CHAPTER 1

INTRODUCTION

Honeybees are vital to our survival. Much of the visual impact of human environments derives from vegetation. And most vegetation is dependent on honeybees for pollination. Thus, as pollinators of crops and natural vegetation, honeybees occupy key position in the web of relationships which sustain the living architecture of our planet.

On the world basis, the annual value of agricultural crops depended on the pollination services of honeybees is estimated at $1590 million (O' Tool and Raw, 1991). In California, the value of agricultural crops pollinated by honeybees has been reported to be $3.3 billion and almost $19 billion in the USA (Levin and Waller, 1989). Commercial honeybees products (e.g., natural honey, beewaxes, royal jelly and pollen) also have a high economic value of industrial beekeeping (Crane, 1990 and Witherell, 1975). In Thailand most honey goes to pharmaceutical factories for making classical Thai medicines. But some is used for making candies, syrup, cosmetics, and some is bottled for grocery stores. Thailand exported huge quantity of natural honey that provide a substantial income to the country (Table 1.1). But even today beekeeping is a major industry which generates significant revenue from the production of honey, wax, and the more costly propolis and royal jelly. As a result, natural honey has economically important values of Thai animal production and the main species for beekeeping is the western honeybees, *Apis mellifera*.

Table 1.1 The imports and exports of natural honey of Thailand in 1992-1997

Year	Import		Export	
	Quantity(Ton)	Value (Baht)	Quantity(Ton)	Value (Baht)
1992	171,664	7,301,116	2,406,569	32,392,121
1993	229,718	10,605,837	2,108,249	28,296,615
1994	264,461	12,237,749	1,894,423	26,939,353
1995	237,589	11,105,838	1,908,476	29,367,298
1996	326,240	16,292,291	2,655,865	44,006,098
1997	228,992	13,291,000	1,943,697	30,061,000

Source : Thai Customs Department, Finance Ministry, Thailand.

1.1 The honeybees in Thailand

Honeybees invaded almost all part of the world. At present in Thailand, five species of honeybees were found. Four native species are the giant or rock honeybees, *A. dorsata*, the dwarf honeybees, *A. florea*, the small dwarf honeybees, *A. andreniformis*, and the eastern honeybees, *A. cerana*. The former three species are open-nesting species that build a single, exposed comb whereas the last species nest in cavities where they construct multiple, parallel combs. The imported species is the western honeybees, *A. mellifera*, which included Italian bees, *A. m. ligustica*, and Carniolans, *A. m. carnica* (Ruttner, 1988; Tingek *et al.*, 1988). The imported *A. mellifera* for beekeeping industry has greatly expanded recently. All honeybees are similar in morphology, social biology, nest architecture, foraging behavior, and the use by foragers of a complex "dance" to signal direction and distance to food sources (Smith, 1991).

1.2 *Apis cerana*

A. cerana, is natively found in Thailand which widely distributed over the different geographic areas of the country. In Thailand, *A. cerana* has been "domesticated" for more than one hundred years (Figure 1.1). Bees were first kept in hollow wooden or coconut logs. At present, some *A. cerana* colonies have been transferred to modern hives with 5-8 frames. Beekeeping with domestic hives is mainly in the Southern part of Thailand (Wongsiri *et al.*, 1989). The apiaries of *A. cerana* mainly are in coconut plantations, more than 2000 traditional hives of *A. cerana* are found on the Samui Island.

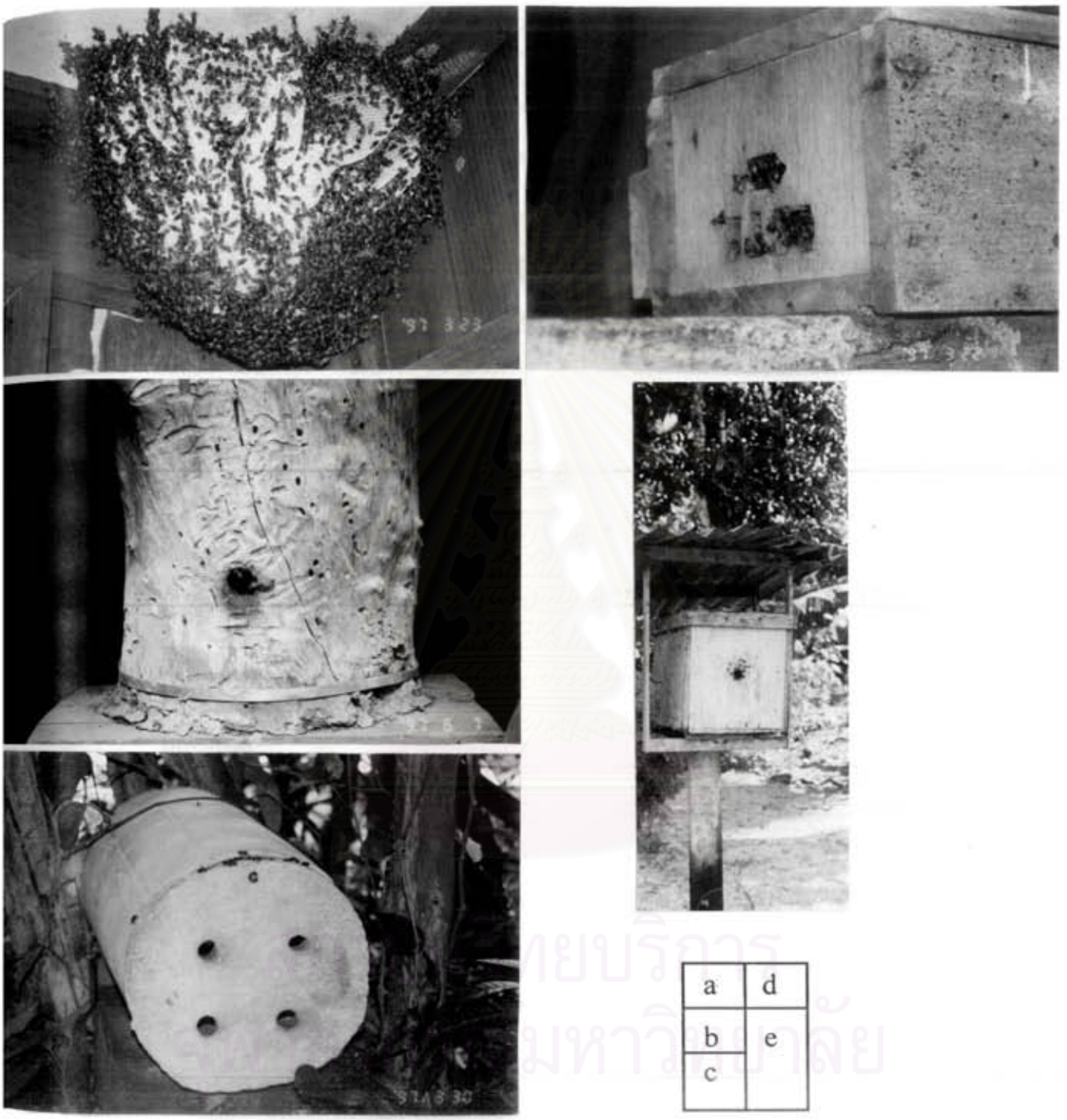

Figure 1.1 The natural colony (a) and the variant domestic hives (b-e) of *A. cerana* in Thailand

A. cerana in Thailand has not been improved in keeping, mostly they are from wild colonies introduced into modern hives. The advantages of *A. cerana* are : fewer enemies, were resistance to *Varroa* mites and don't required sugar feeding. The disadvantages are : they are very prone to swarming and absconding ; they are often more aggressive ; they lay eggs at a rate lower than *A. mellifera*. From these reasons, the genetic basis of *A. cerana* should be further studied for strain improvement.

1.2.1 The classification of *Apis cerana*

The taxonomic definition 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	cerana

Scientific name : *Apis cerana* Fabricius, 1793

1.2.2 The biology of *A. cerana*

The honeybees, *A. cerana*, one of the best studied in Asia (Smith, 1991). They are social insects and live in colonies. Each colony has one, or at most a few queens, her task being to reproduce and regulate

the social structure through secretions she produces in her body. She mates once with a male early in her life, and throughout the remaining of her existence draws on the sperm safely stored inside the spermatheca, an organ near the tip of her abdomen, which releases sperm when required as eggs pass by on their way out. Mating only once saves time and energy for the female, and through various signals she can indicate to males that she is no longer receptive. The life span of the queen is about 1 to 2 years. She also regulate the production of males and females according to the needs of the colony. If more females are required (females in most cases become workers) she lays fertilized eggs with the full complement of chromosome (diploid; 2n=32). If male drones are needed, she prevents sperm reaching the egg and lays unfertilized eggs with a single set of chromosomes (haploid; n=16). Males are reared only at the time of year when their presence for mating is required.

The vast majority of bees in the colony are workers. During early life, the workers feed the developing larvae using honey and pollen kept in the storage cells of the comb. Some days later these workers graduate to their next task, producing royal jelly for the young larvae, prospective queens, and the queen herself. During this stage the worker consume large quantities of pollen and their pharyngeal glands are fully active to produce royal jelly.

In another phase of their lives the workers are absorbed in storing pollen brought to the hive by foraging bees, ventilating the hive by rapid fanning of the wings, producing wax for making combs, removing dead bees and debris. In their final stage of life, workers serve foragers, flying far and wide to search for and bring nectar, pollen and water back to the colony. They live for only ten to twelve weeks.

Flight range

Mating distance of *A. cerana* in Sri Lanka seems not to exceed 500 m (Punchihewa *et al.*, 1990). The flight range of *A. mellifera* drones is reported to vary between 1 and 5 km, and that of queens between 1 and 2 km (Woyke, 1960; Taylor and Rowell, 1988). In isolated areas the mating distance can range up to 16 km (Peer, 1957). There are no data for the *A. cerana* in Thailand.

1.3 The morphology of honeybees

Honeybees posses a 'wasp waist', which is a constriction between the first and second segments of the adult abdomen (Figure 1.2). The first abdominal segment is incorporated into the back of the thorax and is called the propodeum. This means that the apparent first segment of the abdomen is really the second. The 'wasp waist' allows great flexibility of abdominal movement and, without it, the female bee would be unable to bend her abdomen sufficiently to lay an egg at the bottom of a narrow cell.

Honeybees use the sting (Figure 1.3) to defend herself and her nest. The sting is a modified egg-laying tube or ovipositor.

Hymenoptera also possess both sucking and biting mouthparts. This was a prerequisite for the evolution of 'beeness' because bees suck liquid nectar and use their jaws in nest construction (Figure 1.4).

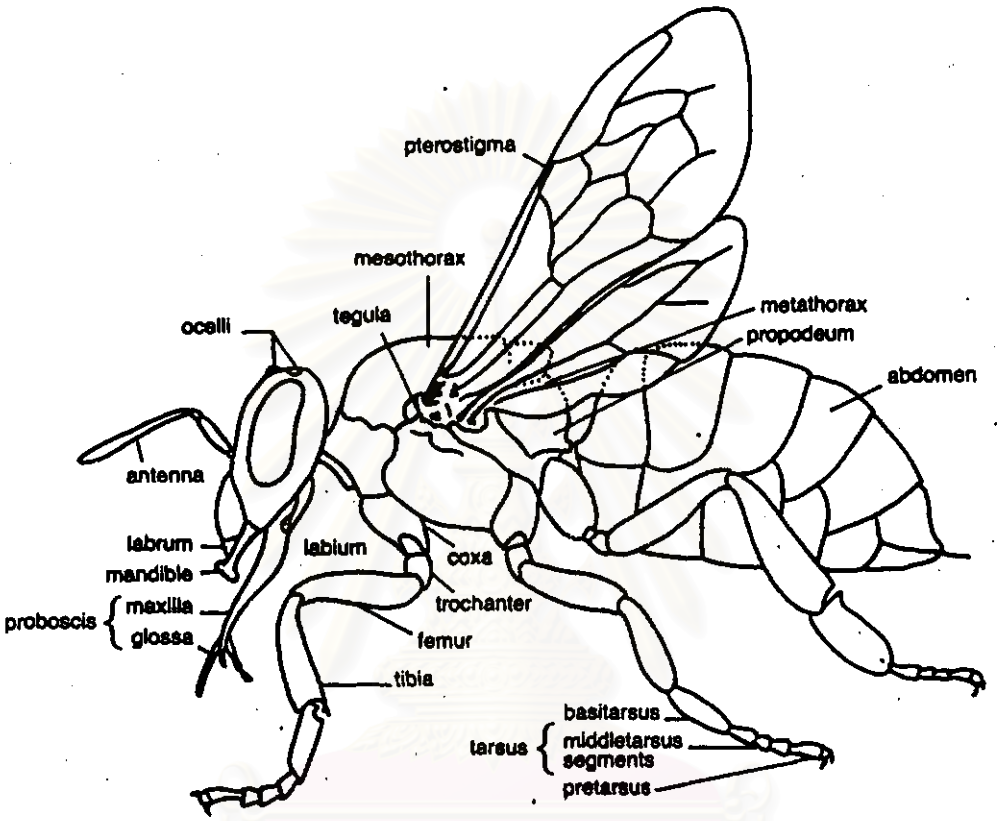

Figure 1.2 Side view of a generalized bee's body to show the main structures (O' Tool and Raw, 1991).

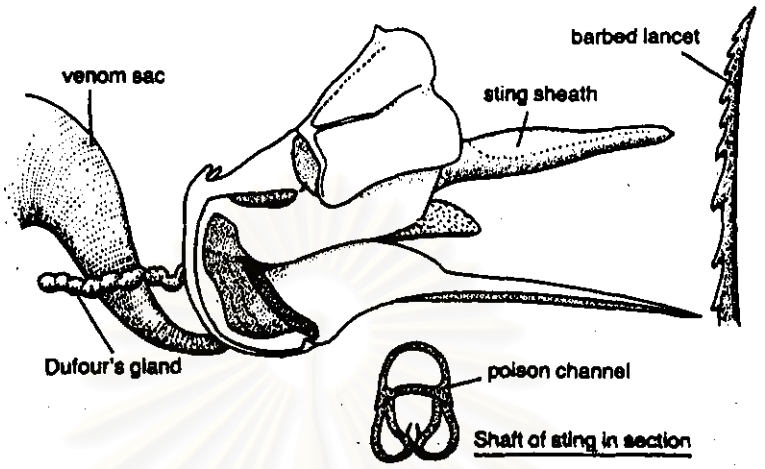

Figure 1.3 Side view of the sting apparatus of a worker honeybee (O' Tool and Raw, 1991).

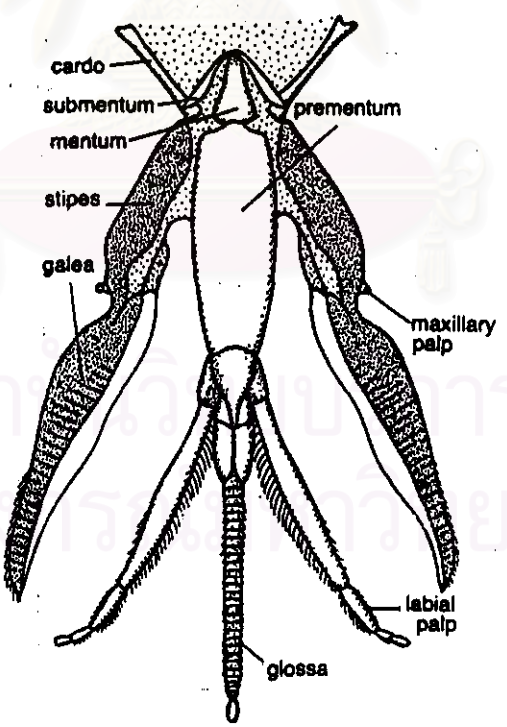

Figure 1.4 The tongue of a worker honeybee, *A. mellifera* (Apidae), to show its constituent parts (O' Tool and Raw, 1991).

1.4 The previous studied of *A. cerana*

A. cerana F. covers a very large area of different climatic conditions in Asia. It is clear differences from *A. mellifera* by statistical analysis of their morphometric data (Peng *et al.*, 1989).

Ruttner (1988) summarized morphometric information and grouped the *A. cerana* populations into four subspecies, whose range are shown in Figure 1.5 : 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 Lan Ka, Bangladesh, Burma, Malaysia, Thailand, Indonesia and the philippines ; a Japanese subspecies, *A. c. japonica*, from Japan and Korea ; and a Himalayan subspecies, *A. c. himalaya*, from Nepal, Thailand (including the mountains in Thailand ; Chiang-Mai) and probably South-Western China

For mtDNA analysis, Smith (1991) estimated of percent sequence divergence from the result of restriction fragment data indicated three main lineages of *A. cerana* mtDNA : one including the samples from southern India, Thailand, Malaysia, Borneo and Japan ; second consisting of the sample from the Andaman Island ; and a third consisting of the samples from Luzon (Figure 1.6).

In Thailand, *A. cerana* was divided into three groups ; Northern latitude bees, from Chiang Rai - Phetchaburi ; Southern latitude bees, from Chumphon - Song Khla ; and Samui Island bees, which base on the multivariate statistical analysis (Canonical Analysis) of morphometric characteristics, such as, proboscis, fore and hind wing, hind leg, third and

Figure 1.5 Dotted lines indicate the approximate ranges of *Apis cerana* subspecies as recognized by Ruttner (1988). A = *A. cerana cerana*; B = *A. c. indica*; C = *A. c. japonica*; D = *A. c. himalaya*. Large dots show approximate location of collection sites.

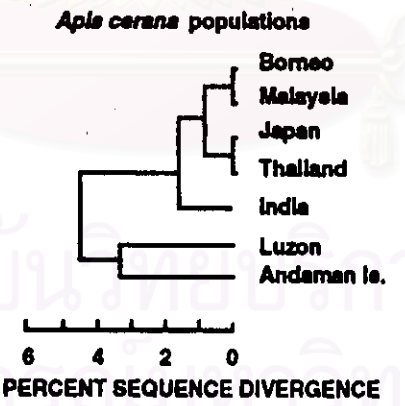

Figure 1.6 Distance phenogram calculated from percent sequence divergence estimates among *A. cerana* mitochondrial haplotypes using UPGMA (Smith, 1991).

sixth sternites, and second, third and fourth tergites etc (Limbipichai, 1990). However using Clustering analysis of above morphometric characteristic, *A. cerana* populations were divided into two groups; Northern latitude bees and Southern latitude bees, Samui Island bees is grouped with Southern latitude bees.

In recent years for nuclear DNA analysis, Uthaisang (1994) constructed DNA probe no. 99 which containing repetitive sequences of *A. cerana*, and used as hybridization probe. Southern blot analysis of *Hae*III digested chromosomal DNA of *A. cerana* male (haploid) collected from five different areas of Thailand showed six different RFLPs patterns.

The above studying of *A. cerana* in Thailand has limited. At present, there has few information about population genetic of *A. cerana*. Population genetic is essential for both of conservation program and improving a selective breeding program in order to show well domesticated population represent the natural variation of the species. Population genetic analysis provided genetic marker to which may be utilized for constructing sophisticated breeding plains. Using molecular data, scientists can developed breeding programs aimed at specific needs. The study of population genetic is necessary to using molecular marker.

1.5 Molecular markers for population genetic analysis

Traditional methods such as comparative anatomy, morphology and physiology have been evaluated genetic variability of various taxa and these methods are not adequate for study genetic variation. During the past decade, traditional methods have been increasing complemented

by molecular techniques, the development of so- called "molecular markers" which are based on polymorphisms found in proteins or DNA.

Protein markers

For the generation of molecular markers based on protein polymorphisms, this method involves separation of native proteins by net charge under influence of an electricurrent, followed by application of histochemical stains to reveal the enzyme or other protein products of particular, specifiable gene. The majority of protein markers are represent by allozymes.

Allozyme

Allozyme electrophoresis remains the predominant tool used for studying genetic variation, although its preeminence is being increasingly challenged by direct DNA analysis. The advantage of allozyme electrophoresis primarily relate to its speed and relatively low cost : data on hundreds of individuals at several loci can be amassed within a short period of time. Equipment demands are modest and personnel can be trained quickly although interpreting gel patterns sometimes requires considerable experience. Disadvantages are the strict requirement of fresh or frozen tissue, the need for more material than DNA methods, protein loci evolve more slowly than non-coding DNA sequences.

The technique of allozyme analysis has been used to study the variation within and among populations of honeybees for almost two decades (Daly, 1991). Sylvester (1982), Nunamaker *et al.* (1984), Page and Erickson (1985), Sheppard and Mcpherin (1986), reported on distinct

subspecies of Africanized bees and European bees by using partial difference in allozymes. Allozyme variation have been studied in Asian honeybees by Nunamaker *et al.* (1984). One intense band of malate dehydrogenase (MDH) was found in *A. florea* but two bands, one intense and one faint were found in both *A. dorsata* and *A. cerana*. In contrast, for non-specific esterases (ETS), *A. florea* and *A. dorsata* shared two bands, but with different position of the faint band whereas *A. cerana* had two faint bands which were not shared with either of the other two species. However, intraspecific polymorphism cannot detected by allozyme.

A brief report on EST enzymes by Tanabe and Tamaki (1985) and Sheppard and Berlocher (1989) also indicated that *A. mellifera* and *A. cerana* had species specific differences. Moreover, a unique banding pattern of EST for each of four species; *A. florea*, *A. dorsata*, *A. andreniformis* and *A. laboriosa* was reported (Li *et al.*,1986).

In the social Hymenoptera, protein variation is limited (Graur, 1985) because of their sex-determination system (haploid males). Therefore, allozyme analysis cannot make certain identifications, especially of hybrids after several generations (Rinderer and Sylvester, 1981).

DNA marker

Nuclear and mitochondrial DNA

The use of allozymes to resolve systematic and population level questions has been supplemented with techniques generating direct or indirect estimates of nucleic acid variation. The nuclear genome is

extremely large and complex that can make interpretation of data difficult. However, nuclear DNA represents a wealth of genetic information. Today, many researchers are attempting to look at sequence variation in the nuclear genome using various strategies : examining introns, looking at repetitive sequences, etc. It is a much more arduous process, but the potential for detecting variation is much greater, and if genetic differences exist, mtDNA studies are more likely to detect them.

The reasons for the increasingly widespread use of mtDNA in studies of animal populations are many ; the matrilineal inheritance, general conservation of gene order and composition, rapid rate of sequence divergence, small size as compared to the nuclear genome, and relative easy of isolation.

In the last nine years the first publications on variation at the DNA level in honeybees appeared. The general technique is restriction fragment length polymorphisms (RFLPs). It has been applied to nuclear DNA (Hall 1986, 1988; Severson *et al.*, 1988) as well as to mtDNA (Moritz *et al.*, 1986; Smith, 1988, 1991; Smith and Brown, 1988, 1990; Smith *et al.*, 1989, 1991). The initially reported by Hall (1986), could distinguished between the European bees (*A. m. ligustica* and *A. m. carnica*) and the Africanized bees by using repetitive nuclear DNA of *A. mellifera* as a probe. Additionally, the genetic variation of Asian honeybees have been studied by using nuclear DNA markers from European honeybee, *A. mellifera.* The result showed that the DNA of these species, *A. andreniformis*, *A. florea*, *A. cerana* and *A. dorsata*, could hybridize to *A. mellifera* probes and there was variation among the species (Sylvester and Wongsiri, 1993).

Mitochondrial DNA has recently been used as a marker to distinguish between male and female influence in the Africanization process in the America (Smith, 1991). Besides its potential discriminating power, mtDNA may provide valuable information on the phylogenetic links between subspecies or populations. So far the restriction maps of a few subspecies have been published (Smith, 1991). Furthermore, the complete sequence of *A. mellifera* mtDNA have been reported (Crozier and Crozier 1993).

Because different regions of the mitochondrial genome evolve at different rates, certain regions of the mtDNA have been targeted for certain types of studies. The cytochrome *b* and NADH dehydrogenase (ND) genes have been examined in a number of species (Carr and Marshall, 1991; Brown *et al.*,1993; Cronin *et al.*,1993; Park *et al.*,1993) as they are reported to exhibit variability on the population level. The D-loop has also been targeted for population studies because it is highly variable in mammals, but this is not necessarily the case with fish (Nielsen *et al.*,1994; Park *et al.*,1993). The mitochondrial ribosomal genes evolve more slowly and have been used for species or even family-level studies (Geller *et al.*,1993; Milinkovitch *et al.*,1993).

A portions of the cytochrome subunit II (COII) gene in *A. mellifera*, *A. cerana*, *A. dorsata* and *A. florea* was sequenced by Cameron and Sheppard and McPheron (1991). Sequence divergence among the *Apis* species ranged from approximately 7% to 11% and *A. mellifera* and *A. cerana* were found to be more closely related to one another than to the other two species (Garnery *et al.*,1991). Additionally, mtDNA restriction site and length polymorphisms identify three lineages of honeybees *A. mellifera* subspecies: an east European group including *A.*

m. ligustica, *carnica*, and *caucasis*, a west European group consisting of *A. m. mellifera* and *iberica* with mellifera-like mtDNA (predominantly from northern Spain), and an African group including South African *A. m. scutellata* and *A. m. capensis* and north Afican *intermissa* and *iberica* with intermissa-like mtDNA (predominanty from southern Spain) (Smith *et al.*, 1989; Smith and Brown,1988; Smith *et al.*,1991). And *Eco*RI site in the large ribosomal (lrRNA) subunit gene and an *Xba*I site in the cytochrome *c* oxidase I (COI) subunit gene are found only in bees of the east European group, and a *Hic*II site in the COI subunit gene is found only in bees of the west European group. Length polymorphisms between the COI and COII subunit genes are found in the mtDNA of west European and African bees. The *Eco*RI polymorphism in the lrRNA subunit gene was among several others used in the studies of neotropical bees (Smith *et al.*, 1989).

From the above list it is apparent that the mtDNA has proved to be an excellent cocoon for the metamorphosis of DNA. Furthermore, the mtDNA RFLP is a commonly used alternative analysis for determination of intra specific population structure. However, only RFLP of the whole mitochondrial genome are not suited for rapid testing of large numbers of samples, but new technical advances greatly facilitate such analyses. In realizing part of that goal, the polymerase chain reaction (PCR) have been employed for more rapid identification of subspecies-specific mtDNA.

Therefore this study was aimed to explore the possibility of using ATPase6-ATPase8 gene of *A. cerana* mtDNA to evaluate genetic variability in *A. cerana* population from five different geographic population in Thailand; North, North-East, Central, South, and the Samui

Island, by PCR-RFLP analysis. The results will provide information on the biology and geographic variation of *A. cerana* in Thailand and provide a basis of future selection and breeding for strain improvement.