CHAPTER I

INTRODUCTION

Honeybees are social insects which they are great economic importance. Honeybees produce important hive products such as honey, beeswax, royal jelly, and pollen that provide both nutritious food and cash income. Moreover, bee pollination helps increasing crop yield and improving quality of seed and fruit. Beekeeping and the use of bee products have a long history in Thailand (Wongsiri *et al.,* 1987). Then, the income of Thailand from the natural honey exportation had a high value since 1984 (Table 1.1).

There are five species of honeybees in Thailand. Among them, the giant on rock honeybees, *Apis dorsata*, the dwarf honeybees, *Apis florea*, the small dwarf honeybees, *Apis andreniformis*, and the eastern honeybees, *Apis cerana* are native species, whereas the european honeybees, *Apis mellifera* has been introduced which included *A.m. ligustica*, and *A.m. carnica* (Ruttner, 1988; Tingek *et al.*, 1988; Wongsiri *et al.,* 1990). Only *A. mellifera* and *A. cerana* could be kept and managed in hives for commercial beekeeping (Figure 1.1).

Modern beekeeping in Thailand started in the early 1940s and the european honeybee *A. mellifera* is the most important species in Thai beekeeping. In the 1970s, the native bee *A. cerana* was the species of choice for beekeeping in Thailand because *A. cerana* has a behavioral and physiological resistance to *Varooa* (Wongsiri *et al.,* 1987). As a result, *A. cerana* survive much better than does *A. mellifera* in the region of the tropics.

The social structure of honeybee colony is a clear distinction between a single queen, large number of workers and a few hundred of drones. The females, the queen and workers, are heterozygotes(diploid 2n = 32) and the drones or males are hemizygotes (haploid individuals) which they came from a fertilized eggs and an unfertilized eggs,

Table 1.1 The imports and exports of natural honey of Thailand in 1986 – 1998.

Year	Import		Export	
	Quantity (Ton)	Value (Million Baht)	Quantity (Ton)	Value (Million Baht)
1986	132	4.03	1,222	21.02
1987	130	3.84	745	11.11
1988	125	4.42	1,750	24.53
1989	146	5.59	704	9.29
1990	166	6.19	2,432	31.11
1991	232	8.79	1,206	16.96
1992	172	7.30	2,407	32.39
1993	230	10.60	2,108	28.30
1994	264	12.24	1,894	26.94
1995	238	11.10	1,908	29.37
1996	326	16.29	2,656	44.01
1997	283	17.66	1,996	32.94
Jan.-Oct.1998	104	7.05	1,053	27.19

Source : Thai Customs Department, Finance Ministry, Thailand.

Figure 1.1 Beekeeping of *Apis cerana* in Thailand.

respectively(Ruttner, 1988). The queen secretes a trans - 9 - keto - 2 - decenoic acid or, simple, queen substance or queen pheromone in her mandibular glands which inhibits development of workers' ovaries. So, the workers never mate and never lay eggs so long as the queen is alive. Mating behavior in honeybee, the males and virgin queen leave the nest on mating flights. Through the queen mates with 7 to 17 drones and stores about 5 million sperms in the spermatheca during her mating period, which is a sufficient supplied for egg laying about 2-3 years. The life cycle of honeybee colonies begins by the colony becoming more populous, the workers make provision to divide it by producing swarms. After the queen cell is capped, the mother queen with some workers leave the colony to establish a new colony. In the parental nest, the first new queen daughters is recognized and becomes the new queen.

For the nest of *Apis*, including the common honeybees, *Apis mellifera* and *A. cerana*, have multiple combs and nest in holes or dark caves and hollow trees. Whereas the nest of the giant honeybee, *A. dorsata* of East Asia, is a single exposed comb hanging from a branch or a rock. For *A. andreniformis* and *A. florea*, they have the small nest which is a single comb and stay on a branch(Rinderer *et al.*, 1996). The nest usually has two parts. The brood chamber contains the queen and the cell in which the larvae are reared. This chamber is surrounded by another, called the involucrum which liter all envelops the brood cells. Honey and pollen are stored in groups of large cells outside the brood chamber. The honeybee comb is a thin sheet of wax covered with hexagonal cell on both sides. It hangs from the roof of the nest cavity and has a high strength-to-weigh ratio : it can support 40 times of its own weight. An amazing about behavior of honeybees, the worker can convey the distance, direction and quality of the food source for the dance language. So, the forages (workers) do not leave the hive to look for food at random. They wait for the scouts to tell them where forages are found.

The taxonomy of *A. cerana* is as follow (Borror *et al.*, 1976 and Gojmerac, 1980);

Kingdom	Metazoa
Phylum	Arthropoda
Class	Insecta
Order	Hymenoptera
Super family	Apoidea
Family	Apidae
Genus	*Apis*
Species	*Apis cerana*

Scientific name: *Apis cerana* Fabricius, 1973.

Apis cerana is a native bee to southern and eastern Asia. Ruttner (1988) has distinguished four subspecies of *A. cerana,* based on morphometric characters, distribution and behavior, to be 1) a northern subspecies; *A. cerana cerana* found throughout Afghanistan, Pakistan, North India, China and North Vietnam, 2) a southern subspecies; *A. cerana indica* distributed from South India, Sri Lanka, Bangladesh, Burma, Malaysia, Thailand, Indonesia and The Philippines, 3) a Himalayan subspecies; *A. cerana himalaya* found in the Himalayan mountains north of India, Nepal and probably in the South-West of China, 4) a Japanese subspecies; *A. cerana japonica* distributed throughout Japan and Korea. Geographic distributions of these subspecies is shown by Figure 1.2.

Limbipichai (1990) divided groups of *A. cerana* In Thailand by morphometric characteristics using two multivariate statistical analyses, Canonical analysis and Cluster analyses. Using the first approach, *A. cerana* populations were divided into 3 groups, 1) Northern latitude *A. cerana* (samples collected from Chiang Rai - Phetchaburi), 2) Southern latitude *A. cerana* (samples collected from Chumphon-Song Khla) and 3) Samui Island *A. cerana* while Cluster analyses can be grouped *A. cerana* into Northern latitude bees and Southern latitude bees. Moreover, the technique of

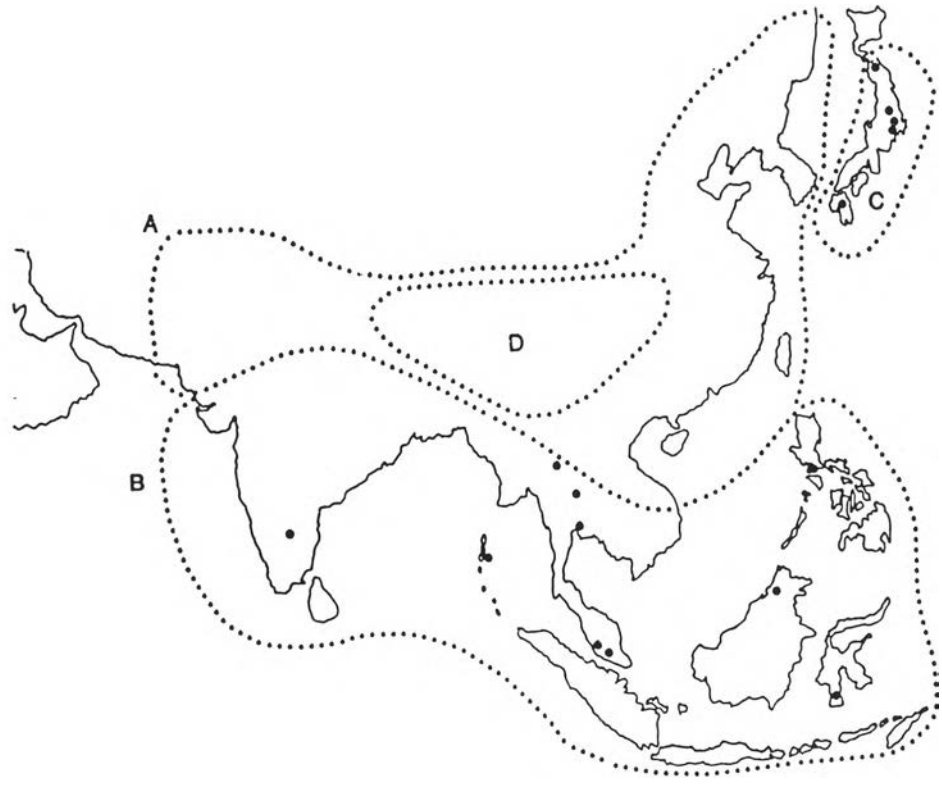

Figure 1.2 Dotted lines indicate approximate geographic ranges of *A. cerana* subspecies as recognized by Ruttner (1988). A= *A. cerana cerana*; B=*A. cerana indica*; C=*A. cerana japonica*; D=*A. cerana himalaya*.

restriction fragment length polymorphism(RFLP) has been used to study DNA variation of *A. cerana* by probed total DNA-*Hae*III digestion with a DNA probe no.99 containing repetitive sequence of *A. cerana*. The result showed six different RFLPs patterns (Uthaisang, 1994).

Since then, genetic variability and population structure of Thai honeybees *A. cerana* was investigated using PCR - RFLP of four mtDNA regions (ATPase6 - ATPase8 gene, sRNA gene, lrRNA gene and inter COI –COII region). The PCR products of ATPase6-ATPase8 gene were digested with three restriction endonuclease (*Taq* I, *Ssp*I, and *Vsp*I) revealed two, five and six haplotypes, respectively. Ten composite haplotypes were generated. In addition, three, five and eight haplotypes were obtained from *Dra*I digestion of PCR - amplified sRNA gene, lrRNA gene and inter COI - COII region, respectively. These three mtDNA regions generated thirteen composite haplotypes. A UPGMA phenogram based on genetic distance among populations clearly allocated 5 geographic samples of *A. cerana* into 2 distinct evolutionary groups: Northern (North, North-East and Central) and Southern (South and Samui Island) and Monte Carlo simulation analyses, South and Samui Island was seperated (Sihanuntavong, 1997 and Songram, 1997).

At present, the beekeeping in Thailand with the european honeybee *A. mellifera* is much popular than with the native due to *A. cerana* an inability of *A. cerana* to rear queens and control absconding(Wongsiri,1992). In addition *A. cerana* always swarming and consequently it gives low honeybee products yield (Wongsiri, 1992). Therefore the number of *A. mellifera* colonies increase rapidly, while the number of *A. cerana* colonies decrease slowly (Figure 1.3) (Wongsiri and Chen, 1995).. This affects on the natural balance among honeybee species in Thailand. Thus, the research need for beekeeping with *A. cerana* is to develop a selective breeding program of *A. cerana* in Thailand. In order to be successful to develop beekeeping and conservation of *A. cerana* in Thailand, intensive studies on basic biology, ecology characteristics and population genetic are necessary.

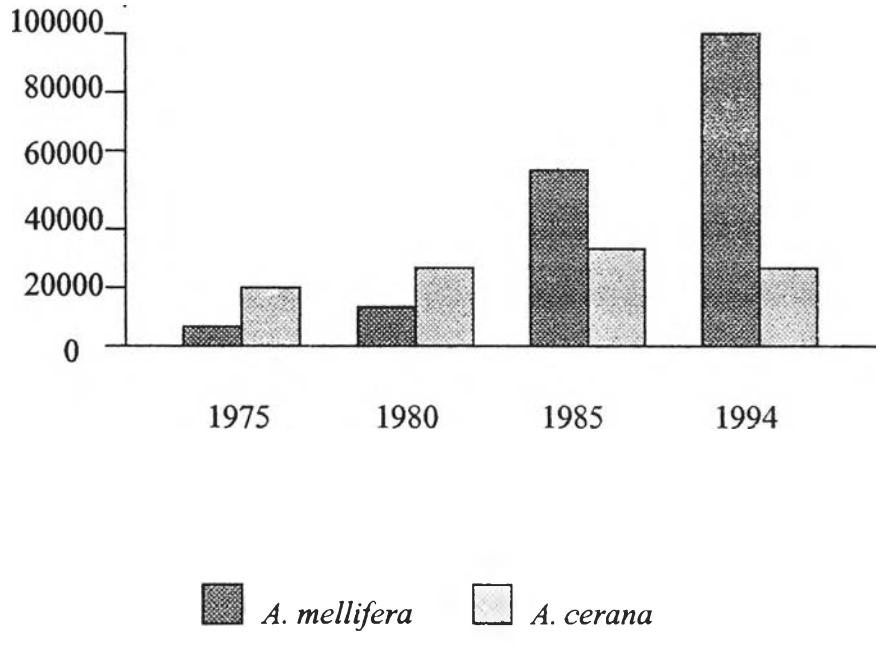

Figure 1.3 Number of *A. cerana* and *A. mellifera* colonies in northern Thailand (Wongsiri and Chen, 1995).

Basic markers for population genetic analysis

1. Protein marker

allozymes

Protein electrophoresis provided a new source of markers and allowed individuals to be identified as homozygotes or heterozygotes at a given locus. The term "allozyme" refers to different allelic form of nuclear - encoded enzymes. The polymorphic of allozyme can be detected by electrophoresis observed based on the properties of enzymes in which different net charges migrate at the different rate through a gel matrix when exposed to an electric field. Homozygotes at a given locus typically yield one band while heterozygotes typically yield two, three or five band, depending on the quaternary structure of the enzyme. This technique is relatively inexpensive and straightforward once the basis procedures have been developed for a given species. Moreover, large number of sample specimen can be analyzed within a limitation of time. Nevertheless, limitation of using allozymes is due mainly to their functional constraints reflecting low mutation rates. Sites and Davis(1989) found higher number of variable markers using restriction site analysis in both mtDNA and nuclear ribosomal DNA than these found using allozymes among central Mexican chromosome races of the lizard *Sceloporus grammicus*. These and other studies (Wetton *et al.*, 1987; Karl *et al.*, 1992; Karl and Avise, 1993) showed a definite lower taxonomic limit to the resolving power of protein electrophoresis. However allozyme analysis is a technique of choice to begin with particularly when the species under investigation has not been reported for any molecular data.

Allozyme have proved to be very useful in many other species but have brought little additional information to our understanding of genetic diversity of honeybees. The main reason is the low level of allozyme polymorphism, as in other Hymenoptera (Packer and Owen,1992) which is generally considered as a consequence of haplodiploidy (Pamilo *et al.*, 1978; Pamilo and Crozier, 1981).

2. DNA markers

The physical aspects of DNA offer several advantages over allozyme studies. DNA is found in nearly all cells of all organisms and it can be recovered from both living and dead tissue. Besides this tissues can be easily stored under field conditions, and in many cases only nanograms are needed for analysis (when amplified by polymerase chain reaction, PCR). The molecule is so stable that recognizable sequences can remain intact for hundreds of millions of year (Cano *et al.*, 1993). Then, analysis of polymorphisms at the DNA level is the direct approach to study genetic variation at both inter- and intraspecific levels. DNA can be classified in two broad categories: chomosomal(nuclear) or extrachomosomal(organelle) DNA.

2.1 Animal mitochondrial DNA (mtDNA)

Mitochondrial DNA is an organelle DNA found in mitochondria. MtDNA is inherited in non - Mendelian fashion, usually with uniparental (maternal) transmission. Animal mitochondrial DNA occurs as a single circular molecule and generally about 16,000-20,000 bp long. This organelle DNA contains 13 protein-encoding genes (the gene for 22 tRNAs and two ribosomal RNA subunit) and a non-coding region containing t he origin of replication. At present, mtDNA is widely employed in evolutionary and population genetic studies because it is small, maternally transmitted, easily purified and consists mostly of coding sequences. In addition, the mutation rate of mtDNA is usually much greater rapidly than that of single-copy nuclear genes. In 1991, Simon has reviewed information on the relative rates of evolution of genes in mitochondrial genome of invertebrates. The non-coding A+T rich region of mtDNA evolves rapidly, while genes such as those coding for the large and small rRNA, or cytochrome oxidase subunits I and II (COI and COII) evolve at the lower rates.

The studies on mtDNA variation can be identified from DNA sequences, restriction fragments (RFLP) obtained either (by hybridizing total DNA with known mitochondrial probes), PCR-PFLP (by amplifying mtDNA with PCR and separating the

cleaved restriction fragments electrophoretically) or conformational polymorphisms. In honeybees, the first studies confirmed the existence of three evolutionary branches of *A. mellifera*, although slightly modifying their subspecies composition and their estimated time of divergence as inferred from morphometry (Smith, 1991; Garnery *et al.*, 1992). For Thai honeybee, mtDNA marker is employed for study genetic variability in *A. cerana* populations using PCR-RFLP in various mtDNA regions such as lrRNA gene, sRNA gene, inter COI-COII region and ATPase 6-ATPase 8 gene and DNA sequencing of mitochondrial lrRNA gene. A large genetic discontinuity of *A. cerana* population in Thailand were divided into 3 groups : Northern (North, North-East, Central), Southern and Samui Island (Sihanuntavong, 1997 and Songram, 1997).

2.2 Nuclear DNA

Nuclear genomes are much larger than mtDNA. The size of nuclear genome range from $<10^6$ bp in some bacteria to $>10^{11}$ bp in some plants (Cavalier and Smith, 1985; Li *et al.*,1985). 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 (introns or intergenic regions). Nuclear DNA (nDNA) contains both unique (single-copy) and nonunique (duplicated or repetitive regions). A single-copy region generally codes for a particular gene product. 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 ribosomal RNA genes

Ribosomal RNA (rRNA) genes are coded for functional RNA molecules (rRNA) participating in the protein biosynthesis machinery of all organisms (Gutell,

Larsen, and Woese, 1994). In insects, rRNA genes are present as tandem repeat as in other eukaryotes. Each repeat unit consists of regions coding for the 18S, 5.8S, and 28S rRNA, two internal transcribed spacer (ITS1 and ITS2) and an external transcribed spacer (ETS) or usually called an intergenic spacer (IGS) at the 3′ end of 28S, RNA gene (Figure 1.4). The rDNA unit has component sequences that evolve at different rates (Apples and Honeycutt, 1986). The coding region evolve slowly and are highly conserved between different species and genera (Turbeville *et al.*, 1991). These can be used for comparing distantly related organisms. The spacer regions (ITS and IGS) evolve rapidly and can be used for comparison of closely related species, subspecies or populations (Black *et al.*, 1989; Chen *et al.*, 1992 and Collins *et al.*, 1990). In the past, DNA variation in these spacer regions was analyzed using restriction fragment length polymorphism (RFLP) (Hillis and Dixon, 1991). Recently, these regions and in particular, the internal transcribed spacers (ITS) have been increasing used through polymerase chain reaction (PCR) technology. Basically, conserved stretches of DNA in the coding rDNA adjacent to the ITS can be conveniently used to design "universal" primers for amplification of variable spacer regions. Sequence and length variation in the ITS regions was used for evolutionary studies of closely related insects in five species of the *Anopheles gambiae* complex (Paskewitz, Wessont and Collins, 1993). In addition, sequence divergence in the internal transcribed spacer region 1 (ITS1) of the ribosomal DNA locus was assessed in subspecies of the coastal North American tiger beetle, *Cicindela dorsalis* (Vogler and Desalle, 1994).

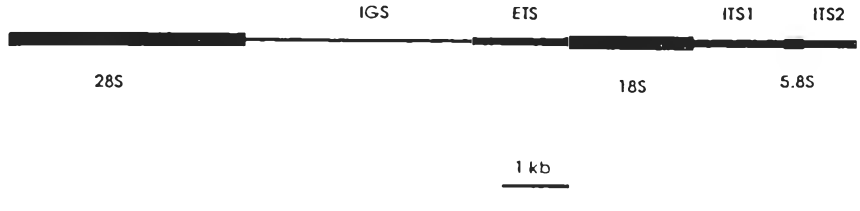

Figure 1.4 Schematic representation of an insect ribosomal DNA repeat unit; IGS (intergenic spacer), ETS (external transcribed spacer), ITS (internal transcribed spacer).

Microsatellites

Microsatellites are a variable number (up to 100) of tandem repeats of 1 - 6 bp. Microsatellites are highly abundant and randomly dispersed in most eukaryote genomes (Valdes *et at.*, 1993; Weissenbach *et al.*, 1992 and Wright, 1993). It was estimated that one microsatellite locus can be found every 10 kb in eukaryotic genomes (Tautz, 1989). The mutation rate of microsatellites is high, approximately 10^{-2} to 10^{-4} per generation (Tautz, 1989; Weber and Wong, 1993). All types of microsatellites have shown variation in the number of repeats and display a level of polymorphism generally greater than that of standard unique-sequence probe. 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. In addition to genomic library or database screening, it is also possible to detect microsatellite loci by using conserved primer sequences from closely related species. Rubinsztein *et al.*(1995) and Garza, Slatkin and Freimer (1995) used this approach studies genetic polymorphism of chimpanzees with human primers and Moritz *et al.*(1995) studies microsatellite variation in *A. dorsata* using locus specific primers developed from *A. mellifera* primers. Therefore, microsatellite loci constitute powerful nuclear markers, increasingly used for identify testing population studies, linkage analysis and genetic mapping. For haplo-diploid, social insects such as honeybees and bumble bees, microsatellites tend to have many alleles and high heterozygosity making them potentially ideal marker population genetic studies in Hymenoptera (Queller, 1993). For example, microsatellite analysis confirmed that *A. mellifera* evolved in three distinct and deeply differentiated lineage previously detected by morphological and mitochondrial DNA studies (Estoup *et al.*, 1995). Ten microsatellite loci of *A. mellifera* was used to investigate genetic differentiation of *Bombus terrestris* (Hymenoptera: Apidae) in Europe which found 8 of the 10 microsatellite loci displayed high levels of polymorphism in most populations (Estoup *et al.*, 1996).

Therefore, the aim of this thesis is to investigate genetic polymorphism among Thai honey bee *Apis cerana* by determination of sequence divergence in the ITS region and by examination of microsatellite variation. The analysis included five different geographic population of *A. cerana* in Thailand ; The North, The North -East, The Central, The South and The Samui Island.