual bee was adjusted to approximately 25 ng/µl.

2.9 PCR amplification

The polymerase chain reaction (PCR) is a technique which allows an exponential increase in the amount of any given DNA template by the simultaneous primers extension of two complementary strands of DNA. This technique has revolutionized molecular biology because it has both simplified existing technology and enabled the rapid development of new applications which would not otherwise have ever been possible. The DNA amplification is achieved by the used of synthetic oligonucleotides, termed primers, that are forward and reverse primer. DNA polymerase is then used to carry out the synthesis of a complementary strand of DNA in the 5′ to 3′ direction of sense and antisense strands using the

oligonucleotides as primer. The power of this technique is that for each heating and cooling cycle, the amount of DNA is doubled, such that after only 20 cycles the yield of PCR product is approximately one million copies from each starting target DNA molecule. The basic reaction is set up in a microcentrifuge tube and placed in a thermocycler machine which is programable to carry out the phases of the PCR cycle automatically. PCR reactions require typically three phases: firstly the DNA template has to be denatured (92-94^{o}C); secondly the primers have to be annealed (40-65^{o}C) and thirdly DNA polymerase extends the annealed primers along the single-stranded template (72^{o}C). The purity of DNA is crucial for any PCR reaction since any contaminant DNA will be amplified alongside if the primers can anneal to it and any protein left attached to the DNA will inhibit the reaction. To avoid any nuclease contamination, gloves should be worn throughout. All pipettes, glassware and solutions should be autoclaved before use. In order to avoid the contamination of foreign DNA in PCR reaction, it is often advisable to keep solutions and equipment used for PCR separate from other laboratory uses. If separate micropipettes cannot be afforded it is often useful to sterilize them under UV light prior to use.

2.9.1 Amplification condition

Four different regions (Intergenic CO I – CO II, Cytb I - tRNA^{Ser}, lrRNA and ATPase6-8) of *A. dorsata* mitochondrial DNA were *in vitro* amplified by PCR, with a set of primers based specifically on sequences from *A. mellifera* mitochondrial DNA as Table 2.1 (Delarua 1998; Crozier and Crozier 1993). Twenty five nanograms of extracted DNA was used as template in each PCR reaction tube, along with the following reagents: 0.1 μM of each primer, 1X PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), 2.0-5.0 mM $MgCl_2$, 100 μM of each dNTPs (dATP, dCTP, dGTP, dTTP) and 0.6 unit of *Taq* DNA polymerase. The total volume was made up to 25 μl with sterile distilled water. The 35 reaction cycles were performed in a thermocycler machine (Mini-cycler^{TM} PTC-150, MJ Research Inc., U.S.A.) using the program : 94^{o}C for 1 minute following by 35 cycles of a 92^{o}C denaturation for 1 minute, a 40-60^{o}C (60^{o}C, 48^{o}C, 50^{o}C and 40^{o}C for Intergenic CO I – CO II, Cytb I - tRNA^{Ser}, lrRNA and ATPase6-8,

respectively) annealing for 1 minute and a 72°C extension for 2 minutes. The final extension was carried out at 72°C for 10 minutes. Three microliters of each PCR product was analyzed by 0.7% agarose gel electrophoresis and visualized on UV light after staining with ethidium bromide. The amplified products were stored at 4°C until used.

Table 2.1 Primer sequences used for PCR amplification in *A. dorsata* mtDNA. Primers sequences were taken from Crozier & Crozier (1993) and Delarua (1998).

Region	Primers	Coordinates
Intergenic CO I – CO II		
-E3	TCTATACCACGACGTTATTC	3090-3109
-COII	CCACAAATTTCTGAACATTGACC	4191-4213
Cytb I-tRNA^{Ser}		
-Cytb I	TATGTACTACCATGAGGACAAATATC	11420-11425
-tRNA^{Ser}	GAAAATTTTATTTCTATATTATATTTTCA	12230-12258
LrRNA		
-L1	CTATAGGGTCTTATCGTCCC	13707-13728
-L2	TTTTGTACCTTTTGTATCAGGGTT	14424-14447
ATPase		
-ATPase6	TTTAATTCCTCAAATAATAC	4440-4459
-ATPase8	TTAATTTGATTCAGAGAAAT	5244-5264

2.9.2 PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism)

Digestion of PCR products with restriction endonucleases that cleave at different recognition sites is the important process of RFLP analysis. Complete digestion is essential to obtaining interpretable RFLP patterns. PCR products of each region were digested with restriction endonucleases that shown in Table 2.2. The reaction mixture was made up to a final volume of 20 μl by adding the following regents in the given order : 500 ng of PCR product,

1X reaction buffer (as recommend by the manufactor), 5 units of restriction endonuclease and adjusted volume to 20 μl with sterile distilled water.

Table 2.2 Restriction enzyme used to determine genetic variation in *A. dorsata.*

Enzyme	Restriction site	Enzyme	Restriction site
Alu I	AG′CT TC′AG	*Hinf* I	G′ANTC CTNA′G
*Bam*H I	G′GATCC CCTAG′G	*Nde* I	CA′TATG GTAT′AC
Bgl II	A′GATCT TCTAG′A	*Pst* I	CTGCA′G G′ACGTA
Dra I	TTT′AAA AAA′TTT	*Sau3*A I	′GATC CTAG′
*Eco*R I	G′AATTC CTTAA′G	*Spe* I	A′CTAGT TGATC′A
*Hin*d III	A′AGCTT TTCGA′A	*Xba* I	T′CTAGA AGATC′T

Mix by flicking the tube repeatedly with finger and then centrifuge for a few seconds to bring the reaction mixture to the bottom of the tube. Incubate at 37°C (or the temperature appropiate for each restriction endonuclease) in the heating block or water bath for 3-4 hours. However, larger amounts of DNA may take longer. Stop reaction by adding one-fifth volume of loading dye. The reaction fragments were separated on 2% metaphor agarose gel which has resolution higher than agarose gel. The metaphor agarose gel was prepared by weighting out an enough amount, dissolved in 1X TBE buffer and melted until a clear. The melted solution was poured into the chamber set. The total digestion volume was loaded into the gel. Electrophoresis using 1X TBE buffer at 10 volts/cm. The gels were stained with ethidium bromide and photographed under UV light.

2.10 Amplification of microsatellite DNA

Microsatellites are a class of DNA marker that involve a variable number of tandem repeats of 1-6 bp. The different alleles are characterized by the exact length in base pairs of a DNA fragment obtained by the polymerase chain reaction (PCR) performed between two fixed sequence motifs flanking the tandem repeat region. Before a specific microsatellite locus can be amplified, one needs sequence information for the flaking DNA to allow the design of specific primers. Primer sequences for the 13 *A. mellifera* microsatellite loci were used to screen for polymorphic loci *in A. dorsata.* These primer sequences were taken from Estoup *et al*. (1994, 1995) and Oldroyd *et al*. (1997, 1998) that shown in Table 2.3.

2.10.1 PCR of microsatellite loci

PCR of microsatellite loci were carried out in 12.5 μl of a mixture containing 12.5 ng of DNA template, 0.4 μM of each primer, 1X PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), 0.6-1.5 mM $MgCl_2$, 100 μM of each dNTPs (dATP, dCTP, dGTP, dTTP) and 0.4 unit of *Taq* DNA polymerase. After a denaturing step of 1 minute at 94^{o}C, samples were processed through 30-35 cycles consisting of 1 minute at 92^{o}C, 30 seconds at an optimal annealing temperature (50-60^{o}C) and 2 minutes at 72^{o}C. The last elongation step was lengthened to 10 minutes at 72^{o}C. It is important to optimize PCR condition of each microsatellite loci (the microsatellite DNA of *A. dorsata* was amplified with the primer Table 2.3 under standard condition used in *A. dorsata* (Oldroyd *et al*., 1995) and the $MgCl_2$, dNTPs, primer, *Taq* DNA polymerlase and DNA template concentration were optimized to used for PCR reaction). After the thermal cycling program was completed, 8 μl of loading dye (95% NaOH, 0.05% bromophenol blue, 0.05% xylene cyanol FF.) was added before loading to 8% polyacrylamide sequencing gel.

Table 2.3 Primer sequences of the 13 *A. mellifera* microsatellite loci used to screen for polymorphic loci in *A. dorsata.* Primer sequences were taken from Estoup *et al*. (1994, 1995) and Oldroyd *et al*. (1997, 1998).

Locus	Core sequence	Primer sequence (5′ to 3′)
A7	$(CT)_3(T)_7CCTTCG(CT)_{24}$	GTT AGT GCC CTC CTC TTG C CCC TTC CTC TTT CAT CTT CC
A8	-	CGA AGG TAA GGT AAA TGG AAC GGC GGT TAA AGT TCT GG
A14	$(CT)_{13}$……$(GGT)_9$	GTG TCG CAA TCG ACG TAA CC GTC GAT TAC CGA TCG TGA CG
A24	$(CT)_{11}$	CAC AAG TTC CAA CAA TGC CAC ATT GAG GAT GAG CG
A28	$(CCT)_3GCT(CCT)_6(CT)_5TT(CT)_4$	GAA GAG CGT TGG TTG CGA GG GCC GTT CAT GGT TAC CAC G
A29	$(GT)_{24}$	AAA CAG TAC ATT TGT GAC CC CAA CTT CAA CTG AAA TCC G
A35	$(GT)_{14}$	GTA CAC GGT TGC ACG GTT G CTT CGA TGG TCG TTG TAC CC
A43	$(CT)_{13}$	CAC CGA AAC AAG ATG CAA G CCG CTC ATT AAG ATA TAT CCG
A79	$(CT)_{14}$	CGA AGG TTG CGG AGT CCT C GTC GTC GGA CCG ATG CG
A81	-	GCC GAG TTC TTC GAC TCC C GGA CTT TGC CAA ATG GGT C
A88	$(CT)_{10}TC(CCTT)_2(CTTT)_3$…$(GGA)_7$	GCG AAT TAA CCG ATT TGT CG GAT CGC AAT TAT TGA AGG AG
A107	$(GCTC)_2(GCT)_2(CT)_{23}$	CCG TGG GAG GTT TAT TGT CG CCT TCG TAA CGG ATG ACA CC
A113	$(TC)_2C(TC)_2TT(TC)_5TT(TC)_8TT(TC)_5$	CTC GAA TCG TGG CGT CC CCT GTA TTT TGC AAC CTC GC

2.10.2 Standard marker of microsatellite loci

DNA vector, pGEM-3Zf(+) is a derivative of the pGEM-3Zf which contains the origin of replication of the filamentous phage f1. The plasmid serves as a standard cloning vector, as a template of *in vitro* transcription, and can be used for the production of circular ssDNA. The ssDNA can be sequenced using pUC/M13 Forward Primer (24 mer). The pGEM-3Zf(+) sequencing marker was prepared using OmniBase™ sequencing kit. For each set, four sequencing reactions contained 4 μg of pGEM-3Zf(+) control DNA, 5 μl of 5X DNA sequencing buffer (250 mM Tris-HCl pH 9.0, 10 mM $MgCl_2$), 4.5 pmol of pUC/M13 Forward primer. The total volume was made up to 16 μl with sterile distilled water and 1.0 μl of Sequencing Grade *Taq* DNA Polymerase (5U/μl) was added to the primer/template mix. Then four microliters of the enzyme/primer/template mix was added in to four separate 0.5 ml microcentrifuge tubes which each tube contained d/ddNTP mix. Then the following cycling program was performed by 60 amplification cycles of 95°C denaturation for 30 seconds and a 70°C annealing/extension for 30 seconds. After the thermal cycling program was completed, 3 μl of DNA Stop Sequencing Solution (95% NaOH, 0.05% bromophenol blue, 0.05% xylene cyanol) was added to the inside wall of each tube. The reaction mixture was heated at 70°C for 2 minutes and immediately snap-cooled on ice before loading of each reactions on a polyacrylamide sequencing gel.

2.11 Preparation of the polyacrylamide gel

Polyacrylamide gel is formed by the polymerization of acrylamide monomers in to long chains cross-linked by *N,N′*-methylenebisacrylamide units. The pore size is determined by the concentration of the acrylamide monomers in the polymerizing solution. The DNA products of sequencing reaction or amplified product of microsatellite DNA was generally separated in denaturing polyacrylamide gel. The gel was run at constant power and then the banding pattern was revealed with silver staining.

Polyacrylamide gel is electrophoresed vertically with the gel enclosed between two glass plates. Typically, the plates are 40 cm long and 20 cm wide. This long enough to achieve a good separation for 200-300 bands, and wide enough to accommodate 32-40 lanes. The two plates are not the same size. One plate is shorter than the other and may have “ears” or “notches” at either side. The inner surfaces of the two plates are totally dust-free, having been cleaned with water and rinsed in ethanol. Silanizing solution (2 μl bind silane, 950 μl 95% ethanol and 50 μl acetic acid) has also been applied to the two inner surfaces. This solution can be a silicone polish, which prevents the gel adhering to the plates when the assembly is taken apart after electrophoresis. The two plates are held apart by plasticard spacers (1 cm in width) laid down either side of the assembly. These spacers determine the thickness of the gel, which in turn has an influence on the sharpness of the bands that are eventually obtained. It is best to hold the assembly together with tape. Make sure that there are no wrinkles in the tape along the sides and the bottom of the plates, and arrange the tape very neatly around the corners to prevent leakage. The gel was prepared by used 50 ml of 8% denaturing acrylamide gel (76% acrylamide, 4% bis-acrylamide, 7.66 M urea) in 1X TBE buffer (89 mM Tris-HCl, 89 mM boric acid and Na_2EDTA pH 8.3) and then added 242 μl of 10% ammonium persulfate and 48 μl of TEMED (*N,N,N′,N′*-tetramethylene diamine) mixed together. Hold the plates at just the right angle, so the gel mix flowed down one side and gradually filled the space between the plates. As soon as the space between the plates was filled, laid the assembly down. Slide the comb into the open end to a depth about 0.5 cm. The acrylamide would have polymerized to be completed for at least 1 hour.

When the gel was fully polymerized the plate assembly were placed to the electrophoresis apparatus. The tape was removed from the plates. Slid the comb out, being careful not to damage the top of the gel. The electrophoresis apparatus has two buffer reservoirs, top and bottom, which were filled 1X TBE buffer after the plates had been clamped in position. Then the sharkstooth comb was re-inserted on the top of the gel. The gel was pre-run at constant power of 50 watts for 30 minutes. Before loading, the samples were heated at

95°C for 10-15 minutes to detach the chain terminated molecules from the template DNA and immediately snap-cooled on ice. The gel was run at constant power of 50 watts for 4-5 hours.

2.12 Detection of microsatellite alleles using silver staining

Silver staining procedure of Bassam *et al*. (1991) was used to detect non-isotopic amplified products. Classical silver stain provides a cheap and sensitive alternative to radioactive detection of nucleic acid. However, sensitivity is too low for reliable detection of a sequencing ladder on gel. Furthermore, strand-specific staining is not possible, resulting in the problems mentioned above. A silver staining involves several washing steps and the use of supporting sheet on which the gels is covalently bond is highly recommended. Alternatively, using binding silane (Phamarcia) to fix the gel on one glass plate. System staining procedure consists of just a few steps. After electrophoresis, the gel was fixed in 10% acetic acid and agitated well for 20 minutes or until the tracking dye was no longer visible to remove electrophoresis buffer and urea from the gel and to prevent diffusion of small extension products. Excess acetic acid and urea/Tris-borate were removed from the gel by rinsing three times (2 minutes each) with ultrapure water. Next, the gel was transferred to staining solution (200 ml of 10% silver nitrate and 300 µl of 37% formaldehyde) and agitated well for 30 minutes. Subsequently, the gel was rinsed very briefly in ultrapure water to remove excess silver and immediately developed in complete preparation of the developing solution (400 ml of 10% sodium carbonate that chilled on ice and immediately before use, 600 µl of 37% formaldehyde and 80 µl of sodium thiosulfate) at 10°C and agitated until the first bands were visible. The developing solution was pored off. The gel was covered again with the new developing solution and continue developing until the desired band intensity was achieved. Under these conditions, The silver ions were reduced to metallic silver by the formaldehyde. The rate of the reaction was temperature-dependent and was stopped by the addition of 10% acetic acid (already used in the fix stop) and incubated with shaking for 2-3 minutes. The gel was then rinsed for 2 minutes each time in ultrapure water to remove excess reactants and kept

on a piece Whatman 3 MM paper. The gel was dried at 80^{o}C under vacuum and photographed for a permanent record. (Brown 1994)

2.13 Size estimation of amplified microsatellite alleles

After amplification process was completed. The size of amplified microsatellite DNA was estimated by using denaturing polyacrylamide gel electrophoresis. The amplified products were electrophoresed on a 8% denaturing polyacrylamide gel and pGEM-3Zf(+) standard sequencing marker was run on the same gel as size standard. Sizes of microsatellite alleles of each locus were estimated by comparing with pGEM-3Zf(+) standard sequencing marker.

2.14 Data analysis

2.14.1 PCR-RFLP analysis

An alphabets name (A, B, C,…..) were used to code for the restriction patterns from each region in order of appearance. The composite haplotypes were generated from combination of restriction patterns of each region with an alphabetical arranging from the lowest to the highest number of restriction patterns. The bands of restriction fragments were organized into 1-0 matrix. The genetic distance between mtDNA composite haplotypes, haplotype and nucleotide diversity within populations, nucleotide divergence between populations, and geographic heterogeneity in frequency distribution among populations were calculated by using Restriction Enzyme Analysis Package, REAP (McElroy *et al*., 1991).

2.14.2 Analysis of microsatellite data

Mutation rates ranging from 10^{-3} to 10^{-6} are high enough to generate sufficient polymorphisms to provide high resolution. Interestingly, the microsatellite mutation pattern very closely fits the step-wise mutation model which has been studied intensively by population geneticists in order to explain patterns of allozyme variation. On this basis it is possible to introduce genetic distance, a widely used measurement in population genetics, to microsatellite analysis. A genotype of each *A. dorsata* individuals that could be divided to

homozygotic and heterozygotic states was scored from an electrophoretic pattern for each microsatellite locus. Based on the fact that stuttered bands were commonly observed in dinucleotide microsatellite. Therefore, scoring of a particular band can be carried out by making an assumption that an actual band of a given allele was the most intense band among the group of stutter band. The size of alleles were estimated in base pair length (bp) by relatively compared to the pGEM3Zf(+) sequencing marker. Each *A. dorsata* individuals was recorded to be either homozygote and heterozygote. The allelic stages were also recorded from each individual for each locus.

Data obtained from the polymorphic loci were included in the analyses. Allele frequencies, mean number of alleles per locus, observed and expected heterozygosity were estimated from a GENEPOP computer package version 2.0 (Raymond and Rousset, 1995).

Allele frequency and genetic variation

The frequency of a particular allele in a population for diploid organisms is given by

$$p = \frac{(2N_{AA} + N_{Aa})}{2N}$$

when p is the frequency of the A allele, N_{AA} and N_{Aa} are the number of homo- and heterozygotes for that allele and N is the number of individuals examined.

Genetic variation within populations was measured in terms of heterozygosity (H) which is the proportion of heterozygous individuals in all investigated samples, therefore observed heterozygosity can be estimated as

$$H = \frac{\sum (N_{Aa} / N)}{n}$$

When *n* is the number of investigated loci. When determined populations conform Hardy-Weinberg expectation, the unbiased estimated of heterozygosity (expected heterozygosity) can be calculated as

$$h = 1 - \sum p\imath^2$$

when *pi* is the frequency of i^{th} allele at a given locus, expected heterozygosity across all loci is the mean of *h* from each locus.

Hardy-Weinberg Equilibrium

Hardy-Weinberg Equilibrium of genotype frequency at a given locus for each investigated population should be examined. The observed genotype frequencies are concordant to Hardy-Weinberg expectation when there are no significantly disturbing force e.g. selection, mutation or migration changing allele frequencies over time and mating is actually occurred at random in a large population. Genotype frequencies in each population at each locus were tested for conformity to Hardy-Weinberg Equilibrium using a Markov chain "approximation to exact test" followed the algorithm of Guo and Thomson (1992) and implemented in GENEPOP version 2.0.

Geographic heterogeneity analysis

The statistically significant difference in genotype frequencies between *A. dorsata* from a pair of geographic sampling locations were tasted using the exact test of differentiation using GENEPOP version 2.0. Results are expressed as the probability of homogeneity between compared populations or regions. To diminish type I error, level of significance was further adjusted using the sequential Bonferroni test (number of population X number of loci).

Estimation of population structure

F-statistics, *F*st is a standard parameter for measurement of population structure (or interpopulation diversity). It can be calculated using the exact test of genotypic differentiation of GENEPOP version 2.0. *F*st and related measures of population differentiation are based on the assumption of relatively low mutation rates.

Genotypic disequilibrium analysis

Only one test on contingency table is available to test for genotypic (for the diploid case) or gametic (for the haploid case) linkage disequilibrium. For a given pair of loci within one population, the relevant information is represented by GENEPOP version 2.0.

CHAPTER III

RESULTS

3.1 DNA extraction

Genomic DNA was extracted from each *A. dorsata* individual using Phenol-Chloroform modification of Hall and Smith (1991). This method gave high molecular weight DNA which was larger than 23.1 kb. An amount of extracted DNA was about 1.5-2.0 μg per individual as estimated by comparing its intensity with that of the λ/*Hin*d III marker in 0.7% agarose gel electrophoresis (Figure 3.1). For subsequent use in the PCR reaction, the DNA solution was adjusted to final concentration of 25 ng/μl.

Figure 3.1 High molecular weight DNA of *A. dorsata* extract from the thorax of each *A. dorsata* worker and subjected to 0.7% agarose gel electrophoresis at 100 V for 50 minutes.

Lane 1 λ/*Hin*dIII DNA standard

Lane 2-13 Total nuclear DNA isolated from *A. dorsata* individuals.

3.2 Optimization of PCR conditions

Four different regions (intergenic CO I – CO II region, Cytb I - $tRNA^{Ser}$ gene, ATPase6-8 genes and lrRNA gene) of *A. dorsata* mtDNA were amplified by using the specific primers designed from *A. mellifera* mtDNA sequence. These regions of *A. dorsata* mtDNA were separately amplified in 25 μl reaction mixture containing 1XPCR buffer, 100 μM each of dNTPs, 0.1 μM each of primers, 0.6 unit *Taq* DNA polymerase and 25 ng total DNA with $MgCl_2$ concentration and annealing temperature varied from 0-5.0 mM and 40-60°C, respectively. The amplified products of all regions were firstly appeared at $MgCl_2$ concentration of 0.5-1.0 mM. At these conditions, non specific products were observed below the specific PCR-amplified DNA but their amounts were not high enough to interfere an interpretation of the restriction enzyme digesting results. Primer dimer was also observed. The amplified products were consistently increased with higher $MgCl_2$ concentration to appropriate $MgCl_2$ concentration of each region and then non specific products were decreased until disappeared. For annealing temperature optimization, at low annealing temperature the amplified products and non specific products appeared because the primers caught non-specifically with DNA temperature. More specific catching was observed when annealing temperature was increased to appropriate annealing temperature. The resulting products of increasing annealing temperature was the same as those of increasing $MgCl_2$ concentration. The $MgCl_2$ concentration and annealing temperature used for amplification of these regions in present study were shown in Table 3.1.

These regions of mtDNA were amplified from five geographic samples of *A. dorsata* in Thailand using primers shown in Table 3.1. When the PCR reaction was completed, the amplified products were electrophoresed through a 1.5 % agarose gel and stain with ethidium bromide. The amplified products obtained from intergenic CO I – CO II region, Cytb I - $tRNA^{Ser}$ gene, ATPase6-8 gene and lrRNA gene were 1040, 845, 820 and 750 bp, respectively. (Figure 3.2)

Table 3.1 PCR conditions and primer sequences used to determine genetic variation in *A. dorsata.*

Region	Primers	Annealing Temp. °C	$MgCl_2$ mM	No. cycle
E3 CO II	TCTATACCACGACGTTATTC CCACAAATTTCTGAACATTGACC	60	2.0	35
Cytb I $tRNA^{Ser}$	TATGTACTACCATGAGGACAAATATC GAAAATTTTATTTCTATATTATATTTTCA	48	2.0	34
lrRNA1 lrRNA2	CTATAGGGTCTTATCGTCCC TTTTGTACCTTTTGTATCAGGGTT	55	2.5	35
ATPase6 ATPase8	TTTAATTCCTCAAATAATAC TTAATTTGATTCAGAGAAAT	40	5.0	35

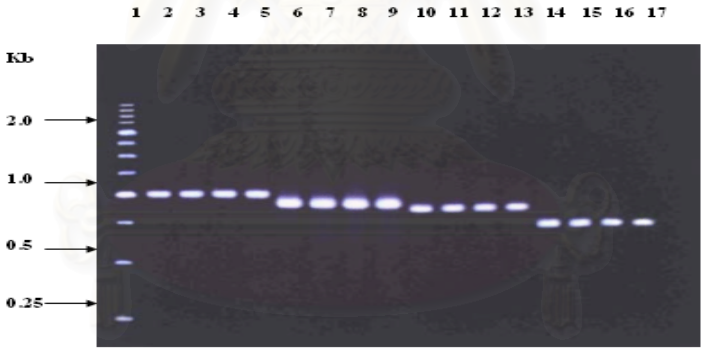

Figure 3.2 The amplified products were electrophoresed through a 1.5% agarose gel at 120 V for 90 minutes.

Lane 1 A 250 bp DNA ladder

Lane 2-17 The amplified products of inter COI-COII region (lane 2-5), CytbI-$tRNA^{Ser}$ gene (lane 6-9), ATPase6-8 gene (lane 10-13) and lrRNA gene (lane 14-17) of *A. dorsata* individuals.

3.3 PCR-amplified DNA and screening of restriction endonuclease

The DNA samples from colonies of *A. dorsata* in Thailand were used for screening of informative restriction endonuclease. After amplification, PCR products of each region were digested with restriction endonucleases. Twelve restriction endonucleases consisting of *Alu* I, *Bam*H I, *Bgl* II, *Dra* I, *Eco*R I, *Hin*d III, *Hin*f I, *Nde* I, *Pst* I, *Sau*3A I, *Spe* I and *Xba* I were screened whether they are polymorphism on the amplified DNA fragments. Among all restriction endonucleases only *Dra* I and *Hin*f I showed polymorphism. Therefore, *Dra* I and *Hin*f I were used to digest PCR products of four different mtDNA regions of *A. dorsata* in Thailand.

3.3.1 Analysis of intergenic COI-COII region polymorphism

For intergenic CO I – CO II region, only one restriction pattern was found after digested PCR products (1040 bp) from all DNA samples with *Dra* I fragments of 500, 210, 160, 90 and 80 bp were obtained (Table 3.2(a) and Figure 3.3). Three different haplotypes (A, B and C) were found in these samples when digested with *Hin*f I (Figure 3.4). Haplotype A which was commonly distributed in all populations of Thailand had discrete bands of 360, 265, 195, 190 and 30 bp whereas haplotype B and C were 360, 195, 190,160, 105 and 30 bp and 360, 195, 190, 190, 75 and 30 bp, respectively. These haplotypes (B and C) interconnected with haplotype A by a single loss (or gain) of *Hin*f I restriction site. A single point mutation step caused a replacement of 165 and 105 bp fragments in haplotype B and 190 and 75 bp fragments in haplotype C. Haplotype B was found in one specimen of central and two specimens of south. Haplotype C was found only in one specimen of central. The relationship among three restriction patterns were shown in Table 3.3(a).

3.3.2 Analysis of Cytb I - $tRNA^{Ser}$ gene polymorphism

One haplotype was found in *Dra* I digestion pattern of Cytb I - $tRNA^{Ser}$ gene. Discrete bands of 250, 235, 220 and 140 bp fragments were shown in Figure 3.5. When these samples were detected for *Hin*f I variation, four distinct haplotypes were observed (Figure 3.6). Haplotype A showed 4 bands of 480, 170, 135 and 60 bp. Only one restriction site loss in 615 bp fragment of haplotype B showed 480 and 135 bp fragments in haplotype A. Haplotype A and C were interelated by a change of 480 and 60 bp fragments in haplotype A to be 540 bp fragment in haplotype C. Finally, haplotype D was different from haplotype A by only one point mutation (a site gain in a 135 bp fragment of haplotype A gave 95 and 40 bp fragments in haplotype D). However, these haplotypes (B, C and D) were found

only one specimen of Central. Haplotype A was a major haplotype which was found in all populations. The summary of four *Hin*f I patterns were shown in Table 3.3(b).

3.3.3 Analysis of ATPase6-8 gene polymorphism

The digestion of ATPase6-8 gene from all samples with *Dra* I showed only one restriction pattern which was haplotype A. This haplotype consisted of two fragments of 520 and 300 bp (Figure 3.7). One restriction pattern was shown after this amplified region was digested by *Hin*f I. *Hin*f I restriction pattern gave two fragments of 550 and 270 bp (Figure 3.8). No different pattern was found among *A. dorsata* on all populations of Thailand when detected with *Dra* I and *Hin*f I.

3.3.4 Analysis of lrRNA gene polymorphism

Two haplotypes were observed among 154 *A. dorsata* detected for *Dra* I variation (Figure 3.9). Haplotype A which had discrete bands of 300, 200, 100, 80 and 70 bp was the most frequent. However, one sample from Central showed different pattern (haplotype B) from haplotype A. A site gain in a 200 bp fragment of haplotypeA gave 110 and 90 fragments in haplotype B (Table 3.2(d)). When these colonies were detected for *Hin*f I variation, undigested pattern was found.

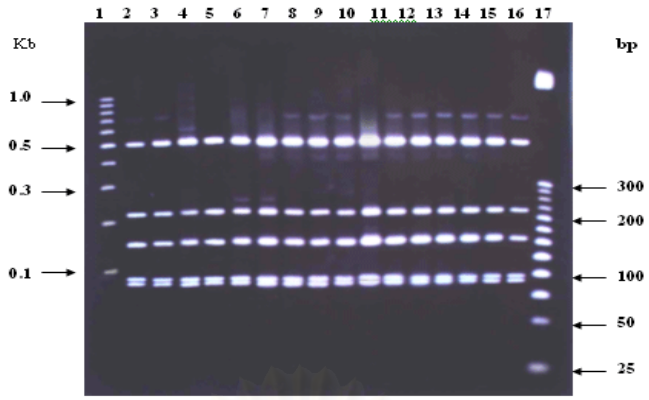

Figure 3.3 One haplotype was observed from *Dra*I digestion of amplified mitochondrial inter COI-COII region of *A. dorsata*.

Lane 1 100 bp DNA ladder

Lane 2-17 An example of restriction pattern from 16 *A. dorsata* individuals (haplotype A).

Lane 18 25 bp DNA ladder

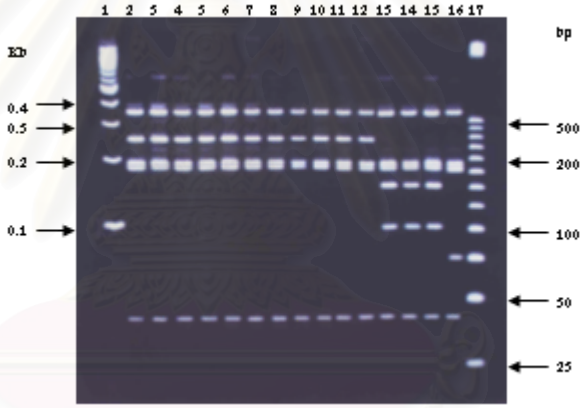

Figure 3.4 Three different haplotypes were observed from *Hin*fI of amplified mitochondrial inter COI-COII region of *A. dorsata*.

Lane 1 ,17 100 bp DNA ladder 25 bp DNA ladder

Lane 2-16 Restriction patterns from 16 *A. dorsata* individuals

(lane 2-12 : haplotype A, lane 13-15 : haplotype B and lane 16 : haplotype C)

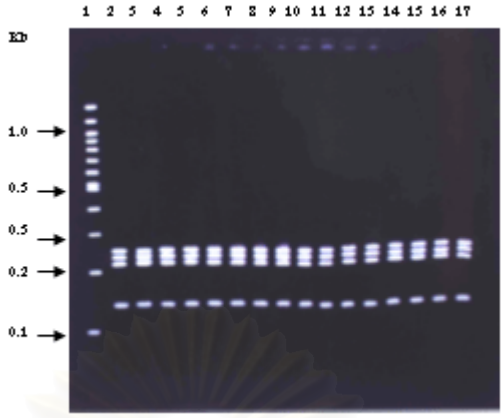

Figure 3.5 One haplotype was observed from *Dra*I of amplified mitochondrial CytbI-RNA^{Ser} gene of *A. dorsata*.

Lane 1 100 bp DNA ladder

Lane 2-17 An example of restriction pattern from 16 *A. dorsata* individuals (haplotype A).

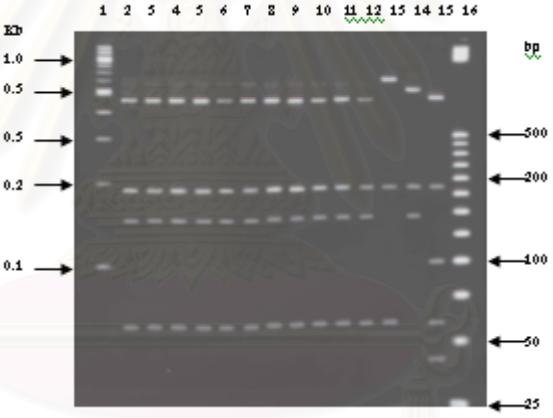

Figure 3.6 Four different haplotypes were observed from *Hin*fI of amplified mitochondrial CytbI-tRNA^{Ser} gene of *A. dorsata*.

Lane 1,16 100 bp DNA ladder, 25 bp DNA ladder

Lane 2-15 Restriction patterns from 14 *A. dorsata* individuals
(lane 2-12 : haplotype A, lane 13 : haplotype B, lane 14 : haplotype C and lane 15 : haplotype D)

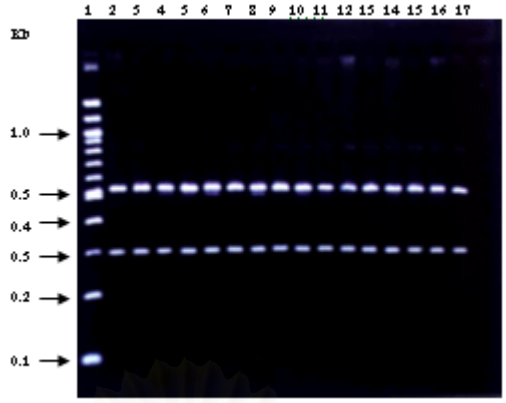

Figure 3.7 One haplotype was observed from *Dra*I of amplified mitochondrial ATPase6-8 gene of *A. dorsata*.

Lane 1 100 bp DNA ladder

Lane 2-17 Restriction pattern from 16 *A. dorsata* individuals (haplotype A).

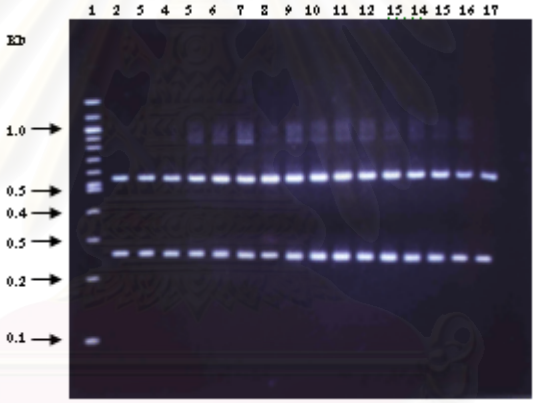

Figure 3.8 One haplotype was observed from *Hin*fI of amplified mitochondrial ATPase6-8 gene of *A. dorsata*.

Lane 1 100 bp DNA ladder

Lane 2-17 Restriction pattern from 16 *A. dorsata* individuals (haplotype A).

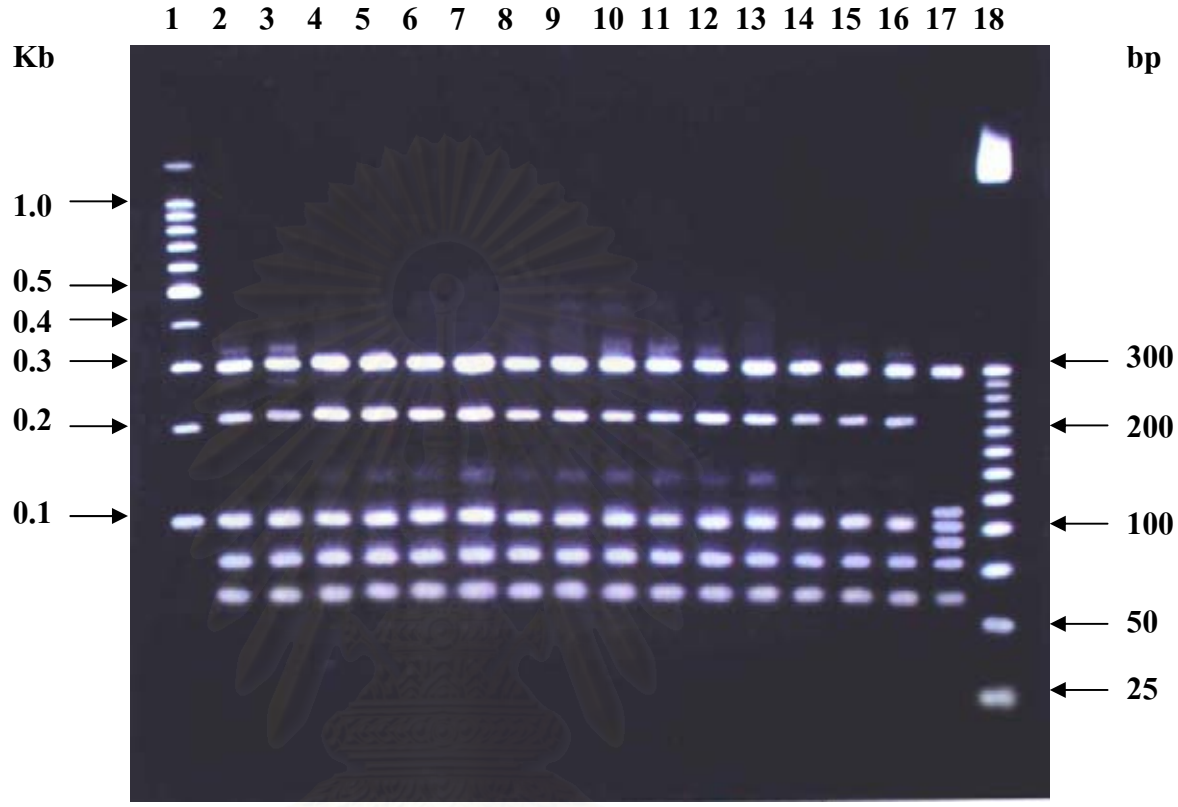

Figure 3.9 Two different haplotypes were observed from *Dra*I of amplified mitochondrial lrRNA gene of *A. dorsata*.

Lane 1 100 bp DNA ladder

Lane 2-17 An example of restriction patterns from 16 *A. dorsata* individuals

: lane 2-16 haplotype A

: lane 17 haplotype B

Lane 18 25 bp DNA ladder

Table 3.2 Summary of restriction patterns of *Dra*I digested amplified DNA from two different regions in mitochondrial DNA.

a) Inter CO I-CO II region

Haplotype	Fragment size (in base pairs)					Total length
	500	210	160	90	80	
A	*	*	*	*	*	100

b) Cytb I-tRNA^{Ser}

Haplotype	Fragment size (in base pairs)				Total length
	250	235	220	140	
A	*	*	*	*	845

c) ATPase6-8

Haplotype	Fragment size (in base pairs)		Total length
	520	300	
A	*	*	820

d) lrRNA

Haplotype	Fragment size (in base pairs)							Total length
	300	200	110	100	90	80	70	
A	*	*		*		*	*	750
B	*		*	*	*	*	*	750

Table 3.3 Summary of restriction patterns of *Hinf* I digested amplified DNA from two different regions in mitochondrial DNA.

a) Inter CO I-CO II region

Haplotype	Fragment size (in base pairs)									Total length
	360	265	195	190	190	160	105	75	30	
A	*	*	*	*					*	1040
B	*		*	*		*	*		*	1040
C	*		*	*	*			*	*	1040

b) Cytb I-tRNA^{Ser}

Haplotype	Fragment size (in base pairs)								Total length
	615	540	480	170	135	95	60	40	
A			*	*	*		*		845
B	*			*			*		845
C		*		*	*				845
D			*	*		*	*	*	845

c) ATPase6-8

Haplotype	Fragment size (in base pairs)		Total length
	550	270	
A	*	*	820

d) lrRNA : uncut

3.4 Analysis of geographic population structure based on mtDNA-RFLP approach

The distribution of *Dra* I and *Hin*f I digestion among the amplified product from *A. dorsata* intergenic CO I - CO II region, Cytb I - $tRNA^{Ser}$ gene, ATPase6-8 gene and lrRNA gene was shown in Table 3.4. Six composite haplotypes were generated from their distribution frequencies. The composite haplotype, AAAAAAA, was found in 147 out of 154 individuals investigated. This haplotype was distributed in all geographic location of Thailand. The composite haplotypes AAACAAA and ACABAAA were only distributed in the north. Besides these haplotypes, only the central was shown composite haplotypes AAADAAA and AAAAAAB. The last composite haplotype (ABAAAAA) was only observed in the central and the south. The AAAAAAA was the most common composite haplotype while the other were the variants composite haplotype. (The symbol of composite haplotypes, the first and second alphabets mean the restriction patterns of intergenic CO I - CO II region digested with *Dra* I and *Hin*f I, respectively. The third and fourth alphabets mean the restriction patterns Cytb I - $tRNA^{Ser}$ gene digested with *Dra* I and *Hin*f I, respectively. The fifth and sixth alphabets mean ATPase6-8 gene digested with *Dra* I and *Hin*f I, respectively. The last alphabet means lrRNA gene digested with *Dra* I.)

Table 3.4 Geographic distribution of 6 composite haplotypes results from *Dra* I and *Hin*f I digestion of inter CO I – CO II region, Cytb I - $tRNA^{Ser}$ gene, ATPase6-8 gene and lrRNA gene.

Composite haplotype	Geographic distribution frequency (no. of individual)					Total
	North	North-East	Central	South	Samui	
AAAAAAA	0.957(45)	1.000(25)	0.933(42)	0.920(23)	1.000(12)	0.955(147)
AAACAAA	0.021(1)	0	0	0	0	0.006(1)
ACABAAA	0.021(1)	0	0	0	0	0.006(1)
AAADAAA	0	0	0.022(1)	0	0	0.006(1)
ABAAAAA	0	0	0.022(1)	0.080(2)	0	0.019(3)
AAAAAAB	0	0	0.022(1)	0	0	0.006(1)
Total	1.000(47)	1.000(25)	1.000(45)	1.000(25)	1.000(12)	1.000(154)

Haplotype and nucleotide diversity within population for 5 populations were illustrated by Table 3.5. The samples from the south showed the highest haplotype diversity (0.1533), whereas that of the north-east and the Samui Island samples showed the lowest haplotype diversity (0.0000). The average haplotype diversity within the *A. dorsata* of Thailand was 0.0735. By the way, nucleotide diversity indicated that the highest nucleotide diversity (0.000585) was observed in the south. Nucleotide diversity of the North and the Central were 0.000531 and 0.000504, respectively, whereas the north-east and the Samui Island were 0.000000. The average nucleotide diversity was 0.000324.

Table 3.5 Haplotype and nucleotide diversity within population for five geographic locations of *A. dorsata* in Thailand.

Population	Haplotype diversity (h ± SE)	Nucleotide diversity
North	0.0842 ± 0.05516	0.000531
North-East	0.0000 ± 0.00000	0.000000
Central	0.1303 ± 0.06782	0.000504
South	0.1533 ± 0.09155	0.000585
Samui Island	0.0000 ± 0.00000	0.000000
Average	0.0735 ± 0.04291	0.000324

Determination of nucleotide diversity between populations of *A. dorsata* can be seen in Table 3.6. Relatively low nucleotide diversity were observed from all pairwise comparisons among the north, north-east, central, south and Samui Island populations. An average nucleotide diversity between populations was 0.0003. Moreover, this Table showed nucleotide divergence. Nucleotide divergence of *A. dorsata* was 0.0000 in average.

Finally, geographic heterogeneity in the distribution frequency of composite haplotype among compared samples was determined with the Markov carlo simulation using chi-square (χ^2) probability values (Table 3.7). Six composite haplotypes did not show any significant differences in the distribution frequency in all pairwise comparisons ($P > 0.05$).

Table 3.6 Nucleotide diversity (above diagonal) and divergence (below diagonal) between populations for five geographic locations of *A. dorsata* in Thailand.

	North	North-East	Central	South	Samui
North	-	0.0003	0.0005	0.0006	0.0003
North-East	0.0000	-	0.0003	0.0003	0.0000
Central	0.0000	0.0000	-	0.0005	0.0003
South	0.0000	0.0000	0.0000	-	0.0003
Samui	0.0000	0.0000	0.0000	0.0000	-

Average Nucleotide diversity = 0.0003 ± 0.00000

Average Nucleotide divergence = 0.0000 ± 0.00000

Table 3.7 Geographic heterogeneity analysis in distribution frequency of composite haplotype among 5 *A. dorsata* locations based on *Dra*I and *Hinf*I digestion of intergenic CO I – CO II region, Cytb I - $tRNA^{Ser}$ gene, ATPase6-8 gene and lrRNA gene.

	North	North-East	Central	South	Samui
North	-				
North-East	$P=1.0000^{ns}$	-			
Central	$P=0.4817^{ns}$	$p=1.0000^{ns}$	-		
South	$p=0.1731^{ns}$	$p=0.4919^{ns}$	$p=0.8126^{ns}$	-	
Samui	$p=1.0000^{ns}$	$p=1.0000^{ns}$	$p=1.0000^{ns}$	$P=0.5466^{ns}$	-

ns = not significant

3.5 Optimization of microsatellite condition

The PCR-RFLP analysis of *A. dorsata* mtDNA in different geographic areas of Thailand showed only one group of bees. In order to confirm the result, microsatellite technique was chosen for further study on genetic variation of *A. dorsata* in Thailand. In this study, *A. mellifera* microsatellite loci were used to screen for polymorphic loci in *A. dorsata*. These microsatellite loci were tested using five populations including the north, north-east, central, south and Samui Island. The $MgCl_2$ concentration and annealing temperature for each *A. mellifera* microsatellite locus was optimized. Three microsatellite loci (A14, A24 and A88), which showed high allele frequencies, were selected from *A. mellifera* microsatellite loci to determine genetic variation in *A. dorsata*. The optimum values for *A. dorsata* were shown in Table 3.8. These conditions were chosen from those provide the most intense band of PCR products.

Table 3.8 PCR conditions of microsatellite primer which was used to determine genetic variation in *A. dorsata*.

Locus	$MgCl_2$ (mM)	Annealing temp. (^oC)	No. alleles observed	Size of allele (bp)
A14	1.1	50	6	206-216
A24	1.8	58	8	98-112
A88	0.6	55	20	132-157

3.6 Genetic variation in Thai *A. dorsata*

Genetic variation of 155 individual colonies of *A. dorsata* from 5 geographic populations (north=46, north-east=28, central=44, south=25 and Samui Island=12) in Thailand were investigated using three microsatellite loci. The A14, A24 and A88 microsatellite loci were polymorphic and gave microsatellite products in all investigated samples which showed in Figures 3.10-3.12, respectively. Their products appeared as the single (homozygote) and double (heterozygote) groups of stutter bands. The actual allele size was determined from the first band within a group of stutter bands. Size of microsatellite product was assigned in base pair length (bp) by comparing with pGEM-3Zf(+) standard sequencing marker.

3.6.1 Diversity within samples

A total of 6 alleles were observed at microsatellite locus A14 with allele size between 206-216 bp. The distribution of allele frequencies of this locus could be summarized and shown in Figure 3.13 and Table 3.9. Only an allele of 212 bp showed highest allele frequencies in the north (0.400), north-east (0.315), central (0.318) and south (0.280). Exception was found in the Samui Island, the highest allele frequency was 0.417 observed in 206 bp allele.

The allele distribution frequencies of microsatellite locus A24 for each population was shown in Figure 3.14 and Table 3.10. A total of 8 alleles were observed. A 100 bp allele was commonly distributed in the north, north-east, central and south with showed allele frequencies of 0.444, 0.423, 0.352 and 0.280, respectively. Whereas, lower allele frequency of this allele was observed in Samui Island (0.042). Samui Island showed the highest 106 bp allele frequency (0.375). Moreover, two alleles (108 and 110 bp) were not found in Samui Island.

For microsatellite locus A88, the highest polymorphic locus showed 20 alleles. All alleles were commonly distributed in all geographic populations 0except a 140 bp allele. This allele showed high allele frequencies in the north (0.196), north-east (0.196) and central (0.091) while lower allele frequencies of 140 bp alleles were found in south (0.020) and not

found in Samui Island. A 132 bp allele was found only in Samui Island with relatively low frequencies. As typically observed in the allele distribution frequencies, several rare alleles were found in each geographically investigated sample along with a few common alleles (Figure 3.15 and Table 3.11).

Genetic variability with five populations at these three microsatellite loci was shown in Table 3.12. The mean numbers of allele per locus were comparable in all geographic populations. The lowest of this was 6.0 ± 0.00 for Samui Island and the highest was 9.0 ± 3.61 for the north. In all samples the observed heterozygosities of five populations fall in a small range, between 0.68 ± 0.082 in the central population to 0.74 ± 0.121 in the north-east population. Difference between observed and expected heterozygosity was observed for all loci in all samples which the observed heterozygosities were lower than the expected value.

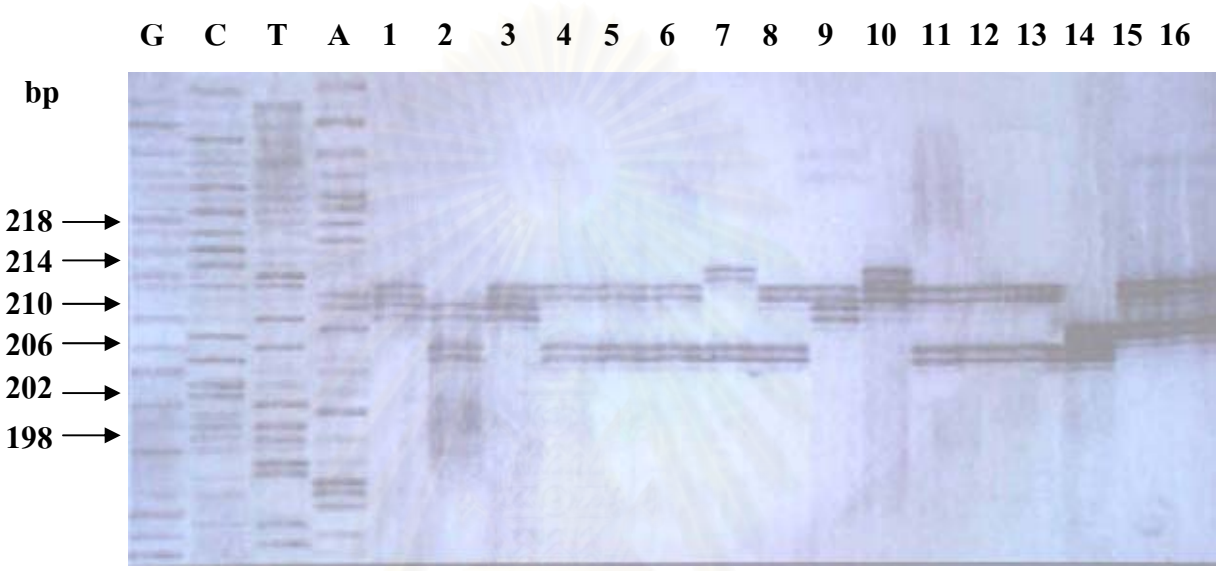

Figure 3.10 Microsatellite patterns of *A. dorsata* individuals at locus A14 (lane 1-16) under the optimal PCR conditions with an annealing temperature at 58 ^{o}C.
The size standard is a sequencing marker of pGEMZ3f(+) vector.

Figure 3.11 Microsatellite patterns of *A. dorsata* individuals at locus A24 (lane 1-16) under the optimal PCR conditions with an annealing temperature at 55 ^{o}C.
The size standard is a sequencing marker of pGEMZ3f(+) vector.

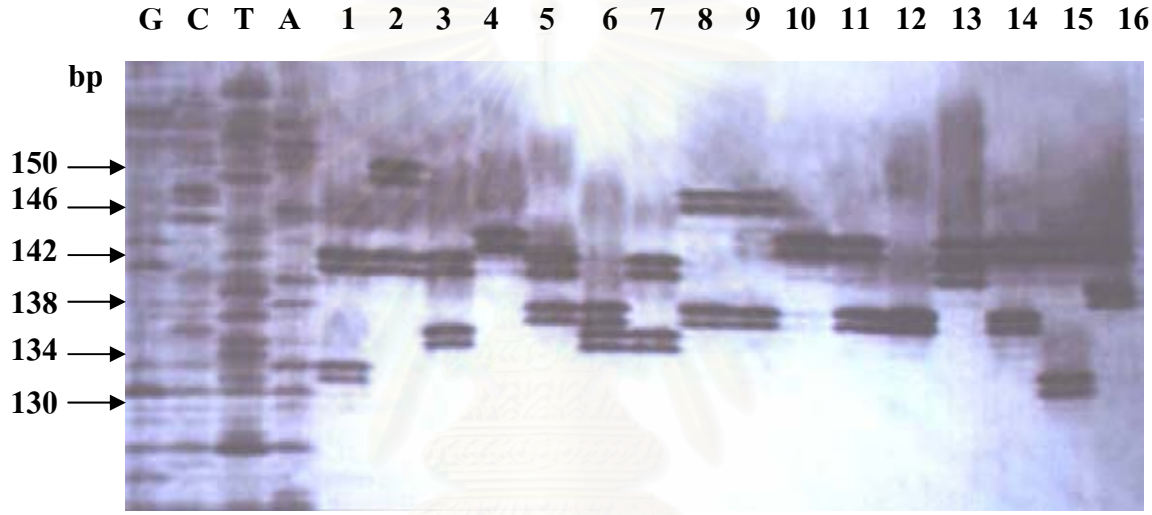

Figure 3.12 Microsatellite patterns of *A. dorsata* individuals at locus A88 (lane 1-16) under the optimal PCR conditions with an annealing temperature at 50 ^{o}C. The size standard is a sequencing marker of pGEMZ3f(+) vector.

Locus A14

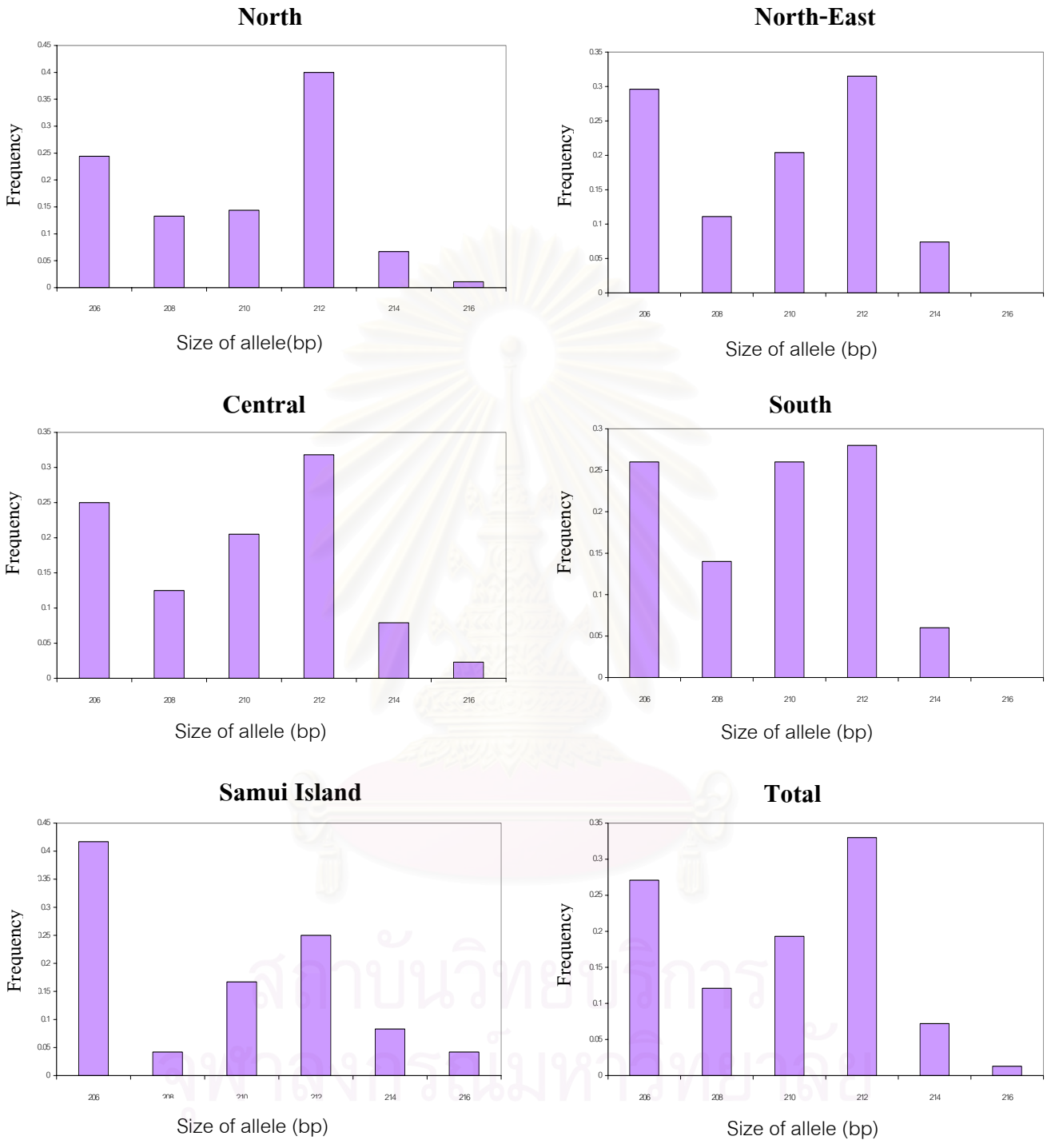

Figure 3.13 Allele frequency distributions at the microsatellite locus A14 from north (n=45), north-east (n=27), central (n=44), south (n=25), Samui Island (n=12) and Total samples (n=153).

Locus A24

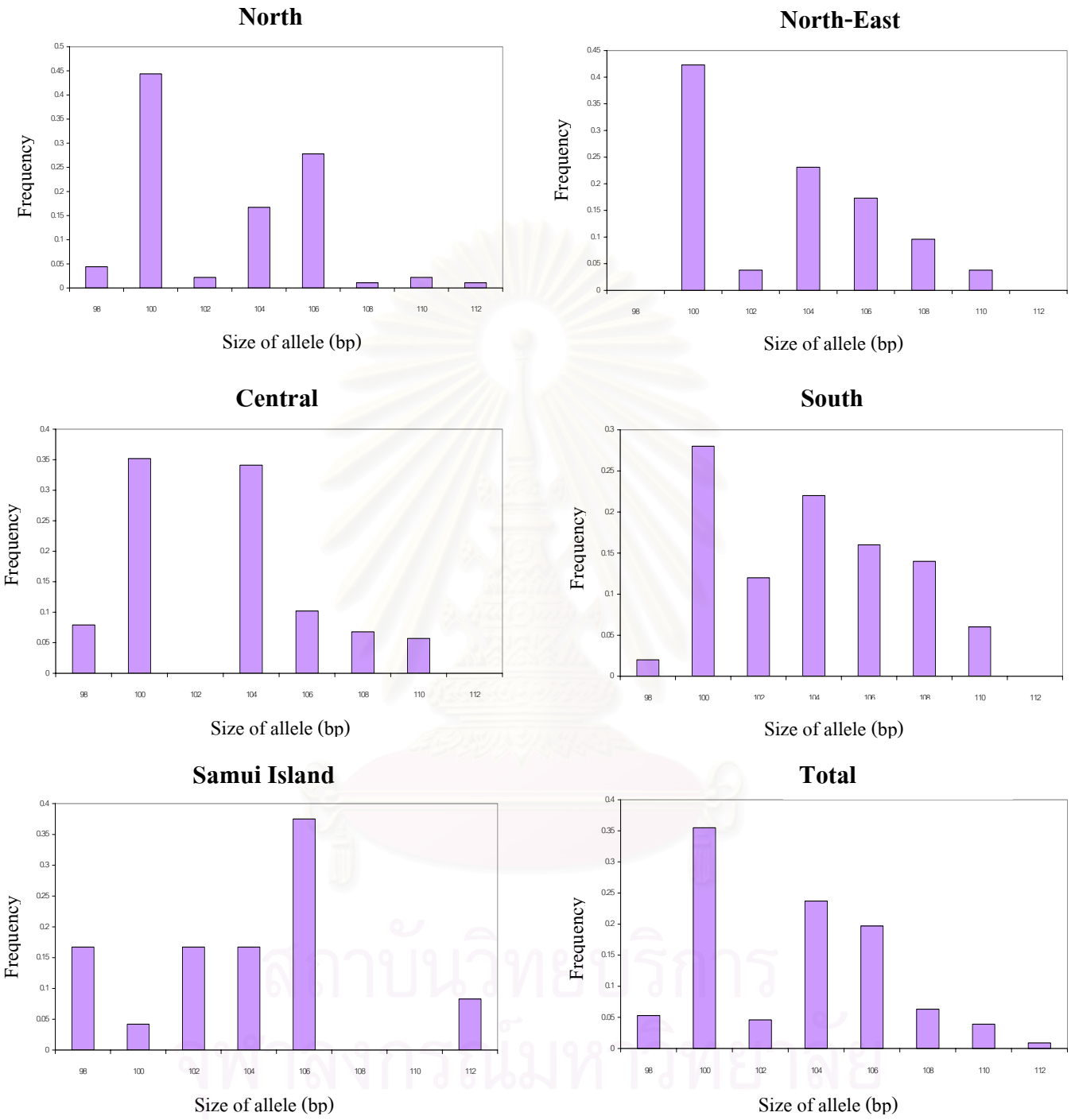

Figure 3.14 Allele frequency distributions at the microsatellite locus A24 from north (n=45), north-east (n=26), central (n=44), south (n=25), Samui Island (n=12) and Total samples (n=152).

Locus A88

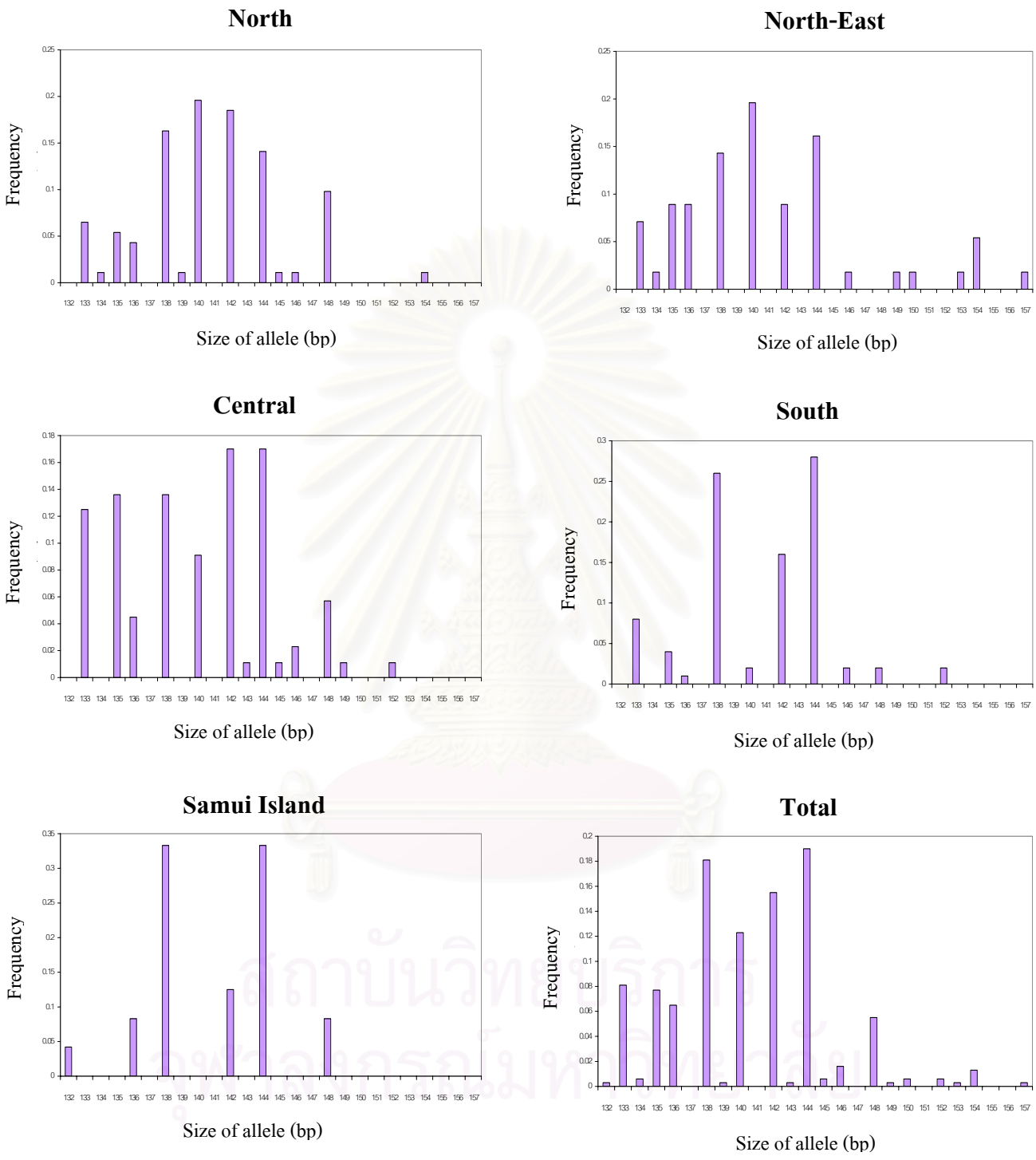

Figure 3.15 Allele frequency distributions at the microsatellite locus A88 from north (n=46), north-east (n=28), central (n=44), south (n=25), Samui Island (n=12) and Total samples (n=155).

Table 3.9 Allele frequencies, number of allele, observed and expected heterozygosity of A14 microsatellite locus in five samples of *A. dorsata* in Thailand.

Allele (bp)	North (N=45)	North-East (N=27)	Central (N=44)	South (N=25)	Samui Island (N=12)	Total (N=153)
206	0.244	0.296	0.250	0.260	0.417	0.271
208	0.133	0.111	0.125	0.140	0.042	0.121
210	0.144	0.204	0.205	0.260	0.167	0.193
212	0.400	0.315	0.318	0.280	0.250	0.330
214	0.067	0.074	0.079	0.060	0.083	0.072
216	0.011	-	0.023	-	0.042	0.013
Number of allele	6	5	6	5	6	6
Observed of heterozygosity	0.756	0.740	0.659	0.600	0.583	0.686
Expected of heterozygosity	0.737	0.754	0.772	0.763	0.725	0.760

Table 3.10 Allele frequencies, number of allele, observed and expected heterozygosity of A24 microsatellite locus in five samples of *A. dorsata* in Thailand.

Allele (bp)	North (N=45)	North-East (N=26)	Central (N=44)	South (N=25)	Samui Island (N=12)	Total (N=152)
98	0.044	-	0.079	0.020	0.167	0.053
100	0.444	0.423	0.352	0.280	0.042	0.355
102	0.022	0.038	-	0.120	0.167	0.046
104	0.167	0.231	0.341	0.220	0.167	0.237
106	0.278	0.173	0.102	0.160	0.375	0.197
108	0.011	0.096	0.068	0.140	-	0.063
110	0.022	0.038	0.057	0.060	-	0.039
112	0.011	-	-	-	0.083	0.009
Number of allele	8	6	6	7	6	8
Observed of heterozygosity	0.711	0.615	0.613	0.680	0.833	0.671
Expected of heterozygosity	0.695	0.726	0.735	0.810	0.767	0.769

Table 3.11 Allele frequencies, number of allele, observed and expected heterozygosity of A88 microsatellite locus in five samples of *A. dorsata* in Thailand.

Allele (bp)	North (N=46)	North-East (N=28)	Central (N=44)	South (N=25)	Samui Island (N=12)	Total (N=155)
132	-	-	-	-	0.042	0.003
133	0.065	0.071	0.125	0.080	-	0.081
134	0.011	0.018	-	-	-	0.006
135	0.054	0.089	0.136	0.040	-	0.077
136	0.043	0.089	0.045	0.010	0.083	0.065
138	0.163	0.143	0.136	0.260	0.333	0.181
139	0.011	-	-	-	-	0.003
140	0.196	0.196	0.091	0.020	-	0.123
142	0.185	0.089	0.170	0.160	0.125	0.155
143	-	-	0.011	-	-	0.003
144	0.141	0.161	0.170	0.280	0.333	0.190
145	0.011	-	0.011	-	-	0.006
146	0.011	0.018	0.023	0.020	-	0.016
148	0.098	-	0.057	0.020	0.083	0.055
149	-	0.018	0.011	-	-	0.003
150	-	0.018	-	-	-	0.006
152	-	-	0.011	0.020	-	0.006
153	-	0.018	-	-	-	0.003
154	0.011	0.054	-	-	-	0.013
157	-	0.018	-	-	-	0.003
Number of allele	13	14	13	10	6	20
Observed of heterozygosity	0.696	0.857	0.773	0.880	0.750	0.781
Expected of heterozygosity	0.862	0.882	0.875	0.819	0.747	0.872

Table 3.12 The number of allele per locus and heterozygosity averaged overall loci.

Sample	Mean number of allele per locus	Mean of heterozygosity	
		Observed ($H_o \pm$ SD)	Expected ($H_e \pm$ SD)
North	9.0 ± 3.61	0.72 ± 0.031	0.77 ± 0.087
North-East	8.3 ± 4.93	0.74 ± 0.121	0.79 ± 0.083
Central	8.3 ± 4.04	0.68 ± 0.082	0.79 ± 0.073
South	7.3 ± 2.52	0.72 ± 0.144	0.80 ± 0.030
Samui Island	6.0 ± 0.00	0.72 ± 0.127	0.74 ± 0.021

3.6.2 Hardy-Weinberg equilibrium

Estimation of Hardy-Weinberg expectation in each *A. dorsata* sample for each microsatellite locus was shown in Table 3.13. This test by locus gave P-value greater than 0.003 for all loci except for the A88 locus in the north (P=0.0001). However, when all microsatellite loci were considered, Hardy-Weinberg expectations were not rejected at all microsatellite loci. Therefore, no significant departure from Hardy-Weinberg equilibrium was detected for all microsatellite loci.

Table 3.13 Estimation of Hardy-Weinberg expectations in each conspecific *A. dorsata* sample for each microsatellite loci.

Population	P-value		
	A14	A24	A88
North	0.3030^{ns}	0.3786^{ns}	0.0001*
North-East	0.3409^{ns}	0.0080^{ns}	0.0331^{ns}
Central	0.0141^{ns}	0.2722^{ns}	0.0282^{ns}
South	0.0441^{ns}	0.0220^{ns}	0.7662^{ns}
Samui Island	0.1065^{ns}	0.7767^{ns}	0.6258^{ns}

ns = non significant * = $p<0.003$

3.6.3 Genotypic disequilibrium

Three microsatellite loci (A14, A24 and A88) were for investigated genotypic disequilibrium of each pair five geographic samples of *A. dorsata* in Thailand by using chi-square test (Table 3.14). Genotypic disequilibrium at all of these microsatellite loci was not observed from all populations ($P > 0.003$). The distribution of allele frequencies at one locus were independent from the distribution of allele frequencies at the other locus.

Table 3.14 Genotypic disequilibrium analysis of each pair five geographic samples of *A. dorsata* in Thailand using three microsatellite loci (A14, A24 and A88).

Sample	A88-A14	A88-A24	A14-A24
	P-value	P-value	P-value
North-North-East	0.9211 ^{ns}	0.2251 ^{ns}	0.7934 ^{ns}
North-Central	0.6666 ^{ns}	0.2319 ^{ns}	0.8944 ^{ns}
North-South	0.1396 ^{ns}	0.2498 ^{ns}	0.4825 ^{ns}
North-Samui	0.9231 ^{ns}	0.2537 ^{ns}	0.5442 ^{ns}
North-East-Central	0.8359 ^{ns}	0.9940 ^{ns}	0.7866 ^{ns}
North-East-South	0.2103 ^{ns}	1.0000 ^{ns}	0.3713 ^{ns}
North-East-Samui	1.0000 ^{ns}	1.0000 ^{ns}	0.4345 ^{ns}
Central-South	0.1225 ^{ns}	0.9940 ^{ns}	0.4856 ^{ns}
Central-Samui	0.8263 ^{ns}	0.9953 ^{ns}	0.5263 ^{ns}
South-Samui	0.2171 ^{ns}	1.0000 ^{ns}	0.2301 ^{ns}

ns = non significant

3.6.4 Geographic heterogeneity and population differentiation

Heterogeneity analysis of allele frequencies among samples was shown in Table 3.15. The A14 and A88 loci did not show any significant differences in the distribution of allele frequencies in all possible pairwise comparisons (all P-value>0.003). Whereas, geographic heterogeneity at the A24 locus was observed from 5 out of 10 possible pairwise comparisons of this locus (P <0.003). These pairwise comparisons were north-central (0.0000), north-Samui Island (0.000), north-east-Samui Island (0.000), central-Samui Island (0.000) and south-Samui Island (0.000). These results indicated that the allele distribution frequencies of *A. dorsata* in the Samui Island were significant difference from other populations. While frequencies among the four populations (north, north-east, central and south) were not significant differences. Besides these, geographic heterogeneity between north and central was observed for A24 locus.

Intraspecific genetic population among *A. dorsata* samples in Thailand was detected by *F*-statistic (F_{ST}) estimate (Table 3.16), but the corresponding value for each pairwise comparison averaging the overall loci was below 0.003. Every pair of populations was not significantly different when allele frequencies of A14 and A88 loci were compared (P >0.003). While A24 locus showed a significant difference for 3 out of 10 possible pairwise comparisons (P <0.003). Comparisons between North, North-East and Central with Samui Island *A. dorsata* revealed significant difference at this locus (P=0.0008, 0.0010 and 0.0012, respectively) whereas, lacked of significant difference was found between south and Samui Island (P=0.0085). The F_{ST} values for overall loci were -0.0066, 0.0347 and 0.0142 for locus A14, A24 and A88, respectively (Table 3.17). Considering for P-value, only A24 locus was significantly larger than 0.003 for five geographic populations of *A. dorsata* in Thailand indicated a significant degree of genetic differentiation within this species.

Table 3.15 Geographic heterogeneity analysis of five conspecific samples of *A. dorsata* using three microsatellite loci (A14, A24, A88).

Population	*P*-value		
	A14	A24	A88
North-North-East	0.748 ns	0.083 ns	0.051 ns
North-Central	0.785 ns	<0.001	0.294 ns
North-South	0.524 ns	0.006 ns	0.007 ns
North-Samui	0.269 ns	<0.001*	0.048 ns
North-East-Central	0.903 ns	0.128 ns	0.128 ns
North-East-South	0.910 ns	0.709 ns	0.013 ns
North-East-Samui	0.610 ns	<0.001*	0.041 ns
Central-South	0.935 ns	0.024 ns	0.099 ns
Central-Samui	0.543 ns	<0.001*	0.127 ns
South-Samui	0.369 ns	0.001*	0.986 ns

ns = non significant * = p<0.003

Table 3.16 *F*-statistics for microsatellite analysis of each pair of five geographic samples of *A. dorsata.*

Sample	Locus A14		Locus A24		Locus A88		Overall	
	*F*st	*P*-value	*F*st	*P*-value	*F*st	*P*-value	*F*st	*P*-value
North-North-East	-0.0050	0.5977^{ns}	0.0058	0.2317^{ns}	-0.0012	0.4890^{ns}	-0.0003	0.4955^{ns}
North-Central	-0.0044	0.6306^{ns}	0.0431	0.0047^{ns}	0.0015	0.3509^{ns}	0.0127	0.0328^{ns}
North-South	0.0019	0.3454^{ns}	0.0245	0.0399^{ns}	0.0270	0.0133^{ns}	0.0182	0.0086^{ns}
North-Samui	0.0140	0.1943^{ns}	0.1065	0.0008*	0.0480	0.0134^{ns}	0.0568	0.0002*
North-East-Central	-0.0134	0.9208^{ns}	-0.0012	0.4053^{ns}	0.0003	0.4179^{ns}	-0.0046	0.7150^{ns}
North-East-South	-0.0168	0.8839^{ns}	-0.0109	0.7435^{ns}	0.0276	0.0130^{ns}	0.0014	0.1464^{ns}
North-East-Samui	-0.0171	0.7216^{ns}	0.1109	0.0010*	0.0540	0.0051^{ns}	0.0524	0.0003*
Central-South	-0.0159	0.9537^{ns}	0.0069	0.2284^{ns}	0.0140	0.0720^{ns}	0.0022	0.2163^{ns}
Central-Samui	-0.0038	0.4673^{ns}	0.1206	0.0012*	0.0369	0.0280^{ns}	0.0529	0.0011*
South-Samui	-0.0084	0.5107^{ns}	0.0666	0.0085^{ns}	-0.0168	0.8282^{ns}	0.0158	0.0807^{ns}

ns = non significant * = p<0.003

Table 3.17 *F*-statistics for microsatellite analysis of five geographic population of *A. dorsata*.

Locus	*F*st	P-value
A14	-0.0066	0.822^{ns}
A24	0.0347	<0.001*
A88	0.0142	0.009^{ns}
Overall	0.0142	-

ns = non significant * = p<0.003

CHAPTER IV

DISCUSSION

In honeybee, mtDNA is a potential marker to investigate genetic structure. Based on the fact that, the mtDNA of honeybees are similar among nest mate. This is an advantage for sampling only one sample from each colony. This mtDNA contains several regions such as 13 protein-encoding genes, 2 rRNAs subunits, and a non-coding region containing the origin of replication. Several studies have been done on several species of the *Apis* and on various regions of mtDNA.

The intergenic CO I - CO II region in mtDNA has been widely used for studies of genetic polymorphism at the intraspecific level (Cornuet *et al*., 1991; Crozier and Crozier, 1992 Garnery *et al*., 1993: *A. mellifera* and Deowanish *et al*., 1996; Sihanuntavong *et al*., 1999: *A. cerana*). In addition, Cytb (Cytochrome b) gene was chosen for investigation of phylogenetic relationships among Bumble Bees (*Bombus*, Latreille) at the interspecific level (Koulianis and Schmid-Hempel, 2000). Songrum (1997) studied genetic variability of *A. cerana* with the ATPase6-8 gene in mtDNA. Moreover, genetic variability of *A. cerana* at the intraspecific level was examined by RFLP in Cytb I - $tRNA^{Ser}$ gene and lrRNA gene in mtDNA (Deowanish *et al*., 1996). From the complete mitochondrial genome sequence of *A. mellifera* (Crozier and Crozier, 1993), the expected amplification products for intergenic CO I - CO II region, Cytb I - $tRNA^{Ser}$ gene, ATPase 6 - 8 gene and lrRNA gene were 1123, 858, 824 and 739 bp, respectively. However, the resulting products in *A. dorsata* were 1040, 845, 820 and 750 bp which is slightly different than those expected from the *A. mellifera* mtDNA sequence. The intergenic CO I - CO II region in *A. dorsata* was typically shorter than that of *A. mellifera*.

In the restriction enzyme screening step, twelve restriction enzymes (tetranucleotide, pentanucleotide and hexanucleotide cutting enzymes) were used in order to produce large DNA fragments that could be rapidly examined using agarose gel electrophoresis. However, all restriction endonuclease showed only one haplotype for each region except *Dra* I and *Hin*f I, which showed polymorphism. Therefore, *Dra* I and *Hin*f I were selected to be employed for analysis of genetic variation in this study. It has been reported that *Dra* I was used in PCR-RFLP analysis of *A. mellifera* and *A. cerana* genetic variation by Delarua *et al*., (1998) and Sihanuntavong *et al*., (1999), respectively. Besides this, *Hin*f I is one of the enzymes that Sheppard et al (1996) used to examine mtDNA variation in the intraspecific level of *A. mellifera*.

In this study, the *Dra* I digestion of these regions (intergenic CO I - CO II region, Cytb I - $tRNA^{Ser}$ gene, ATPase 6 - 8 gene and lrRNA gene) in mtDNA of *A. dorsata* showed the lowest polymorphism. All samples showed the same pattern in each region except one sample from central, which showed another haplotype (with single lost or gain mutation step) after digestion of the lrRNA gene with *Dra* I. However the *Hin*f I digestion of the mtDNA of *A. dorsata* showed more polymorphism than the *Dra* I digestion. But only 7 samples (one, one and two samples from north, central, and south, respectively, for intergenic CO I - CO II region, two samples from north and one sample from central, for Cytb I - $tRNA^{Ser}$ gene) showed other haplotype. The most common composite haplotype, AAAAAAA, was distributed in all geographic location of Thailand. The other composite haplotypes were the variants composite haplotype. These variants occurred by mutation in recognition site of *Dra* I and *Hin*f I.

Usually a non-coding sequence is free to evolve rapidly and provides information analyzable at the intraspecific level. The non-coding region between CO I – CO II was found to be longer in the cavity - nesting bees (89 - 97 in *A. cerana*, 94 in *A. koschevnikovi*, ~ 200 - 900 in *A. mellifera*) than other honey bee species, whereas in *A. florea*, *A. andreniformis* and *A.*

dorsata were only 24 - 32 bp in this region. Therefore, there was no different restriction pattern of *A. dorsata* in this region.

The mitochondrial DNA regions of *A. dorsata*, Cytb I - $tRNA^{Ser}$, ATPase6-8, lrRNA and intergenic CO I – CO II were more conserved than these regions in *A. mellifera* and *A. cerana*. This may be because of the migratory behavior of *A. dorsata* (Koeniger and Koeniger, 1980), which could generally explain high gene flow of mtDNA. The mtDNA could be distributed from one population to other population and may be distributed across the sea to Samui Island by bees. Other reason for no different restriction pattern in *A. dorsata* may be due to low mutation rate of mtDNA. Mutation of nucleotide was not found in this generation. Therefore, only one haplotype was found in each region in all populations except the variants.

The samples from north, central and south showed high haplotype diversity while north/east and Samui Island showed the lowest of this. The lowest haplotype diversity in north/east and Samui Island indicated limited genetic diversity in mtDNA of these populations. Effective population may be one of reason to explain this circumstance. Beside this reason, it may be caused by the screening of restriction enzymes did not appropriate.

A chi-square analysis was used to statistically indicate genetic differentiation form composite haplotype distribution frequencies. Five geographic locations of *A. dorsata* could not be statistically allocated genetically different groups or there was no intraspecific differentiation in *A. dorsata* in Thailand.

Although sampling strategies and laboratory techniques are relatively simple when mtDNA is employed, contribution of the male component on population genetic sense cannot be inferred from mtDNA. Nuclear DNA is inherited from female and male. This was used to investigate population genetic information from both male and female. Based on the fact that

microsatellite markers are inherited in a co-dominant fashion. Therefore, microsatellite DNA from nuclear genome of *A. dorsata* was chosen for further study.

The first application of nuclear DNA analysis to the population genetics of honeybees is one based on microsatellite variation (Estoup *et al*., 1995). Estoup *et al.* (1995) found microsatellites analysis confirmed that *A. mellifera* evolved in three distinct and deeply differentiated lineages previously detected by morphological and mitochondrial DNA studies. Microsatellite which show a low number of alleles is potentially useful for population structure and genetic differentiation analysis, whereas microsatellite that show a high number of alleles should be used for studying of kin structure within colonies (Moritz *et al*., 1995; Oldroyd *et al*., 1995).

In the screening process of microsatellite primers, thirteen microsatellite primers originally developed from *A. mellifera* were used to amplified loci in *A. dorsata* DNA using the PCR conditions optimised from those used to amplify these regions in *A. mellifera*. The $MgCl_2$, dNTPs, primers, DNA template and *Taq* DNA concentration were all optimized for PCR of each microsatellite loci. Three microsatellite loci (A14, A24 and A88) were successfully amplified in *A. dorsata* DNA whereas other microsatellite loci could not be optimized to give reliable product.

The three loci A14, A24 and A88 were selected to determine genetic diversity within five geographic populations of *A. dorsata* in Thailand. A14 and A88 are compound microsatellites whereas A24 is a dinucleotide repeat microsatellite. Core sequences of A14, A24 and A88 are $(CT)_{13}$…$(GGT)_9$, $(CT)_{11}$, and $(CT)_{10}TC(CCTT)_2(CTTT)_3$…$(GGA)_7$, respectively.

Amplified alleles were separated by electrophoresis on an 8% denaturing polyacrylamide sequencing gel. Allele sizes were determined by comparing products with those of the DNA sequence of pGEM-3Zf(+). After electrophoresis, the banding patterns were detected by silver

stain (Bassam *et al*., 1990). Detection of amplified microsatellire alleles with silver stain is a simple and convenient method, which is cheaper and more safety than that of radio isotopic method by radioisotopic labelling of microsatellite DNA.

Allelic size differences by one single nucleotide were found in locus A88. This may be the effect of single base mutation in the flanking region of the repeated motifs or may occur because of mutations occurring within the cluster of repetitive sequences. Besides these, it may have occurred because of dinucleotide, trinucleotide and tetranucleotide repeats in core sequence of this locus. Alleles at loci A14 and A24 did not suffer from single base differences. However locus A24 showed the appearance of shadow or stutter bands had been shown to be due to slipped-strand miss-pairing during PCR.

For locus A14 of *A. dorsata*, 6 alleles (206 - 216 bp in size) were observed. Only one of these (216 bp) was also observed among 13 alleles (216 - 248 bp in size) in *A. mellifera*. Eight allelic sizes (98 - 112 bp in size) of *A. dorsata* at A24 locus were also found in *A. mellifera* (98 - 110 bp in size), with the exception of allele 112 bp not found in *A. mellifera*. In addition, *A. dorsata* had a high number of alleles (20 alleles: 132 - 157 bp in size) compared with *A. mellifera* (15 alleles: 138 - 159 bp in size) at the A88 locus. Ten alleles (138, 143, 144, 146, 149, 150, 152, 153, 154 and 157 bp) were overlapping between these two species. The numbers of share alleles of each loci that were observed in all investigated populations were 5 (206, 208, 210, 212 and 214 bp), 3 (100, 104 and 106 bp) and 4 alleles (136, 138, 142 and 144 bp) for A14, A24 and A88 loci, respectively. The most common genotype at locus A24 of *A. dorsata* was a 100 bp allele, carrying a high frequencies in all mainland samples whereas a 106 bp allele carrying a high frequencies in Samui Island samples.

The mean of observed heterozygosity of north/east and Samui Island *A. dorsata* was high (*Ho* = 0.74 and 0.72, respectively). Considering the number of alleles per locus and level of heterozygosity and the high level of genetic diversity of the north/east and Samui

Island, the results of *A. dorsata* inferred from microsatellite loci were in contrast to results from mtDNA - RFLP (haplotype diversity of these populations = 0.00). This may arise because of the fact that mtDNA is inherited only from the female line while nuclear DNA is inherited from both female and male parents. The mating behavior of *A. dorsata* may amplify this difference. Usually *A. dorsata* drones fly to drone congregation areas (DCAs) for mating with a virgin queen (Koeniger and Koeniger, 2000). Queen may be mated with many drones from several unrelated colonies, for example Wattanachaiyingcharoen (2001) found *A. dorsata* queens can mate with more than 100 males. This means that colonies have many nuclear alleles derives from all the drones that the queen mate with, whereas they only have one mitochondrial mitotype from the queen. Therefore sampling only one worker from a colony may not be representative of all the nuclear alleles in the colony.

Significant different of geographic heterogeneity at locus A24 was observed between north-Samui Island, north/east-Samui Island, central-Samui Island, south-Samui Island and north-central ($P<0.003$), whereas locus A14 and locus A88 did not show significant differences in geographic heterogeneity ($P>0.003$). The significant differences in geographic heterogeneity between the mainland and Samui Island may be the result of firstly if these two populations of bees did not mate each other, and secondly if there was genetic drift in the later as it suffered from low gene flow. Genetic drift may have occurred in Samui Island since the population of Samui Island is smaller than the mainland, therefore the allele frequency could be different to the mainland. The *P* value of *F*st between populations in mainland were high (>0.003) and between populations in mainland and Samui Island were low (<0.003). This clearly showed that high gene flow level of *A. dorsata* occurred within the mainland but restricted gene flow found between mainland and Samui Island groups. In this circumstance, the sea may be a geographical barrier for significant migration events and hence gene flow from the mainland to the Samui Island (the distant from the mainland coast to Samui Island about 18 km; Sylvester et al., 1998). Thirdly, the sampling error of Samui Island bees may have contribute, since there was only 12 colonies sampled and just 1 bee from 1 colony was

analysed. However significantly different geographic heterogeneity between two population of mainland (north-central) was also observe. This may have occurred because of the different N_e (effective number of alleles) between of these two populations. Further studies with using an increased number of microsatellite loci are required to clarify this difference since this result was from only one microsatellite locus (A24). In addition, the significant difference of geographic heterogeneity between mainland and Samui Island was supported by the *P* value ($P<0.003$) of *F*st (more than zero).

The results from this study show that genetic structure of *A. dorsata* from mainland and Samui Island in Thailand could be differentiated using geographic heterogeneity analysis of microsatellite locus A24.

Microsatellite locus A24 is the only locus that was used to produce the results for analysis of genetic variation and population structure of *A. dorsata* in Thailand in this study. More studies should be performed using more microsatellite primers. The sample size of 12 individuals on 12 colonies may not cover high number of alleles per microsatellite locus A88 (20 alleles). Therefore, larger sample sizes from Samui Island are needed.

CHAPTER V

CONCLUSIONS

1. No genetic differentiation of *A. dorsata* in Thailand was examined by PCR - RFLP analysis of amplified mitochondrial intergenic CO I - CO II region, Cytb I - $tRNA^{Ser}$ gene, ATPase 6 - 8 gene and IrRNA gene with *Dra* I and *Hin*f I.

2. Three polymorphic microsatellite loci: A14, A24 and A88 showed 6, 8 and 20 alleles per locus, respectively.

3. The average heterozygosity of *A. dorsata* in Thailand estimated from three microsatellite loci was 0.68 - 0.74 indicating a high genetic variation levels in this honeybees.

4. From microsatellite analysis, locus A24 was used to detect a genetic differentiation within the *A. dorsata* in mainland and Samui Island of Thailand.

5. Geographic heterogeneity analysis of all microsatellite loci were not showed a significant degree of genetic differentiation within the mainland populations.

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APPENDICES

APPENDIX 1

Collection data and the allelic sizes of 3 microsatellite loci of *A. dorsata* from the north of Thailand

Code of Colonies	Sampling area	Date of collection	Size of allele (bp.)		
			Locus A14	Locus A24	Locus A88
N1	Mae Jo University, Chiang Mai	01/08/96	208/208	100/106	133/142
N2	Mae Jo University, Chiang Mai	05/11/97	ND	ND	ND
N3	Sarapee, Chiang Mai	05/01/98	210/212	104/104	139/145
N4	Sarapee, Chiang Mai	05/01/98	210/212	100/100	148/148
N5	Sa Moeng, Chiang Mai	09/04/97	206/206	100/100	142/142
N6	Sarapee, Chiang Mai	08/12/97	212/212	102/104	140/140
N7	Sarapee, Chiang Mai	08/12/97	210/212	100/106	144/144
N8	Mae Rim, Chiang Mai	08/12/97	206/210	106/106	138/138
N9	Mae Rim, Chiang Mai	08/12/97	206/210	106/106	140/142
N10	Sa Moeng, Chiang Mai	02/04/99	206/208	100/106	138/144
N11	Sa Moeng, Chiang Mai	02/04/99	ND	ND	ND
N12	Sa Moeng, Chiang Mai	10/04/99	ND	ND	ND
N13	Sa Moeng, Chiang Mai	04/04/99	208/212	100/104	138/142
N14	Maung, Nan	23/04/99	212/214	100/100	142/142
N15	Pua, Nan	23/04/99	206/212	100/106	135/140
N16	Doi Moo-Ser, Tak	23/03/99	212/212	100/104	138/140
N17	Mae Sod, Tak	14/05/99	212/212	100/106	140/140
N18	Lomsak, Phetchaboon	23/03/99	208/208	100/110	140/142
N19	Lomsak, Phetchaboon	23/03/99	208/212	100/100	135/140
N20	Phitsanulok	21/02/99	210/216	106/106	134/140
N21	Phitsanulok	21/02/99	206/212	106/106	133/142
N22	Noenmaprang, Phitsanulok	23/04/99	206/212	100/102	140/140
N23	Phichit	22/02/99	212/214	100/106	144/144
N24	Phichit	22/02/99	206/212	100/110	138/144
N25	Phichit	24/02/99	212/212	98/108	133/138
N26	Phichit	24/02/99	206/212	98/100	133/136
N27	Phichit	08/03/99	206/210	100/106	142/144

Code of Colonies	Sampling area	Date of collection	Size of allele (bp.)		
			Locus A14	Locus A24	Locus A88
N28	Muang, Utraradit	18/05/99	206/210	104/106	144/146
N29	Muang, Utraradit	28/05/99	212/212	100/100	148/148
N30	Klong Lan, Kamghaengphet	14/03/99	206/214	100/106	138/148
N31	Muang, Kamphaengphet	26/07/99	206/210	100/106	140/142
N32	Tak	29/03/99	206/210	104/104	138/142
N33	Maung, Phetchaboon	22/11/99	208/212	100/106	136/136
N34	Lomsak, Phetchaboon	10/10/99	208/208	100/106	138/142
N35	Hangchat, Lampang	18/02/00	ND	ND	138/154
N36	Phitsanulok	17/02/00	208/212	104/106	135/135
N37	Chiang Dao, Chiang Mai	20/02/00	206/212	98/106	136/144
N38	Chiang Dao, Chiang Mai	20/02/00	212/214	100/106	140/142
N39	Chiang Dao, Chiang Mai	20/02/00	208/212	100/112	138/140
N40	Chiang Dao, Chiang Mai	20/02/00	212/212	100/100	135/144
N41	Phusang, Pha-yao	06/06/00	206/212	100/106	140/148
N42	Phusang, Pha-yao	06/06/00	212/214	104/106	138/148
N43	Phusang, Pha-yao	06/06/00	210/212	98/100	133/140
N44	Phusang, Pha-yao	06/06/00	206/212	100/104	142/142
N45	Phusang, Pha-yao	06/06/00	206/214	100/104	140/144
N46	Khuntan, Chiang Rai	07/06/00	206/212	100/104	133/144
N47	Wiang Kaen, Chiang Rai	07/06/00	206/212	100/104	138/144
N48	Mae Sai, Chiang Rai	08/06/00	206/212	100/104	138/142
N49	Klong Lan, Kamphaengphet	29/04/00	210/210	100 /100	148

Collection data and the allelic sizes of 3 microsatellite loci of *A. dorsata* from the north-east of Thailand

Code of Colonies	Sampling area	Date of collection	Size of allele (bp.)		
			Locus A14	Locus A24	Locus A88
NE1	Nakhon Ratchasima	10/12/96	212/212	100/100	136/140
NE2	Nakhon Ratchasima	10/12/96	206/206	100/106	138/140
NE3	Nakhon Ratchasima	07/06/99	208/214	ND	140/144
NE4	Khonburi, Nakhon Ratchasima	26/06/99	ND	ND	138/144
NE5	Pakchong, Nakhon Ratchasima	03/07/99	206/212	104/104	133/144
NE6	Nongbunnak, Nakhon Ratchasima	10/12/96	206/206	104/104	140/140
NE7	Kudbak, Sakolnakhon	01/12/99	210/214	108/108	136/142
NE8	PhuPhan, Sakolnakhon	05/12/99	210/210	100/106	133/133
NE9	Chumphuang, Nakhon Ratchasima	06/12/99	206/212	100/104	140/142
NE10	Namsome, Udon Thani	20/12/99	210/210	100/100	138/140
NE11	Sangkom, Nong Kai	26/12/99	206/212	100/100	136/144
NE12	Phu Kheaw, Chai Ya Phum	22/05/00	212/212	104/106	135/140
NE13	Phu Kheaw, Chai Ya Phum	23/05/00	206/208	100/104	135/136
NE14	Srangkom, Udon Thani	24/05/00	212/214	104/108	133/142
NE15	Phonphisai, Nong Kai	25/05/00	206/208	102/104	135/150
NE16	Fao Rai, Nong Kai	25/05/00	210/212	106/108	138/144
NE17	Fao Rai, Nong Kai	25/05/00	206/212	100/102	138/140
NE18	So Phi Sai, Nong Kai	25/05/00	206/212	100/104	136/154
NE19	Sriwilai, Nong Kai	26/05/00	206/210	100/100	135/144
NE20	Srisongkram, Nakhon Phanom	26/05/00	212/214	100/106	144/157
NE21	Tha U Tain, Nakhon Phanom	26/05/00	210/210	100/106	154/154
NE22	Thatphanom, Nakhom Phanom	27/05/00	206/210	100/106	136/138
NE23	Muang, Mukdahan	27/05/00	206/212	100/108	135/144
NE24	Sawang Wirawong, Ubol Ratchathani	28/05/00	210/212	100/100	140/144
NE25	Somdet, Karasin	28/05/00	208/212	110/110	142/142
NE26	Som, Karasin	28/05/00	208/212	104/106	138/149
NE27	Muang, Khon Kaen	29/05/00	206/208	104/106	134/138
NE28	Muang, Khon Kaen	29/05/00	206/212	100/100	140/153

Collection data and the allelic sizes of 3 microsatellite loci of *A. dorsata* from the Central of Thailand

Code of Colonies	Sampling area	Date of collection	Size of allele (bp.)		
			Locus A14	Locus A24	Locus A88
C1	Muang, Saraburi	15/07/99	ND	ND	133/138
C2	Koksomrong, Lopburi	15/07/99	210/212	104/106	135/135
C3	Koksomrong, Lopburi	15/07/99	212/214	98/108	135/135
C4	Donjedee, Suphanburi	15/04/99	206/212	104/104	142/144
C5	Sriprachan, Suphanburi	24/04/99	206/206	100/100	133/148
C6	Muang, Suphanburi	23/04/99	212/214	106/110	144/148
C7	Danchang, Suphanburi	22/05/99	206/210	98/110	138/140
C8	Bangkok	15/03/99	212/214	104/106	138/142
C9	Bangkok	23/06/99	208/212	100/100	136/144
C10	Soi Dao, Chanthaburi	01/12/96	206/206	100/100	136/146
C11	Soi Dao, Chanthaburi	01/12/96	206/206	98/104	135/152
C12	Soi Dao, Chanthaburi	02/12/96	206/206	100/108	143/145
C13	Laemsing, Chanthaburi	14/05/99	208/208	104/110	140/140
C14	Muang, Trat	03/12/96	210/212	98/100	ND
C15	Krathumban, Samutt Songkhram	17/03/99	212/214	100/100	135/142
C16	Krathumban, Samutt Songkhram	11/04/97	210/212	100/104	135/144
C17	Muang, Samutt Songkhram	08/03/97	206/206	100/100	142/142
C18	Muang, Samutt Songkhram	17/12/98	210/210	100/106	138/142
C19	Muang, Samutt Songkhram	10/07/99	212/212	98/108	142/144
C20	Muang, Samutt Songkhram	15/07/99	206/212	100/106	133/148
C21	Kaeng Kra Chan, Phetburi	11/07/99	210/210	100/104	133/142
C22	Muang, Prachup Khiri Khan	27/05/96	206/208	100/104	138/138
C23	Thapsakae, Prachup Khiri Khan	16/05/99	206/208	100/100	140/144
C24	Bang Sa Phan, Prachup Khiri Khan	15/05/99	208/210	104/104	142/144
C25	Bang Sa Phan, Prachup Khiri Khan	01/06/99	210/212	100/100	135/138
C26	Bang Sa Phan, Prachup Khiri Khan	01/06/99	206/206	104/110	144/144
C27	Bang Sa Phan, Prachup Khiri Khan	01/06/99	214/216	104/104	144/144
C28	Sam Roi Yod, Prachup Khiri Khan	11/07/99	210/212	104/106	135/142
C29	Muang, Samutt Songkhram	26/07/99	212/212	104/106	133/144
C30	Muang, Samutt Songkhram	20/08/99	206/212	100/108	142/144

Code of Colonies	Sampling area	Date of collection	Size of allele (bp.)		
			Locus A14	Locus A24	Locus A88
C31	Bang Sa Phan, Prachup Khiri Khan	21/08/99	210/212	104/104	138/138
C32	Tha Klee, Nakhon Sawan	10/09/99	210/210	100/100	138/149
C33	Bang Pra Kong, Cha Choeng Sao	13/09/99	206/210	104/104	136/142
C34	Muang, Samutt Songkhram	13/10/99	206/212	98/100	133/144
C35	Laemsing, Chanthaburi	17/09/99	212/212	104/106	140/140
C36	Kumphaengsan, Nakhom Pathom	25/05/00	206/212	100/104	133/148
C37	Lamnarai, Lopburi	06/12/99	ND	ND	ND
C38	Laemsing, Chanthaburi	31/03/00	208/212	104/104	133/140
C39	Laemsing, Chanthaburi	31/03/00	212/216	104/110	140/144
C40	Khao Yai, Nakhon Na Yok	04/04/00	210/212	104/104	142/146
C41	Khao Yai, Nakhon Na Yok	04/04/00	214/214	104/104	142/148
C42	Bangkok	19/04/00	208/208	104/108	133/135
C43	Muang, Rayong	08/05/00	208/210	100/108	138/142
C44	Muang, Rayong	18/05/00	208/212	100/106	133/138
C45	Muang, Rayong	18/05/00	206/212	100/100	133/136
C46	Wisetchaichan, Aungthong	17/05/00	210/212	98/100	135/135

Collection data and the allelic sizes of 3 microsatellite loci of *A. dorsata* from the South of Thailand

Code of Colonies	Sampling area	Date of collection	Size of allele (bp.)		
			Locus A14	Locus A24	Locus A88
S1	Sawee, Chumphorn	29/05/96	210/212	100/110	142/144
S2	Prathew, Chumphorn	16/05/99	206/212	100/104	142/144
S3	Muang, Chumphorn	05/06/99	206/214	102/108	133/152
S4	Hatyai, Song Kla	09/07/99	212/214	100/106	136/138
S5	Muang, Song Kla	10/07/99	206/208	104/104	138/138
S6	Hatyai, Song Kla	10/07/99	210/210	98/100	135/138
S7	Muang, Song Kla	10/07/99	206/208	100/106	144/148
S8	Muang, Song Kla	10/07/99	206/206	100/100	136/142
S9	Thachana, Surat Thani	16/03/96	206/210	108/108	138/142
S10	Sawee, Chumphorn	25/03/96	210/210	100/104	138/144
S11	Donsak, Surat Thani	29/10/99	206/206	104/106	133/138
S12	Thachana, Surat Thani	02/05/00	212/212	102/104	138/146
S13	Thachana, Surat Thani	02/05/00	206/212	100/108	140/144
S14	Thalang, Phuket	07/05/00	210/212	102/110	144/144
S15	Thalang, Phuket	08/05/00	208/208	106/106	136/142
S16	Thalang, Phuket	08/05/00	206/208	100/100	142/142
S17	Thalang, Phuket	08/05/00	210/210	104/104	138/144
S18	Thalang, Phuket	08/05/00	210/210	103/104	138/144
S19	Thachana, Surat Thani	12/05/00	206/212	100/106	133/144
S20	Bang Klom, Song Kla	11/05/00	212/212	100/106	142/144
S21	Bang Klom, Song Kla	11/05/00	208/210	102/102	136/138
S22	Hatyai, Song Kla	10/05/00	210/214	108/108	134/144
S23	Chai Ya, Surat Thani	12/05/00	208/212	106/110	138/144
S24	Surat Thani	10/03/00	206/212	100/104	135/138
S25	Surat Thani	11/03/00	212/212	104/108	133/144

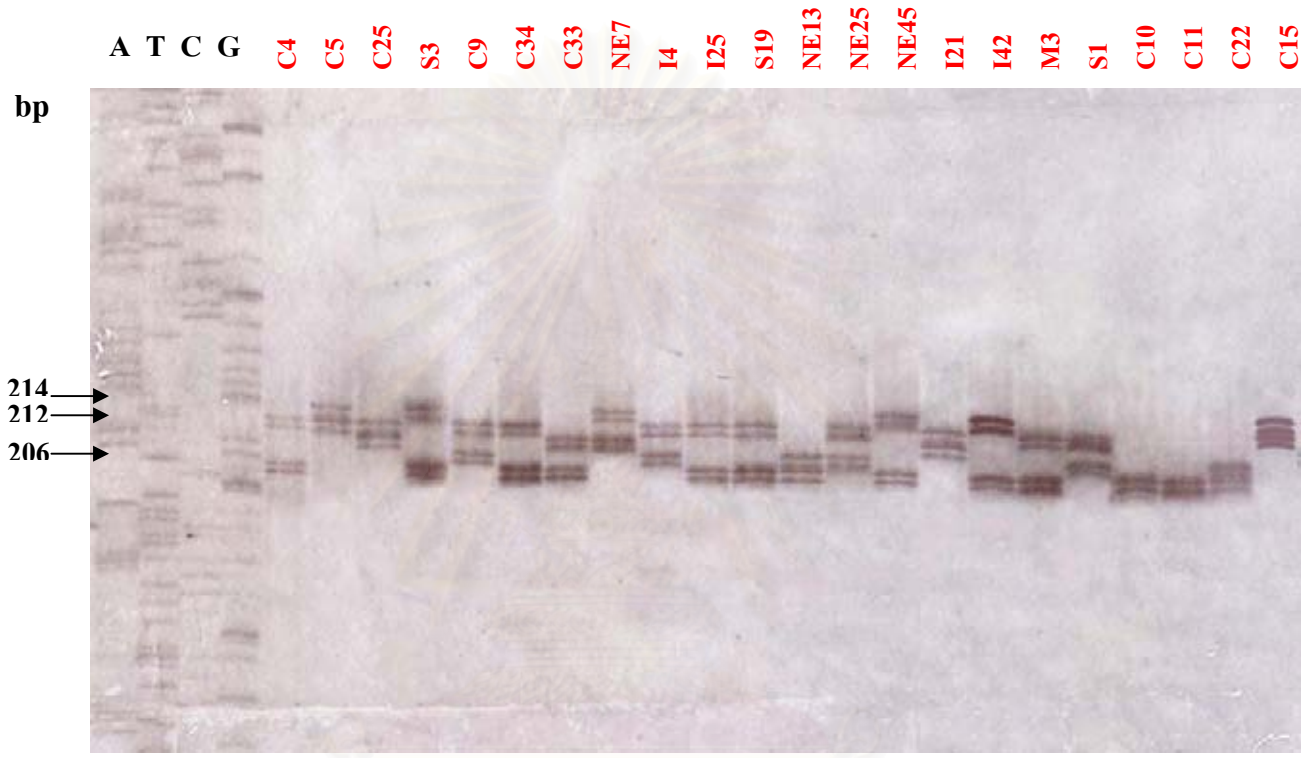
Collection data and the allelic sizes of 3 microsatellite loci of *A. dorsata* from the Samui Island of Thailand

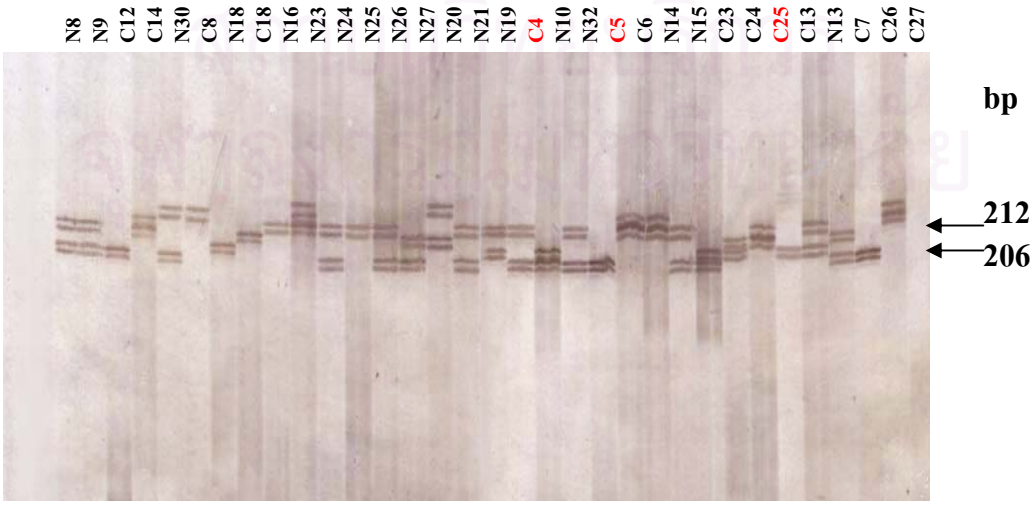
Code of Colonies	Sampling area	Date of collection	Size of allele (bp.)		
			Locus A14	Locus A24	Locus A88
I1	Tham Bon Aungthong	04/05/00	206/212	106/106	144/144
I2	Tham Bon Aungthong	04/05/00	208/212	98/106	132/144
I3	Tham Bon Aungthong	04/05/00	206/206	106/112	138/144
I4	Tham Bon Aungthong	04/05/00	206/206	106/112	142/144
I5	Tham Bon Aungthong	04/05/00	212/212	98/102	138/138
I6	Tham Bon Aungthong	04/05/00	206/210	98/106	138/144
I7	Tham Bon Lipanoi	04/05/00	206/210	98/104	144/144
I8	Tham Bon Maret	05/05/00	210/210	106/106	138/148
I9	Tham Bon Mae Nam	05/05/00	206/212	102/104	138/148
I10	Tham Bon Mae Nam	05/05/00	212/214	100/102	136/142
I11	Tham Bon Mae Nam	05/05/00	206/206	102/104	136/138
I12	Tham Bon Aungthong	05/05/00	214/216	104/106	138/142

APPENDIX 2

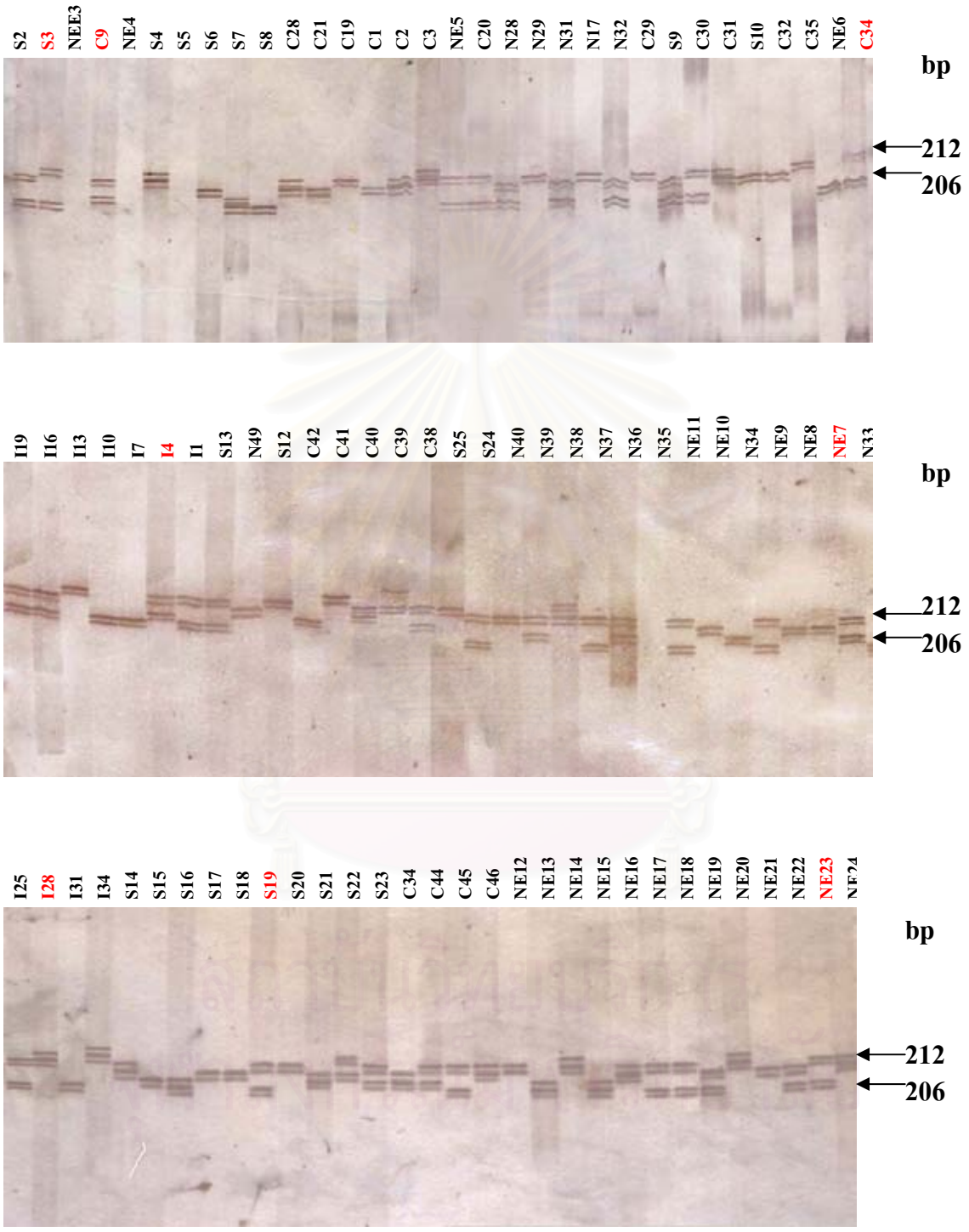
PCR amplification patterns of the three microsatellite loci (A14, A24 and A88) for 155 *A. dorsata* individuals collected from five geographic location in Thailand.

Microsatellite locus A14

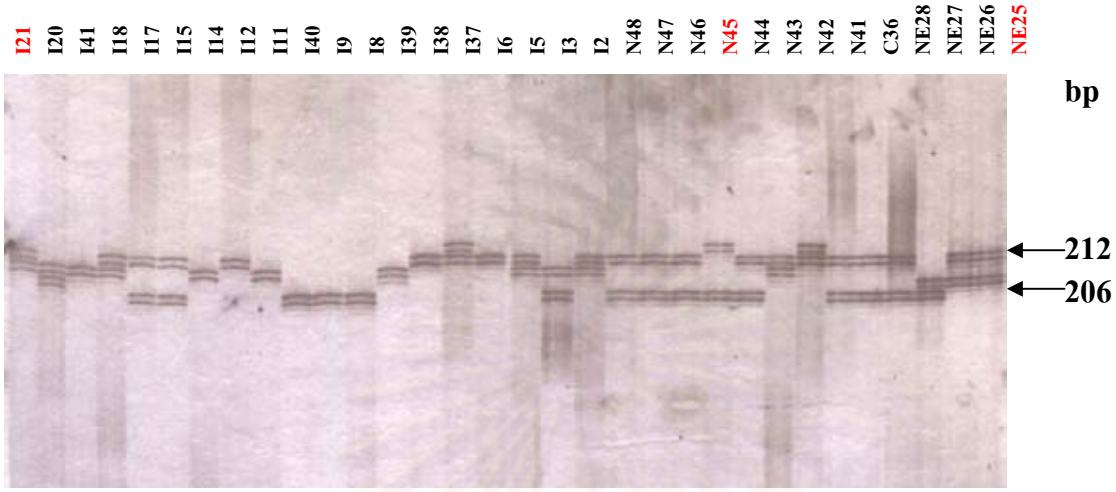


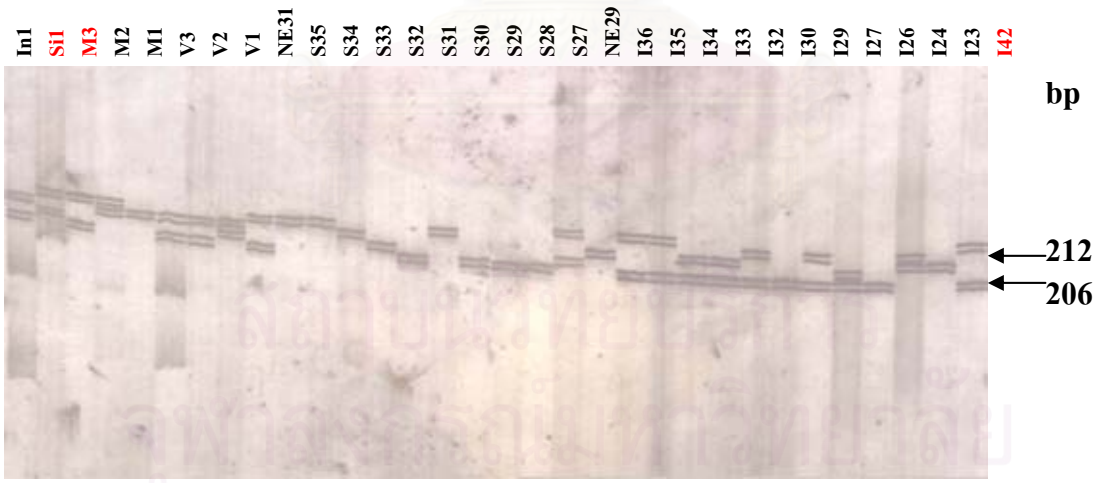


Microsatellite locus A14 (continued)

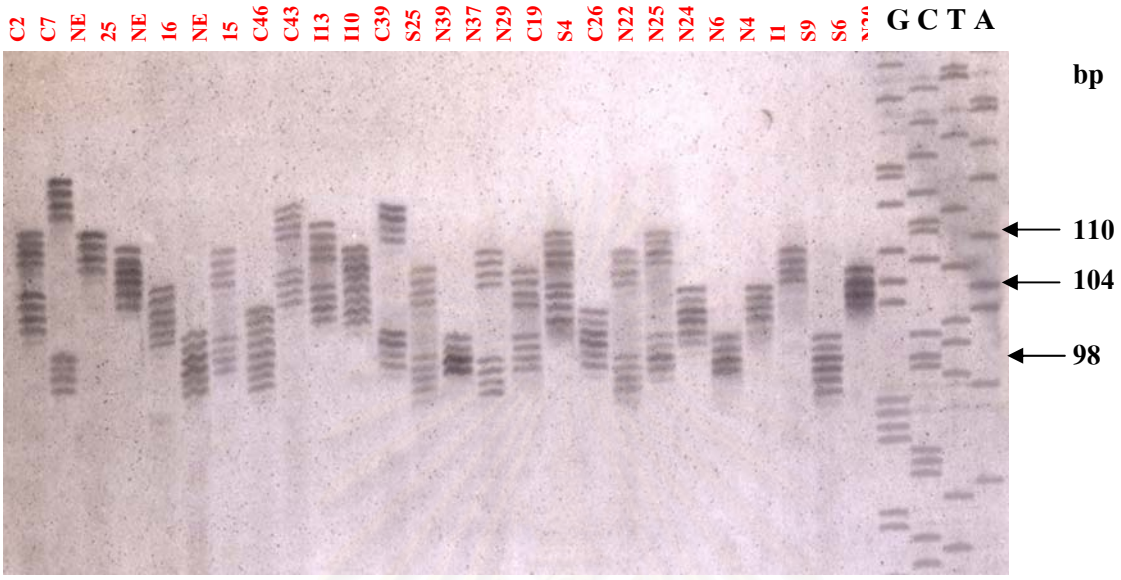


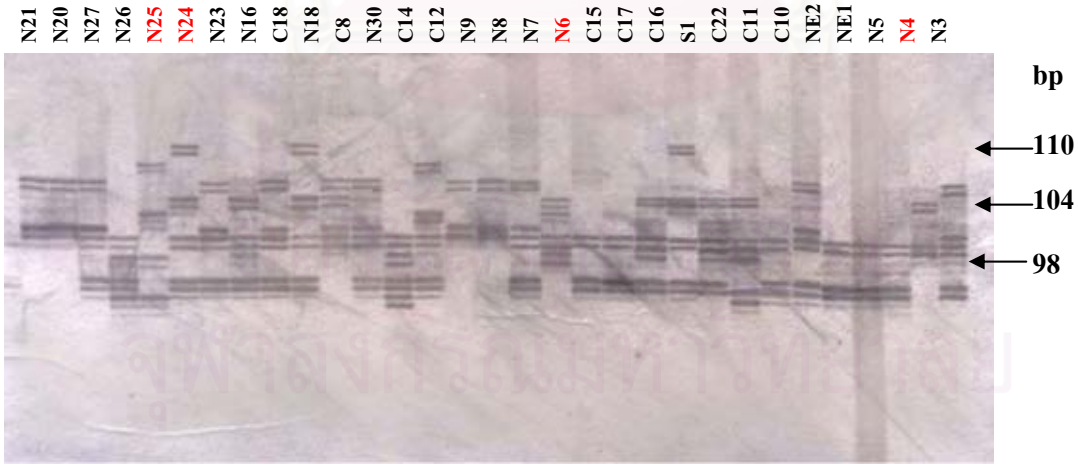
Microsatellite locus A14 (continued)



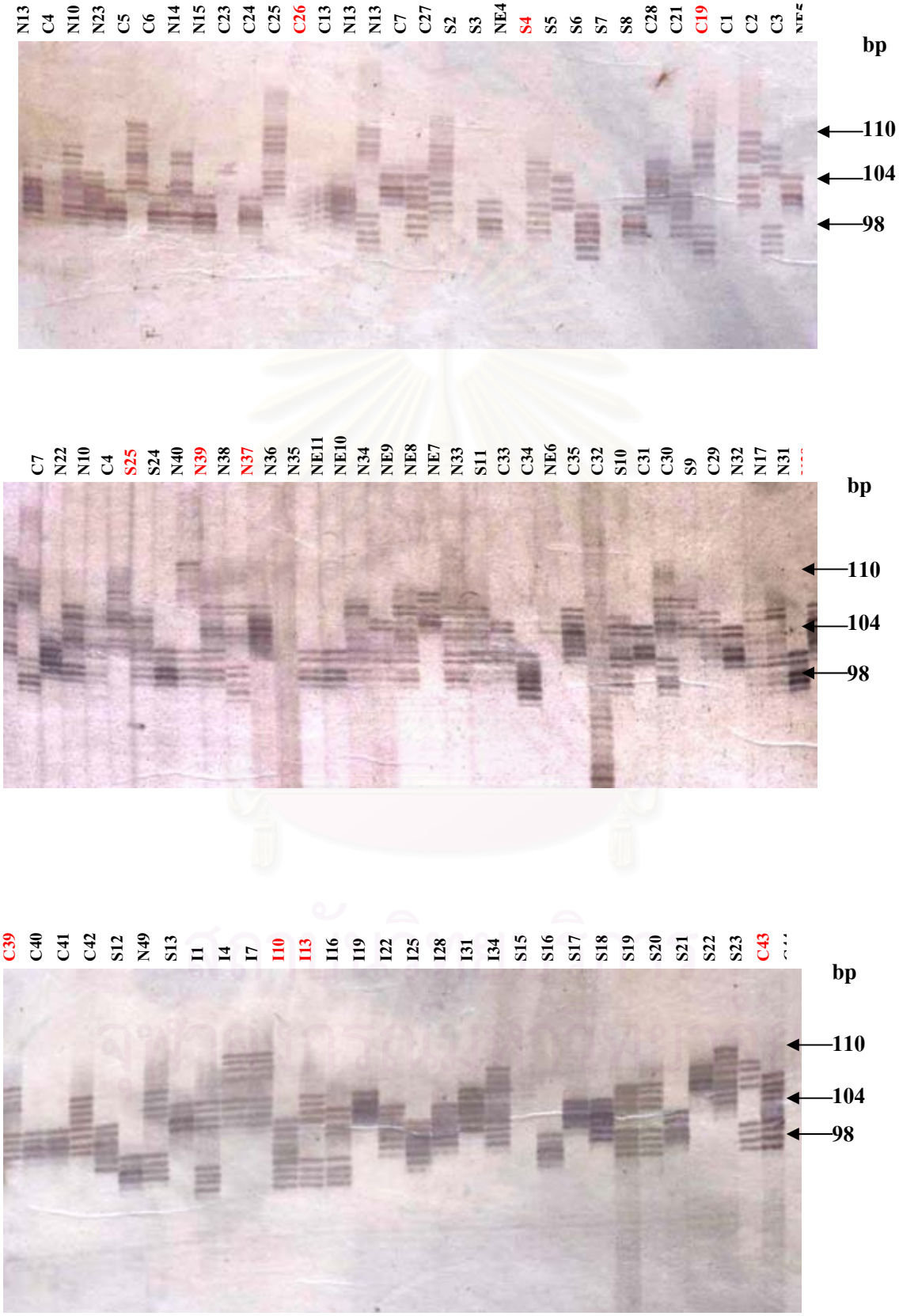


Microsatellite locus A24 (continued)

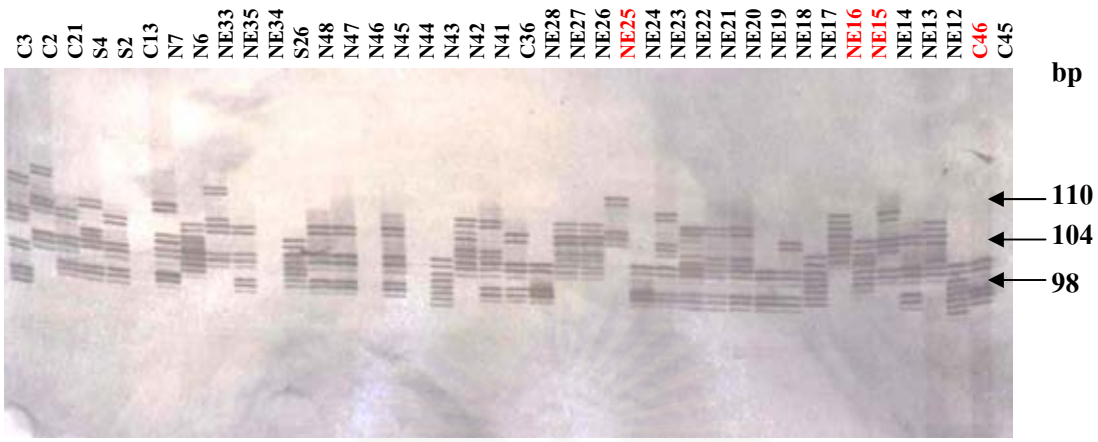


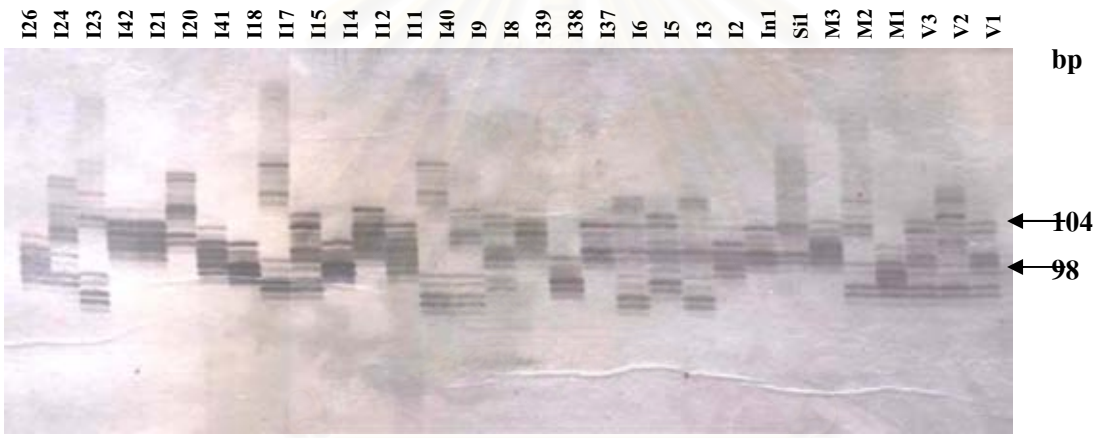


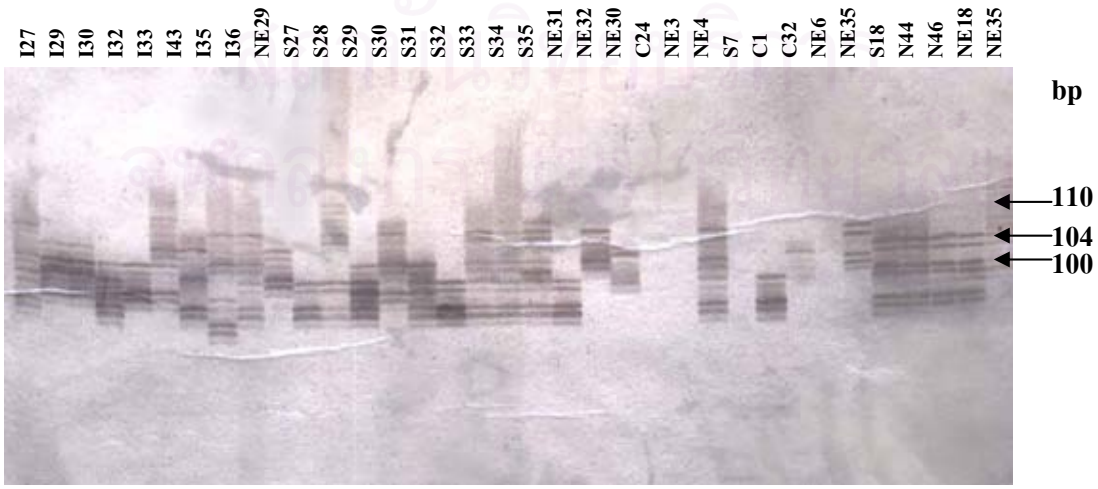
Microsatellite locus A24 (continued)



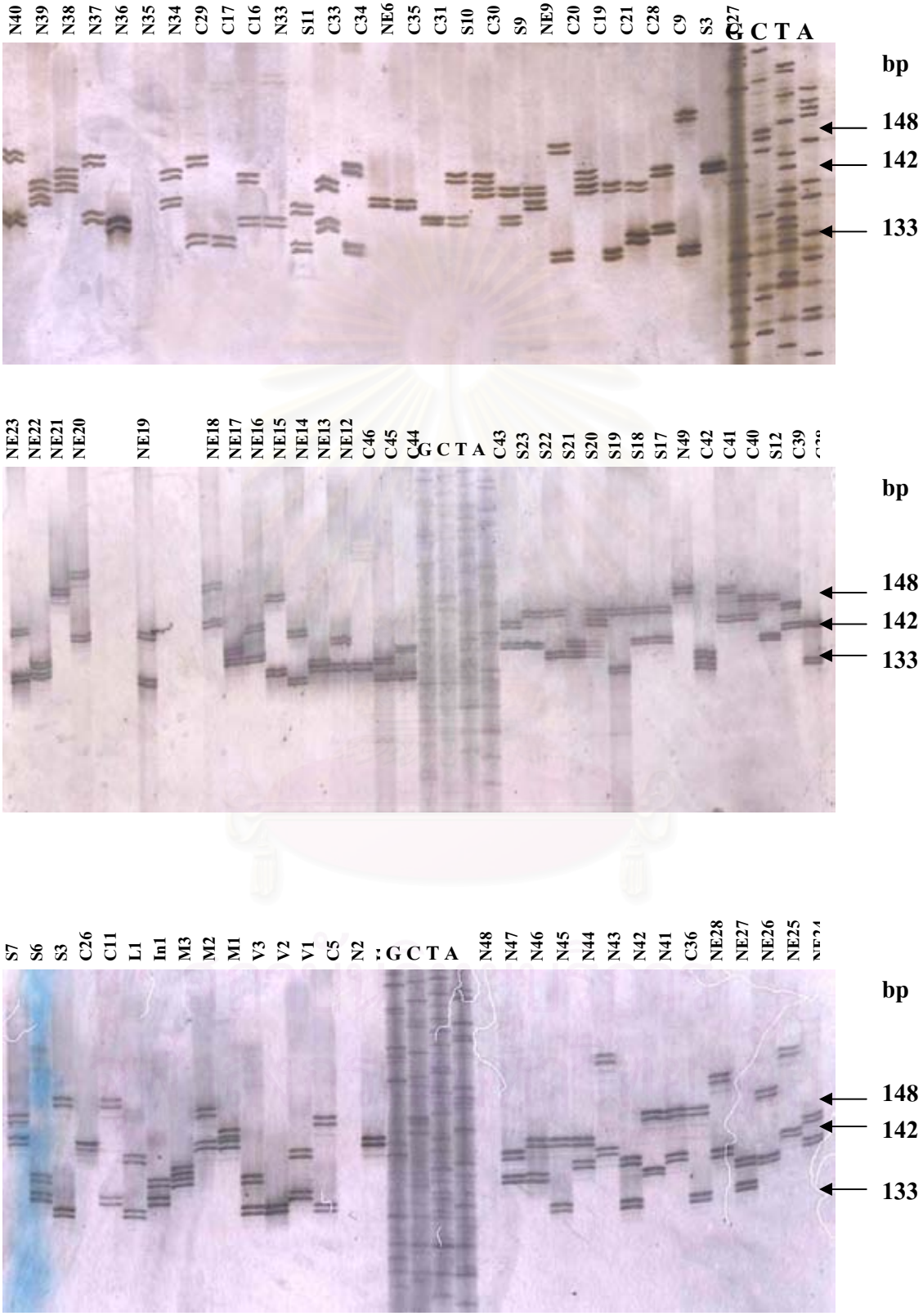
Microsatellite locus A24 (continued)



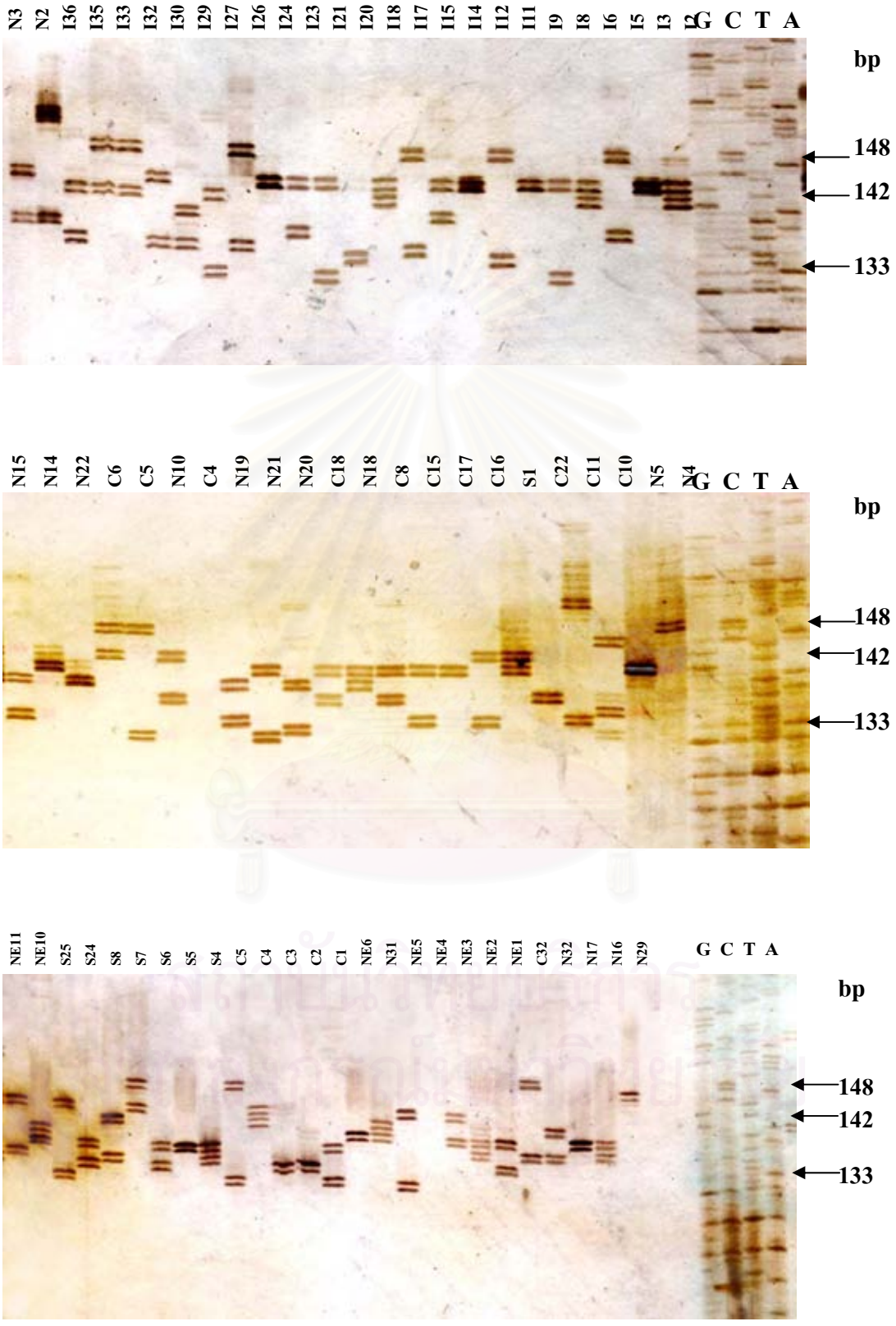




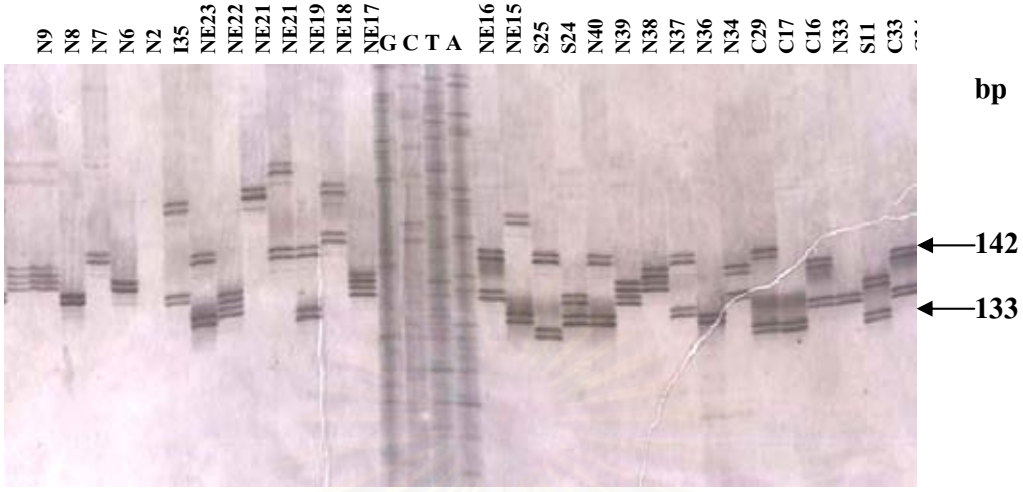
Microsatellite locus A88 (continued)

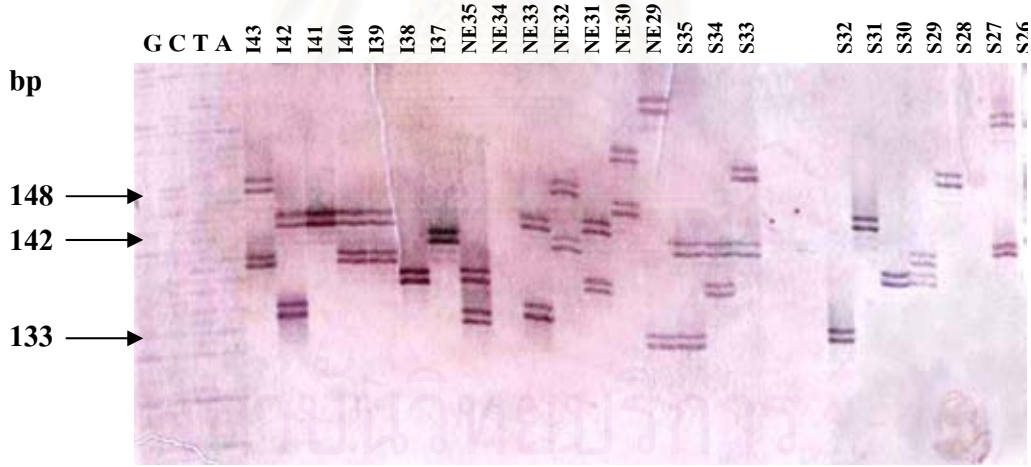


Microsatellite locus A88 (continued)



Microsatellite locus A88 (continued)





BIOGRAPHY

Miss Sucheera Insuan was born on September 8, 1973. She graduated with the Bachelor degree of Science in Biochemistry from Chulalongkorn University in 1993. She continued her study in Biochemistry Department, Chulalongkorn University in 1998.