CHAPTER 1

INTRODUCTION

The honeybee is one of the most importance insect. It increases crop plant production by mediation of pollination. When the honeybee gathers nectar her body becomes dusted with pollen. As she moves from flower to flower, the pollen passes from male to female stigma that causes of cross-pollination. The honeybee produces natural honey, beeswax, pollen, royal jelly and propolis which are of high economic value.

In United States, the annual value of honey produced in North America is estimated at about 25 million dollar (Anonymous, 1993) and estimated approach 57 billion dollars for the value of pollination (Southwick and Southwick, 1992). In Thailand during 1986 to 1997, the honey exported and earning in country is sharply increased up to 44 million baht. (Table 1.1)

The honeybees are social insects and live together in colonies. In one colony consists of one queen, serveral thousand of workers and a few hundred of drones. The queen is the mother of all members of the colony. The size of queen is the largest bee and each normal colony has only one. A normally mated queen is capable of laying two kinds of eggs, fertilized eggs from female bees arise, and unfertilized eggs which, through the phenomenon of parthenogenesis, produce males (Wongsiri and Deowanish, 1995). Both the workers and queen are heterozygous diploid reproduced from sexual fertilization, whereas the drone are the

Table 1.1 The import and export of natural honey of Thailand during 1986-Sept. 1998.

Year	Import		Export	
	Quantity (Ton)	Value (Million Baht)	Quantity (Ton)	Value (Million Baht)
1986	132	4.03	1222	21.02
1987	130	3.84	745	11.11
1988	125	4.42	1750	24.53
1989	146	5.59	704	9.29
1990	166	6.19	2432	1.11
1991	232	8.79	1206	16.96
1992	172	7.30	2407	32.39
1993	230	10.60	2108	28.30
1994	264	12.24	1894	26.94
1995	238	11.10	1908	29.37
1996	326	16.29	2656	44.01
1997	284	17.66	1996	32.94
1998 (Jan-Sept)	104	7.05	1053	27.19

Source: Thai Customs Department, Finance Ministry, Thailand.

future fathers. Their only task is to mate with the queen bee and have hemizygotes (haploid individuals) arising from unfertilized eggs.

The honeybees use their dance language for communication such as indicate distance of food to other bees in the colony. In 1961, Lindauer studies the dance language of vary honeybee species and found that *Apis florea* in performed the waggle dance on a horizontal surface, and straight run portion of the dance is pointed right in the direction foragers should fly when they leave the nest. The other honeybee species are performed the dance on the vertical face of a honey comb, and directional cues are presented with reference to the pull of gravity.

The honeybees in Thailand

In Thailand, five honeybee species are found. Four of them are the native species. The giant or rock honeybee, *A. dorsata;* the dwarf *(A. florea*) and small dwarf honeybee (*A. andreniformis*) are open-nesting species that build a single, exposed comb. The eastern honeybee (*A. cerana*) and the imported species, the western honeybee (*A. mellifera*) are nested in cavities where they construct multiple exposed comb. The eastern honeybee, *A. cerana,* has been used in domestic beekeeping in Thailand for along time. Mostly they are introduced from wild colonies and kept in domestic hives (Figure 1.1). For industrial beekeeping, *A. mellifera* is more sucessful than *A .cerana* because the imported bee is not aggressive, produces high quantity of honey products and lay eggs at higher rate than *A. cerana.* However, *A. cerana* has some better biological features such as resistance to honeybee mites, do not required sugar feeding and has better climatic adaptability. The disadvantages of *A. cerana* are their aggression,

Figure 1.1 Beekeeping of *Apis cerana* inThailand

low quantity of honey production and slowly rate of egg laying.

The classification of *Apis cerana*

The honeybees are species belonging to the superfamily Apoidea, order Hymenoptera and genus *Apis* .The taxonomic definition of the honey bee is as follow (Borror *et al.*, 1976 and Gojmerac, 1980);

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

Scientific name: *Apis cerana Fabricius*, 1793.

The studied of *Apis cerana*

A. cerana, the Asian cavity-nesting bees, occurs over a wide range of climates and habitats in Asian. They have been subdivided into subspecies on the basis of morphological difference. In 1953, Maa placed these bees in their own subgenus, *Apis* (Stigmatapis) and recognized 11 species in this group. Maa's scheme was not widely accepted, and until recently, most authors used *A. cerana* to refer to all of these populations. Ruttner (1988) reexamined the morphometric information on the eastern cavity-nesting bees, which he considered as

one species, *A. cerana*. He grouped the *A. cerana* population into four subspecies; a northern subspecies, *A. cerana cerana* from Afghanistan, Pakistan, north India, China and north Viet Nam; a southern subspecies, *A.c. indica*, from south India, Sri Lanka, Bomgladesh, Burma, Malaysia, Thailand, Indonesia and the Philippines; a Japanese subspecies, *A. c. Japonica*; and Himalayan subspecies, *A. c. himalaya*. Limbipichai (1990) studied morphological characters (such as: proboscis, fore and hind wing, hind leg, third and sixth sternites etc.) of the eastern honeybee (*A. cerana*) in Thailand and the Malaysian peninsula. The analysis was performed by Multivariate Statistical Analysis Software (SAS) with two methods, the clustering analysis and the cononical discriminant analysis. The former method is capable to discriminate into two groups; Northern latitude bees, from Chiang Rai-Phetchaburi and Southern latitude bees, from Chumphon-Songkhla which include Samui Island bees. The last method is able to separate Samui Island bees from Southern latitude bees.

However, the morphometric parameters are subject to environmental effect, and their genetic basic is undefined (Daly, 1998). Thus, the discovery of numerous electrophoretic allozymes opened up a new field of molecular taxonomy. For allozyme analysis, the western honeybee, *Apis mellifera* has been the subject of a great number of allozyme studies such as; studies of the bees from different geographic regions, others have demonstrated the possibility of using allozymes for racial discrimination (Nunamaker *et al.*, 1984b; Daly, 1991). The studies of allozymes in Asian honeybee were done by Nunamaker, Wilson and Ahmad (1984). They used specimens of *A. cerana* , *A. dorsata* and *A. florea* from Pakistan and studied with two enzymes: malate

dehydrogenase (MDH) and esterase (EST). MDH produced one intense band for *A. florea* and one intense and faint band in both *A. dorsata* and *A. cerana*. While, for EST, *A. cerana* had two faint band which different to the other two species. In a brief report on EST by Tanabe and Tamaki (1985) indicated that *A. mellifera* and *A. cerana* had species specific differences. Unfortunately; the low level of allozyme polymorphism in social insects, especially honeybees (Parker and Owen, 1992) limits applied ability of this technique. This may be caused by the effect of haplo-diploid sex determination system in social insects (Pamilo *et al.*, 1978; Graur, 1985).

Recently, variation at the nuclear and mtDNA level have been effectively investigated to determine genetic variation and population structure among honeybee populations. The nuclear DNA of honeybee has a diploid chromosome number of 16 (Petrunkewitsch, 1901) and a total size of about 180 megabase pair (Jordan and Brosemer, 1974). In 1990, Hall analysed of introgressive hybridization between African and European honey bees using nuclear DNA of *A. mellifera* as a probe. This report showed paternal gene flow between African and European honeybee but suggested asymmetrics in levels of introgressive hybridization. In addition, the eastern honeybee, *A. cerana* which collected from five different areas in Thailand, have been studied the genetic variation using constructed DNA probe number 99 which containing repetitive sequence of *A. cerana*. After digested chomosomal DNA of *A. cerana* male (haploid) with *Hae*III. The Southern blot analysis showed six different RFLPs patterns of *A. cerana* from five different area in Thailand. (Uthaisang, 1993)

Mitochondrial DNA (mtDNA) has recently been focused on number of molecular genetic and evolution studies. (Wilson *et al.*, 1985; Tzagologg and Myer, 1986; Avise *et al.*, 1987; Moritz *et al.*, 1987). In multicellular animals, mtDNA is a small circular molecule of appoximately 16 kb carrying a set of highly conserved genes. (Wilson *et al.*, 1985). It generally encodes 13 proteins, two ribosomal RNAs, 22 tRNAs and control region. Animal mtDNA is typically (though not in variably) maternally inherited without recombination (Lansman *et al.*, 1983; Brown, 1985; Gyllensten *et al.*, 1985), effective haploid, conservative gene order and rapid rate of evolution more than single copy nuclear DNA about 5-10 fold (Brown and George, 1979). Different parts of mtDNA mutate at different rates. For example, the control region change very rapidly, both within and between species, whereas ribosomal RNA genes change slowly (Moritz *et al.*, 1987). Thus, analysis of mtDNA became a powerful tool in population and evolutionary genetics studies (Moritz, 1994).

The first published of mtDNA polymorphisms in honeybee mtDNA (Moritz *et al.*, 1986) was the comparison of mtDNA restriction fragment length polymorphisms (RFLPs) in Australian honeybees derived from three European subspecies: *A. m. carnica*, *ligustica* and *caucasica*. The Western European group ; *A. mellifera* and the African group containing *A. m. intermissa*, *A. m. scutellata*. The mtDNA were prepared and digested with 16 restriction enzymes (6 base pair cutter) and the fragment were labeled radioactively with ^{32}P. The nineteen different *A. mellifera* mtDNA haplotypes were observed and percent sequence divergence were calculated. A cluster analysis using UPGMA

revealed three main groups of *A. mellifera* haplotype; an eastern Mediterranean, a Western European group and an African group.

In 1991, Hall and Smith employed the PCR-RFLP to detected RFLPs of three major groups (African, west European and east European) of *A. mellifera*. Three polymorphic regions (IsRNA, CO-I and Inter-CO-I/CO-II) were amplified and digested with three restriction endonuclease (*Eco*RI, *Hinc*II and *Xba*I).The PCR-RFLP analysis showed that an *Eco*RI site founded in the large ribosomal subunit gene of east European bees, a *Hinc*II site in the cytochrome C oxidase subunit I gene of west European bee and an *Xba*I site in the inter-CO-I/CO-II region of east European. This result showed that there are three polymorphic regions specific to a different subspecies groups. In 1991, Smith classified *A. cerana* species by analysed mtDNAs. The samples of *A. cerana* were collected from Southern India; the Andaman Island and India, Northern and Southern Thailand, Malaysia, Northern Borneo, Indonesia, Leuzon Island and Japan. Mitochondrial DNAs were prepared and surveyed with the 6-base restriction enzymes, The estimation of percent sequence divergence from the resulting restriction fragment data indicated three main lineages of *A. cerana* mtDNA. First include the samples from southern India, Thailand, Malaysia, Borneo and Japan; second consist of the sample from the Andaman Islands; and a third consist of the sample from Luzon. In addition, the mtDNA variation of *A. cerana* was examined by RFLP analysis. Using ten restriction endonucleases, all geographically investigated sample could be allocated to 6 different groups composed of 1) Japan 2) Nepal, Vietnam and north-to-central Thailand 3) Korea-Tsushima 4)Taiwan 5) south Thailand and 6) The Philippines (Deowanish *et al.*, 1996)

In recent years, PCR-RFLP of 3 regions were analyzed by Sihanantavong (1997). Three mtDNA regions (intergenic region of CO-I and CO-II, IrRNA and sRNA) were amplified following by digestion the PCR products with restriction enzyme *Dra*I. From these PCR-RFLP analysis *A. cerana* in Thailand was divided into three populations, the Northern (North, North/East and Central), South and Samui Island and the UPGMA phenogram of population derived from PCR-RFLP data can divide *A. cerana* in Thailand into 2 lineage. In 1997, Songram employed the PCR-RFLP analysis of ATPase 6-ATPase 8 gene of *A. cerana* in Thailand with three restriction enzyme (*Ssp*I, *Taq*I and *Vsp*I). The UPGMA phenogram of populations derived from PCR-RFLP data allocated five geographic locations of *A. cerana* in Thailand into two evolution lineage (Northern, Southern) same Sihanantavong (1997). While, based on Monte Carlo simulation, five geographic locations of *A. cerana* in Thailand could be genetically divided into three groups included of the Northern, the South and the Samui Island.

In addition, there is control (D-loop) region, a part of the animal mitochondrial genome which encompasses the site of initiation of heavy strand replication as well as both heavy strand and light strand transcription (Chang *et al.*, 1987; Clayton, 1991a,b). This region is organized very differently, without an obvious control region. In sea urchins, this region is under 200 bp length. In fish, it tends to be very long and is often full of repeated sequences. In insect, it is called the AT- rich region and can also be long and full of repeated sequences (Hillis, Moritz and Mable, 1996). The control region is the most rapid evolution (Brown, 1985) which more than another part on mtDNA approximate two to five fold (Aquadro, Kaplan and Risko, 1984).

Therefore, the control region is used as genetic marker for study of inter- and intra- specific taxonomy in animals. Norman, Moritz and Lympus (1994) used the control region as genetic marker for ecological studies of marine turtles. The control region was amplified and digested with *Mse*I and sequenced this PCR product. They found that only 2 of 12 different Indo-Pacific rookeries serveyed could not be differentiated, indicating that the Indo-Pacific *C. mydon* include a number of genetically differentiated populations.The control region also used to study levels of inter- and intra- populational variation of the harbour porpoise, *Phocoena phocoena* on interoceanic (Rosel, Dizon and Haygood, 1995).

In 1992, Crozier and Crozier showed the complete sequence of honeybee (*A. mellifera*) mtDNA. Two non-coding region were illustrated, the first region is the small region between the $tRNA^{Leu}$ and CO-II genes which is 92.2% AT. Cornuet, Garnery and Solignae (1991) argued that this region may contain an origin of replication. However, it is unclear that the gap between the $tRNA^{Leu}$ and CO-II gene represents essential functions of the region because in other bees if this region is often reduced and sometimes absent. The other region is the large region which situates between the small rRNA and $tRNA^{Glu}$ genes which is 96.0% AT and lacks any apparent signals for the initiation of replication such as those of vertebrate.

The genetic variation study of control region of *A. cerana* has been limited. The aim of this study was investigation of genetic variation among Thai honeybee, *A. cerana* from six geographic area in Thailand (North, North-East, Central, South, Samui Island and Phuket

Island) using PCR-RFLP analysis of control region . The results will provide information on the biology and geographic variation of *A. cerana* in Thailand, a basic of further selection and breeding for strain improvement.