CHAPTER I
LITTERATURE REVIEWS

1.1 STINGLESS BEES BIOLOGY

1.1.1 Division of labor

The stingless bees are eusocial insects. Thus, they involve division of labor, which are similar to that found in honey bees (Kerr and Santos Neto, 1953; Bassindale, 1955). In stingless bees, the females can be divided into queens and workers. Very young bees can produce wax and work in the brood nest and they move further away from nurse bee as they age until they leave the nest to become foragers. According to Wille (1983), stingless bees were generally divided into four stages: callow, nurse bee, housekeeper and forager. The duration of each stage depends on the species and on the condition of the colony. The tasks involved with these stages are as follows: (1) incubation and repairs of the brood chamber; (2) construction and provisioning of cells, cleaning of the nest, and feeding young adults and the queen; (3) further cleaning of the nest, reconstruction of the involucrum, reception and ripening of nectar, and guard duty at the entrance of the nest; (4) foraging for pollen, nectar, propolis and other materials.

1.1.2 Nest architecture of stingless bees

Stingless bees are able to construct their nest with cerumen, a mixture of wax with resins and propolis collected from plant exudates and brought to the nest (Sakagami, 1982; Wille, 1983), but nests of honey bees are made of pure wax. Nesting site of stingless bees is diverse such as those established within nests of termites and ants (Sakagami *et al.*, 1989), those constructed in underground cavities, and nests in preexisting holes in trees (Darchen 1992; Roubik, 1979; Roubik, 1983; Sakagami *et al.*, 1983; Salmah *et al.*, 1990) (Figure 1.1). A stingless bee, they keep pollen and honey in large egg-shaped pots made of beeswax, but honey bees in hexagonal cells of pure beeswax. In stingless bee nest wax typically mixed with various types of plant exudates, and the central brood is also sealed within a layer of cerumen, except for the entrance cavity (Sakagami, 1982; Sakagami *et al.*, 1983; Sakagami and Yamane, 1984). These closed brood cells are often arranged surround

a central set of horizontal brood combs, where the larval bees live. The larvae are not fed directly, but the pollen and nectar are placed in a cell, an egg is laid, and then the cell is enclosed until the adult bee emerges after pupation (Sakagami, 1982). Stingless bees reveal high variability in the mechanisms of caste determination and queen rearing method (Engels and Imperatriz-Fonseca, 1990; Figure 1.2). In the genus *Trigona*, the rearing method of queens and workers is similar to that of honey bees. Caste is determined by nutrition and queens are reared from special large cells (Darchen and Delage-Darchen, 1971; Camargo, 1972; Hartfelder and Engels, 1989; Engels and Imperatriz-Fonseca, 1990; Figure 1.2). By contrast, queens and workers of stingless bees are reared in similarly provisioned cells with the same size, and caste is determined genetically in *Melipona* (Kerr, 1950; 1969; Velthuis and Sommeijer, 1991; Ratnieks, 2001).

1.1.3 Foundation of new colonies and mating

New colonies are founded by swarming which are different from that of the honey bee (Spinola, 1840; Nogueira-Neto, 1954; Kerr, 1951; Kerr *et al.*, 1962; Juliani, 1972). In stingless bees, new colony forming is a progress process. Nest material and food are first transferred from the old nest. When the new queens emerge, they together with swarms of workers leave to new nest sites, and males wait there in anticipation (Nogueira-Neto, 1997; Velthuis *et al.*, 2005). Afterward, the new queens fly out for a single mating, followed by hundreds of males (Peters *et al.*, 1999; Palmer *et al.*, 2002), and most are killed by predators (Michener, 1961; Paxton *et al.*, 2003) on such mating attempts, while brood cell construction and oviposition are started in the new nest (Moure *et al.* 1958, Sakagami 1982, Inoue *et al.* 1984). Beside these, workers can oviposit male producing eggs (Kerr, 1950; Machado *et al.*, 1984; Imperatriz-Fonseca and Kleinert, 1998; Sommeijer and van Buren, 1992; Sommeijer *et al.*, 1999; Koedam *et al.*, 2001; Tóth *et al.*, 2002; Chinh *et al.*, 2003; Paxton *et al.*, 2003; Sommeijer *et al.*, 2003). It was confirmed with recent publications revealing that male production by workers where commonly found in stingless bees (Tóth *et al.*, 2004). Several weeks or even months can take for lasting relationship between the mother and daughter nests (Wille, 1983), because the dispersal radius of stingless bees is

more restricted than in honey bees (to a few hundred meters), (Nogueira-Neto, 1954; Sakagami, 1982).

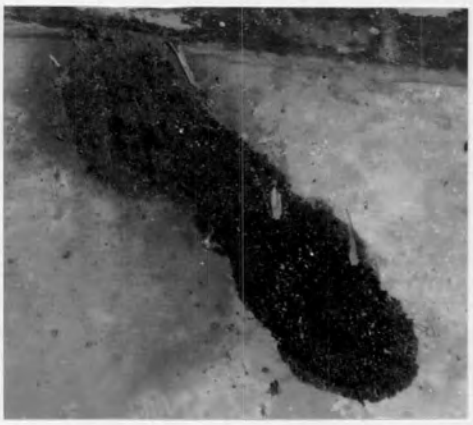
1.2 DISTRIBUTION OF STINGLESS BEES

The stingless bees belonging to the tribe Meliponini, are known as the stingless honeybees, in the family *Apidae* (Winston, 1977) which also include the common honeybees, carpenter bees, orchid bees and bumblebees. The stingless bees apparently originated in and dispersed from Africa, and migrated to tropical or subtropical regions worldwide, such as Southeast Asia, Australia , as well as into parts of Mexico and Brazil (Michener, 1974; Sakagami, 1982; Michener, 1990; 2000) (Figure 1.3), where their status are considered as the major pollinators (Michener, 1974). Due to spreading over a whole continent with highly varied environments, the stingless bees can be evolved various (Wille, 1979). Stingless bees are the most diverse in morphology and behavior of the eusocial corbiculate bees (Michener, 2000). Unlike other bees, stingless bees live in perennial colonies with queens, workers and males (drones) (Michener, 2000). Their colony sizes are quite diverse containing stingless bees ranged from a few dozen to 100,000 or more individuals, depending on species (Michener, 2000). Additionally, all stingless bees are able to build complex nests with structures that are usually specific to each species (Michener, 1974; Sakagami, 1982) (Figure 1.4).

Worldwide approximately 400-500 species are reported and allocated into 23 genera and 18 subgenera (Velthuis, 1997; Costa *et al.*, 2005). Of these, the most important genera are *Melipona* and *Trigona*. *Melipona*, including approximately 50 species restricted to the neotropic regions, has more complex communication systems (Nieh and Roubik, 1995). It is able to buzz pollination (ejecting pollen grains by vibration of the pollen-bearing anthers of flowers that dehisce pollen through pores) (Buchmann, 1995). *Trigona* is the largest and most widely distributed genus classified through the world more than 120 species into ten subgenera from the Indo-Malayan/Australasian and Neotropical Regions (Michener, 2000).

(A)

(B)



(C)

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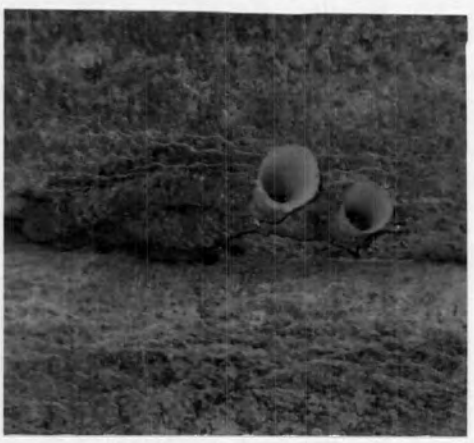

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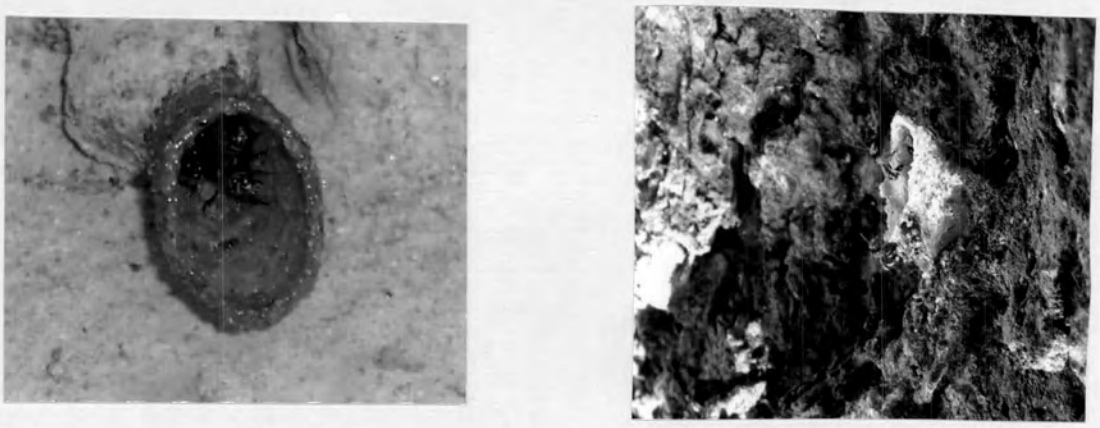

(G) (H)

Figure 1.1 Pictures of nest entrances of stingless bees; *T. pagdeni* (A-C), *T. minor* (D-E) and *T. terminata* (F-H).

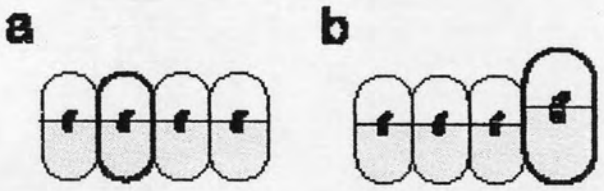

Figure 1.2 Queen rearing method in stingless bees; (a) In the genus *Melipona*, queens and workers are the same size and develop in identical, similarly provisioned cells, (b) In the genus *Trigona*, caste is determined by nutrition and queens are reared from larger royal cells. Bold lines = queen cells; fine lines = worker cells (Ribeiro *et al.*, 2006).

1.3 EXPLOITATION AS NATIVE SPECIES

Stingless bees are general pollinators which may visit flowers of more plant species than solitary bees, honey bees and wasps (Heithaus, 1979). They are friendly to human and domesticated animals. In addition, they are capable of foraging effectively in glasshouses (Kakutani *et al.*, 1993; Amano, 2000), and they are resistant to the diseases and parasites of honey bees (Delfinado-Baker *et al.*, 1989). Like honey bees, stingless bees are able to pollinate well in different plant species and show some characteristics that influence their ability as pollinators are. For example, (a) they are able to adapt and learn rapidly in exploitation of the natural resources (Guibu *et al.*, 1988; Kleinert-Giovannini *et al.*, 1987), (b) a worker usually visits only one plant species on a single trip (Ramalho *et al.*, 1994), (c) colonies can be cultivated in boxes that are convenient to propagate , feed and control for transportation and management (Nogueira-Neto, 1997), (d) workers can forage continuously within climatic constraints due to they live in perennial colonies, (e) workers collected food beyond immediate needs and stored in nests allowing colonies to survive long periods (Roubik *et al.*, 1986), (f) decreasing pollen transfer between plants of self-incompatible species in hives, (g) workers can help nest mates by providing information on the position of those floral resources. Recently, it becomes popular to cultivate in hive boxes for using in plant pollination and for commercial production (Slaa *et al.*, 2006). They show a potential in foraging, such as nectar and pollens including accumulation of propolis from plant exudates for construction, protection and adaptation of their nests (Velthuis, 1997; Velikova *et al.*, 2000). Subsequently, the study of Meliponinae propolis provides information on the chemical composition of these stingless bee propolis, as well as the plants which they use as a resource for collecting propolis to support the medicinal properties of stingless bees propolis (Bankova and Popova, 2007).

Therefore, the ability to transfer colonies into boxes increases the potential in crop pollination (Figure 1.5). The artificial hives can be propagated (Heard, 1988; Nogueira-Neto, 1997; Roubik, 1995) and transported conveniently where needed for pollination. This development may provide high improvement in management of domesticated colonies based on their biology. Hive boxes may be opened for extraction of honey. This may give

the potential in increasing honey production, feeding and treatment from enemies. The traditional beekeeping of many stingless bees has been reported by Crane (1992). This could lead to improve this native stingless bees providing an additional source of income for farmers in rural communities.

1.4 STINGLESS BEES IN THAILAND

A total of 32 stingless bees grouped into a genus *Trigona* have been reported in Thailand (Klakasikorn *et al.*, 2005; Michener and Boongird, 2004) (Table 1.1). Of these, *Trigona* (*Tetragonula*) *pagdeni* Schwarz, an indigenous stingless bees belonging to tribe Meliponini, is distributed covering vast geographic locations in Thailand, peninsular Malaysia and the Indochina region (Sakagami, 1978; Sakagami and Khoo, 1987). *T. pagdeni* is more frequently found nesting in various artificial structures in close contact with human (Franck *et. al.*, 2004). In recently years, it is popular to cultivate in boxes for its use in plant pollination (Figure 1.5), with a potential in foraging, such as nectar used to produce honey and pollens including accumulation of propolis and resins from plant exudates for construction, protection and adaptation of their nests.

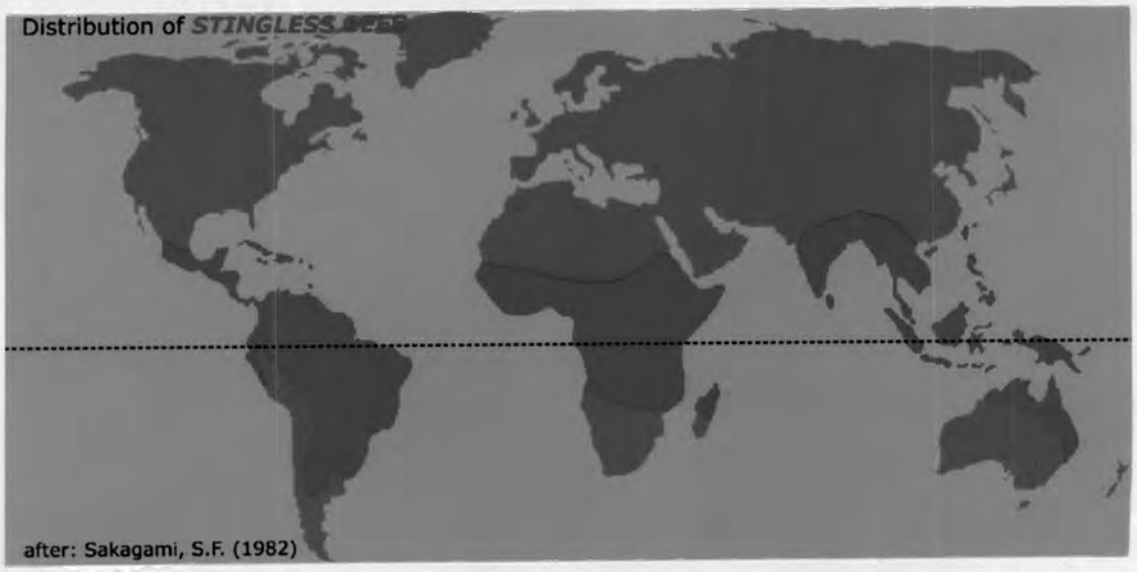

Figure 1.3 Distribution of stingless bees (marked in red) (http://www.b-lab.at/Bilderchens/Distribution-of-stingless-bees.jpg)

(A) (B)

Figure 1.4 Nest of stingless bees; *T. pagdeni* nesting (A), *Lisotrigona furva* (B).

Figure 1.5 The artificial hives of *Trigona pagdeni* in the orchard of Thailand.

1.5 *Trigona* (*Tetragonula*) *pagdeni* Schwarz IN THAILAND

The taxonomy of *Trigona* (*Tetragonula*) *pagdeni* Schwarz was identified according to Michener (2000).

Kingdom: Animalia

Phylum: Arthropoda

Class: Insecta

Order: Hymenoptera

Superfamily: Apodea

Family: Apidea

Subfamily: Apinae

Tribe: Meliponini

Genus: Trigona

Subgenus: Tretragonula

Scientific name: *Trigona pagdeni* (Schwarz, 1939)

Common name: Stingless bee

Morphological structure of stingless bees were shown in Figure 1.6 and 1.7. *Trigona* (*Tetragonula*) *pagdeni* Schwarz is one of the commonest stingless bees in Thailand, and has been recorded in peninsular Malaysia and the Indochina region (Sakagami, 1978; Sakagami and Khoo, 1987). Color on some body parts of *T. pagdeni* reveals variation in various areas of Thailand (Sakagami, 1978). According to Schawarz, anterior corbicular fringe is mainly silvery gray. Its coloration was just as pale as average specimens from Thailand, and fronts were provided with whitish plumose hairs. *T. pagdeni* increases in size toward northern Thailand. The samples from north of Thailand are fairly large workers. However, *T. pagdeni* from Malaysia (Sakagami and Khoo, 1987), the color body is generally darker than described above. The clypeus is black except for the area near the distal margin which tends to be testaceous. The tegulae, anterior vein, anterior corbicular bristles, mesoscutal and mesososcutellar bristles and the legs (except for trochanter and distal tarsal segments) are black to blackish brown. The metasoma is generally darker.

The Indomalayan stingless bees have been reported that many of the species of the *Trigona* subgenus *Tetragonula* were not easily distinguished from each other (Sakagami, 1978). The subgenus *Tetragonula* of the *Trigona* includes *Trigona melina*, *Trigona latigenalis*, *Trigona fuscobalteata*, *Trigona gressitti*, *Trigona hirashimai*, *Trigona minor*, *Trigona pagniformis*, *Trigona iridipennis*, *Trigona pagdeni*, *Trigona geissleri*, *Trigona zucchini*, *Trigona laeviceps*, *Trigona sarawakensis* and *Trigona sirindronae*. Of these, *Trigona sirindronae*, *Trigona melina*, *Trigona fuscobalteata*, *Trigona pagdeni*, *Trigona laeviceps*, *Trigona minor*, *Trigona sarawakensis*, *Trigona pagniformis*, *Trigona hirashimai*, *Trigona geissleri*, *Trigona iridipennis* and *Trigona latigenalis* have been reported in Thailand (Klakasikorn *et al.*, 2005; Michener and Boongird, 2004).

Table 1.1 *Trigona* species found in Thailand (Klakasikorn *et al.*, 2005)

Trigona species	Michener and Boongird (2004)	This study (2003)	Rajitparinya et al. (2000)	Sakagami et al. (1985)	Schwarz, (1939)
T. sirindhornae Michener and Boongird, 2004	*				
T. collina Smith, 1857		*	*	*	*
T. terminata Smith, 1878		*	*	*	*
T. apicalis Smith, 1857		*	*	*	*
T. doipaensis Schwarz, 1939		*			*
T. laeviceps Smith, 1857		*	*	*	
T. minor Sakagami, 1978		*			
T. thoracica Smith, 1857		*		*	*
T. binghami Schwarz, 1939		*			
T. fimbriata Smith, 1857		*	*	*	
T. fuscobalteata Cameron, 1908		*		*	*
T. itama Cockerell, 1918				*	*
T. melanoleuca Cockerell, 1929			*	*	*
T. peninsularis Cockerell, 1927				*	*
T. canifrons Smith, 1857				*	*
T. aliceae Cockerell, 1929					*
T. ferrea Cockerell, 1929					*
T. pagdeni Schwarz, 1939				*	*
T. geissleri Cockerell, 1918				*	*
T. iridipennis Smith, 1854					*
T. valdezi Cockerell, 1918					*
T. melina Gribodo, 1893				*	*
T. sarawakensis Schwarz, 1937					*
T. flavibasis Cockerell, 1929					*
T. ventalis Smith, 1857			*	*	*
T. scintillans Cockerell, 1920			*	*	*
T. nitidiventris Smith, 1857				*	
T. atripes Smith, 1857				*	
T. fuscibasis Cockerell, 1920				*	
T. hirashimai Schwarz, 1939				*	
T. pagdeniformis Sakagami, 1978				*	
T. latigenalis Cockerell, 1969				*	
Total	**1**	**10**	**8**	**22**	**20**

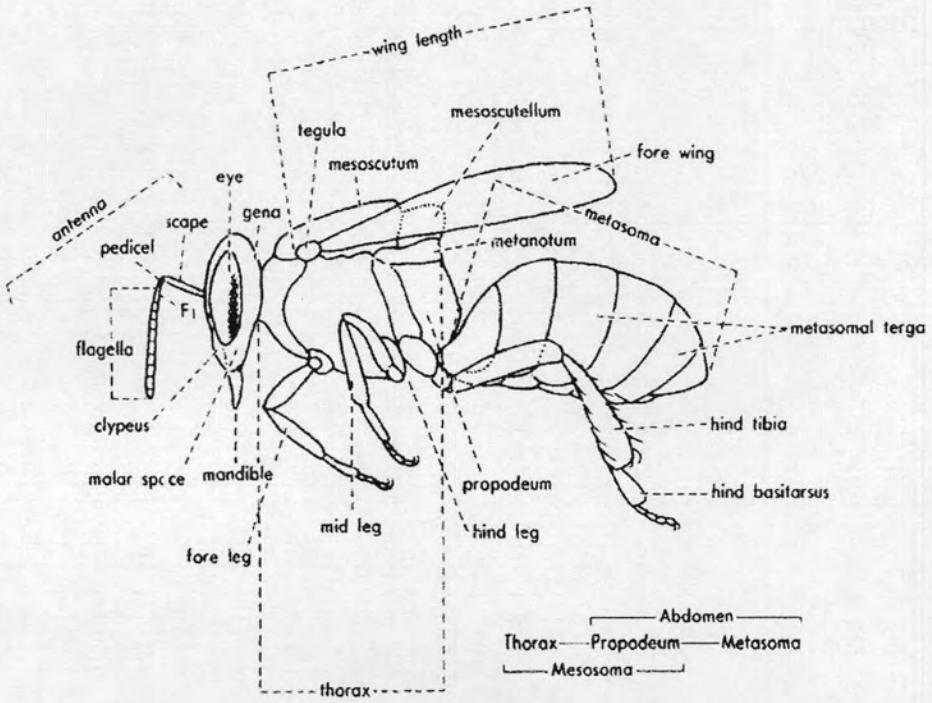

Figure 1.6 Morphological structures of stingless bees

(A) **(B)**

Figure 1.7 Morphological structures of *Trigona pagdeni* (A) and *Trigona laeviceps* (B).

1.5.1 Identification of *T. pagdeni*

Nowadays, the stingless bees are increased exploitation especially in agriculture. Thus, identification of morphological and nest architecture characters of stingless bees have taken place over the years (Michener, 1961; Sakagami, 1978; Dollin *et al.*, 1997). The identification of stingless bees, especially in subgenus *Tetragonula* is complicated (Sakagami, 1978). Taxonomic identification, however, remains unclear and requires experienced scientists.

Since wrong classification can be lead to extensive losses, a reliable and reproducible method of classification is therefore imperative. Proper mean of classification is therefore required to identify species. In order to certify the authenticity of a species, taxonomical studies have been conducted. Nest architecture characters are usually relevant but are reported that they are not sufficient criteria for authentication of species origin of Australian stingless bees (e.g. *Trigona hockingsi* and *Trigona davenporti*; Franck *et al.*, 2004). Despite the problems caused by apparent characters, morphology is still the most commonly used method of classification. Although, these studies could classify the species based on their morphology, they were unstable and not reproducible due to the influence of various experimental factors such as a variety of habitats and environmental conditions. Thus, species-diagnostic markers are required to unambiguously identify the correct species of specimens used for population genetic studies of *T. pagdeni*.

To date, no method of classification of *T. pagdeni* has thus proved to be reliable or reproducible. The development of molecular biology techniques, such as DNA-base markers, has given a new opportunity for genetic characterization, allowing the direct comparison of different genetic material without environmental influences. Various molecular marker techniques, such as DNA fingerprinting are available to detect diversity at the DNA level. One of these techniques, AFLP, has been proven to be valuable to genotype characterization in many crop species (Vos *et al.*, 1995). Likewise, AFLP is a popular technique for identification of DNA markers in many several marine species (Griffiths *et al.*, 2000). AFLP can generate high-resolution markers that exhibit such a purpose, where no data in stingless bees are reported at present.

1.5.2 Genetic diversity of *T. pagdeni* in Thailand

Genetic diversity is a level of the variation of the nucleotides, genes, chromosomes within the cells or organelles of any organism. Genetic diversity enables them to survive and adapt to changing in their environment including new pests, diseases and new climatic conditions. The variation is introduced through harmless mutation of gene or the result of sexual reproduction, especially if the parents come from different population and gene pools, chromosomes of the offspring are then copied and genes can be exchanged by sexual recombination. These may provide the evolution of new characteristics within a single species for survival and adaptation in their environment. Genetic diversity can provide the potential use to agricultural developments, such as to raise commercially breeding new varieties of plant species (Yong *et al.*, 2006). Furthermore, genetic diversity has been studied in several social insects. Genetic diversity can prevent severe infections and promote colony growth in social insects, because of the evolution of polyandry (many females mate with more than one male) in social insects (Arnqvist and Nilsson, 2000). The queen that mates multiply can produce genetically diverse workers that carry different genes for resistance to a particular disease (Tarpy, 2002). Genetic diversity can be determined by many different ways. Traditionally, protein marker had been used to survey genetic diversity within several organisms such as in plants (Ahmed, 2008). A DNA marker was also be used as a marker of genetic diversity within and among individuals of any organisms such as in plants (Fikiru *et al.*, 2007, Terrab *et al.*, 2007), in animals (Klinbunga *et al.*, 2007) and in insects, especially *Thai Apis cerana* (Subrananian and Mohankumar, 2006; Sittipraneed *et al.*, 2001). In Thailand, *Trigona (Tetragonula) pagdeni* Schwarz is one of the most common and widely distributed of the stingless bees (Sakagami, 1978), where they are an important pollinator (Del Sarto *et al.*, 2005). The loss of native pollinators may influence population loss and decline of commercial plants. Likewise, farmers may loss a lot of money to introduce other honey bees for the commercial plant pollination. Research on the genetic variation within and among populations of *T. pagdeni* has not been reported to date. Information about the intraspecific genetic variation of this native species is fundamental to

designing appropriate management strategies for genetic improvement and efficient conservation programs.

1.6 GENETIC MARKERS

A genetic marker is a specific segment on a genomic DNA used as chromosomal or mitochondrial landmarks for genome analysis (Kumar, 1999). According to Kumar (1999), two types of genetic markers; morphological and molecular markers are recongnized. The morphological markers are based on visually assessable traits without specialized molecular techniques. Although, the morphological marker is cheap and fast, it is influenced by several environmental conditions including the relatively low levels of polymorphisms (Paul *et al.*, 1997), whereas molecular markers can reveal greater polymorphisms at the protein level (protein markers) or at the DNA level (DNA markers) without environmental factors.

In the past, protein markers have been well known as molecular markers. For example, glycerol-3-phosphate dehydrogenase variation has been reported in Brazilian stingless bees by Machado and Contel (1991). However, the use of protein as molecular markers can be complicated by dimeric or multimeric enzymes. Likewise, protein markers revealed lower levels of polymorphisms than DNA markers. Nowaday, DNA makers are normally used as either chromosomal DNA or mitochondrial DNA landmarks, which are not influenced by environment and expressed in all tissue of each organism.

Chromosomal DNA is employed as molecular markers for population genetic studies and phylogenetic relationship studies in several organisms. Fernandes-Salomao and coworkers (2005) examined phylogenetic relationships of eight stingless bee species (*Melipona quadrifasciata anthidioides*, *M. mandacata*, *M. bicolor bicolor*, *M. quinquefasciata*, *M. rufiventis*, *M. scutellaris*, *M. compressipes*, *M. marginata*) by using. ITS-1 sequences. Likwise, Franck and coworkers (2004) studied genetic diversity of the *cabonaria* species (*Trigona carbonaria*, *T. hockingsi* and *T. davenporti*) from eastern Australia using 13 microsatellite loci.

Mitochondrial DNA (mtDNA) is also widely employed as molecular markers in systematic, species characterization, population structure, and phylogenetic studies. Animal mtDNA is a double-stranded, circular molecule and inherited from maternal line. Generally, mtDNA reveals large size differences between animal, fungal, and plant species. The average size of animal mtDNA is approximately 16 kb (Boore, 1999), whereas plant mtDNA is vary from 200 to 2500 kb (Palmer, 1990), and fungal mtDNAs range from 19 kb to 176 kb (Hudspeth, 1995).

MtDNA reveales a high evolution rate and much conserved gene order comprising 13 protein-encoding genes (Oki-moto *et al.*, 1992). The genes contain 6 subunits of NADH dehydrogenase (subunit 1, subunit 2, subunit 3, subunit 4, subunit 4L, subunit 5 and subunit 6), cytochrome b, 3 subunits of cytochrome c oxidase (subunit 1, subunit 2 and subunit 3) and 2 subunit of ATP synthase (subunit 6 and subunit 8). In addition to protein coding genes, the mtDNA includes the large and small mitochondrial ribosomal subunits (lrRNA and srRNA, respectively), 22 tRNA genes (Taanmam, 1999; Lavrov *et al.*, 2005), large non coding region involving replication and transcript initiation, variously designated the major control region, displacement loop (D-loop), as well as major non-coding region or A+T rich region (in insects and other hexapods) (Brown, 1985).

Gene order or gene organization is very among vertebrates; birds differed from mammals and amphibians. Furthermore, the move of tRNA gene position was known to very within insects between the orders Diptera (Clary and Wol-Stenhome, 1985) and Hymenoptera (Crozier *et al.*, 1989) and within the order Diptera, with differences observed between *Aedes* (Hsuchen *et al.*, 1984; Hsuchen and Dubin, 1984) and *Drosophila yakuba* (Clary and Wol-Stenhome, 1985). In recent years, Chandra *et al.* (2006) analyzed and compared insect mtDNA. This study revealed differences in gene organization of mtDNA in insects. However, prior to the present study, several mtDNA have been sequenced directly of a complete sequence from insects, including *Apis mellifera* (Crozier and Crozier, 1993), *Drosophila melanogaster* (Lewis *et al.*, 1995), *Anopheles gambiae* (Beard *et al.*, 1993), *Anopheles quadrimaculatus* (Mitchell *et al.*, 1993), *Drosophila simulans* (Ballard, 1999), *Gryllotalpa orientalis* (Kim *et al.*, 2005), *Drosophila mauritiana* (Ballard, 1999),

Locusta migratoria (Flook *et al.*, 1995), *Pyrocoelia rufa* (Bae *et al.*, 2004), *Crioceris duodecimpunctata* (Stewart and Beckenbach, 2005), *Tribolium castaneum* (Friedrich and Muqim, 2003). Of these, *A. mellifera* provides a large source of data for exploring sequence evolution and relationships in several honey bees and stingless bees.

In the past decades, the complete mtDNA sequence from honey bees, *A. mellifera*, was reported with approximately 16,000-17,000 bp long (Crozier and Crozier, 1993; Figure 1.8) comprising 43.2% A, 41.7% T, 5.5% G and 9.6% C. Non- coding region between tRNA-Leu and COII genes presents high A-T content. The mtDNA of *A. mellifera* contains NADH dehydrogenase complex (subunit 1, subunit 2, subunit 3, subunit 4, subunit 4L, subunit 5 and subunit 6), cytochrome b, three subunits of cytochrome c oxidase subunit I-III, and ATP synthase complex (subunit 6 and subunit 8).

Nowadays, mtDNA sequences are widely used for phylogenetic analyses (particularly in animals), and play a important role in evolutionary, population genetic, biodiversity, and conservation studies in several organisms (Moritz *et al.*, 1987; Patarnello *et al.*, 1994; Weinlich *et al.*, 2004) due to its characteristics, such as high-mutation rates, maternal inheritance, absence of recombination, and small molecular size (Brown *et al.*, 1979; Moritz *et al.*, 1987; Harrison, 1989).

MtDNA is commonly used in study of honey bees, *A. mellifera* for example, to study the genetic polymorphisms and natural range origin of *A. mellifera* relied on PCR-RFLP method (Smith and Brown, 1988; 1990; Smith *et al.*, 1989; Hall and Smith, 1991). Additionally, mtDNA has been employed in biodiversity and biogeographic studies of *A. mellifera* to reveal mitochondrial lineages in *Apis mellifera* (Hall and Muralidharan, 1989; Smith *et al.*, 1989; Cornuet and Garnery, 1991). Furthermore, mtDNA is used to investigate the ancestry of individual colonies, gene flow patterns among hybridizing populations, such as the spread of African-derived honey bees from Brazil to other parts of South, Central and North America (Sheppard and Smith, 2000). The favorite region of mtDNA genes proved in the genus *Apis* is a non-coding sequence between cytochrome c oxidase I (COI) and cytochrome c oxidase II (COII) (Cournuet *et al.*, 1991).

The sequence of a non-coding region between tRNA-Leu and COII of the mitochondrial genome was also used to investigate *Apis cerana* in Asia base on PCR-RFLP method, and abled to divide it into six groups; (1) Japanese group, (2) Nepal, Vietnam and north to central Thailand group, (3) Korea-Tsushima group, (4) Taiwan group, (5) south Thailand group and (6) Philippines group (Deowanish *et al.*, 1996).

Moreover, the intraspecific population structure and genetic diversity of *Apis cerana*, honey bee in Thailand, was investigated using mtDNA genes (e.g. ATPase6–ATPase8, 16S rRNA and COI–COII genes) based on PCR-RFLP (Sihanunthavong *et al.*, 1999; Sittipraneed *et al.*, 2001; Songram *et al.*, 2006). Interestingly, strong biogeography between bees from northern and southern Thailand were found, particularly between samples from north and south of 11°N in the Isthmus of Kra (Warrit *et al.*, 2006) (Figure 1.9), where attribute to the transition between seasonal evergreen and mixed moist deciduous (Hughes *et al.*, 2003).

Additionally, a non-coding sequence between tRNA-Leu and COII (Smith and Hagen, 1997) was used to indicate four groups of mitochondrial haplotypes; (1) an Asian mainland group (Japan, Korea, China, Nepal, northern and southern Vietnam, northern Thailand, and some of the bees from India), (2) a Sundaland group (peninsular Thailand and Malaysia, Samui and Phuket islands, Borneo, Java, Bali, Lombok, Timor and Flores, and Sulawesi), (3) a Palawan group, and (4) a Luzon-Mindanao group (Smith and Hagen, 1996; 1999; Smith *et al.*, 2000; Hepburn *et al.*, 2001).

In stingless bees, most of the mtDNA studies were conducted on *Melipona* based on molecular markers suitable to investigate aspects of the biology and evolution of this group of bees (Peters *et al.*, 1998; Paxton *et al.*, 1999; Francisco *et al.*, 2001; Costa *et al.*, 2003). Recently, the partial mtDNA sequences for *Melipona* have been reported with approximately 14,422 bp long comprising 13 % of GC content (Silvestre *et al.*, 2008).

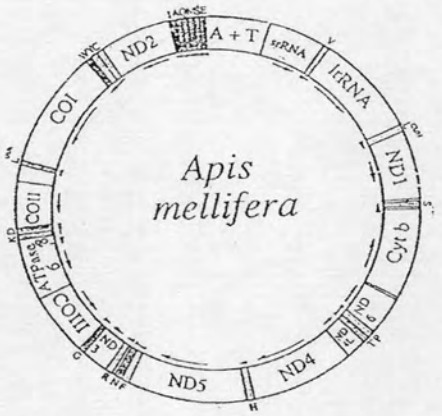

Figure 1.8 Complete mitochondrial genome of *Apis mellifera* (Crozier and Crozier, 1993)

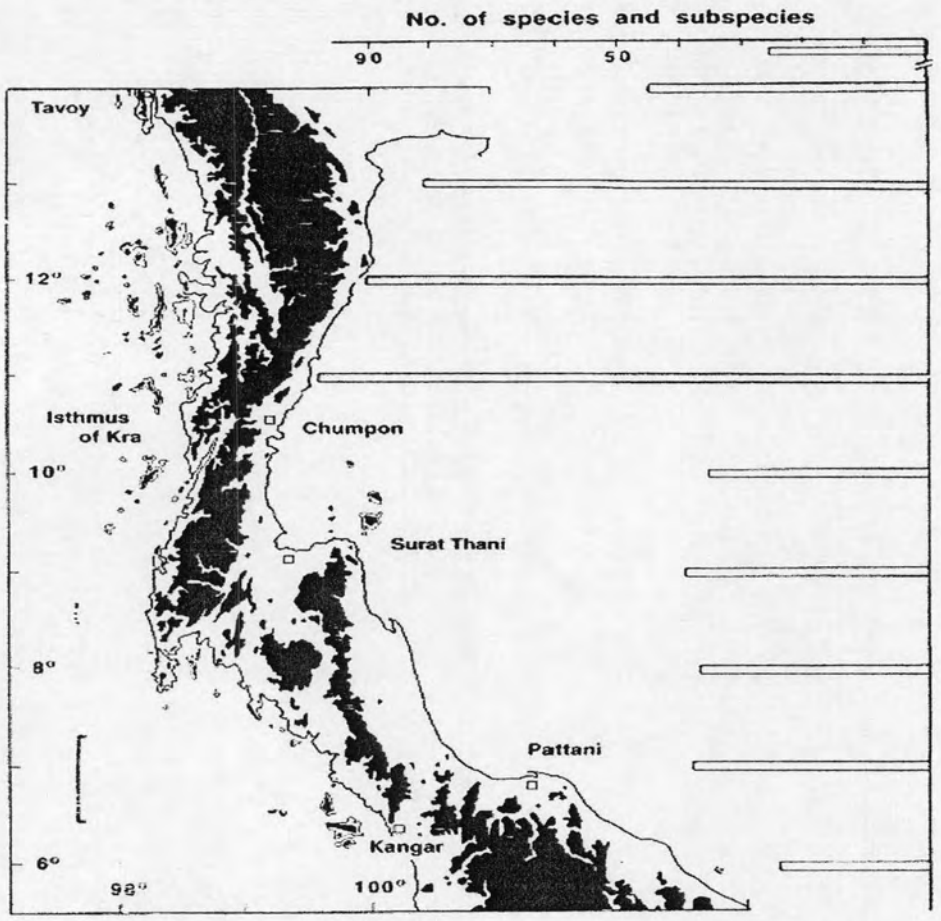

Figure 1.9 Geographic map of Thailand (Hughes *et al.*, 2003)

Subsequently, the mitochondrial gene order between *M. bicolor* and *Apis mellifera* (Crozier and Crozier, 1993) was compared by Silvestre and Arias (2006), eleven tRNA genes located in different genome positions or different strands were found (Figure 1.10). The number of genes rearrangements between these two tribes was much higher than that usually found between pairs of *Diptera* families.

MtDNA has also been employed for the characterization of species and subspecies within the Meliponini. Restriction map data have been published for species belonging to the genera *Plebeia* and *Melipona*. Francisco and coworkers (2001) characterized mtDNA polymorphism of 5 species of *Plebeia* (*P. droryana*, *P. emerina*, *P. remota*, *P. saiqui* and *Plebeia* sp.; tribe Meliponini) for further used in population genetic, phylogenetic and biogeographic studies of these species. The mtDNA of each species was analyzed by 17 restriction enzymes, the result revealed high interspecific genetic variability. The enzyme *Bam*HI and *Xba*I cut the mtDNA of *P. droryana* and *P. remota* only once and can be used as species-diagnostic markers to distinguish these from the remaining species. Weinlich and coworkers (2004) characterized the restriction and partial genomic map of seven *Melipona* species (*M. bicolor*, *M. compressipes*, *M. marginata*, *M. melanoventer*, *M. quadrifasciata*, *M. rufiventris* and *M. subnitida*) by RFLP and PCR-RFLP analysis using 15 restriction enzymes. The results indicated a high level of genetic diversity among those species, and estimated the total size of mtDNA with 18,500 bp long.

Additionary, Moretto and Aries (2005) successfully applied PCR-RFLP of mtDNA genes for differentiation of limited sample sizes of *M. quadrifasciata* and *M. q. anthidiodes* (4 colonies from each species). The patterns generated by *Hin*fI- and *Nde*I-digested COI and *Dra*I-digested cyt b clearly distinguish these subspecies unambiguously. Barni and coworkers (2007) also used PCR-RLFP methods to detect genetic marker patterns of nine mtDNA regions between *M. rufiventris* and *M. mondury*. The patterns generated by three restriction enzymes (*Eco*RI, *Eco*RV and *Hin*fI) out of four could discriminate *M. mondury* and *M. rufiventris*.

Furthermore, Costa and coworkers (2003) used 16S rRNA sequences studied the phylogenetic relationships among 22 of the 56 genera of stingless bees (Meliponini tribe)

previously recognized by Camargo and Pedro (1992) and Roubik and coworkers (1997). Parsimony and maximum likelihood analyses were performed. Four main clades were recognized in the parsimony consensus tree: (A) *Hypotrigona*, (B) *Austroplebeia*, (C) remaining African genera (*Plebeina*, *Meliplebeia*, and *Axestotrigona*) plus the two Oriental genera (*Lepidotrigona* and *Heterotrigona*), and (D) Neotropical genera (Figure 1.11).

In recently studies, Rasmussen and Cameron (2007) constituted the framework for a revised classification of stingless bees (Meliponini tribe) by examining the inter- and infrageneric relationships of Old World Meliponini (e.g. Afrotropical, Indo-Malayan and Australasian; Olson *et al.*, 2001) using mitochondrial 16S rRNA, nuclear long-wavelength rhodopsin copy 1 (opsin), elongation factor-1a copy F2 (EF-1a) and arginine kinase (ArgK) sequences. Meliponini phylogeny (Figure 1.12) estimated from maximum parsimony (MP) analysis of the four gene fragments. The result indicated the three major (M) groups (M-I, M-II, M-III). *Trigona* from the Indo-Malayan/Australasian Regions forms a large clade distantly related to the Neotropical *Trigona*. A separate clade comprises the Afrotropical meliponines, and includes the 'minute' species found in the Afrotropical, Indo-Malayan and Australasian Regions. The Neotropical genus *Melipona*, by contrast with previous investigations, is not the sister lineage to the remaining stingless bees, but falls within the strongly supported Neotropical clade. In Figure 1.12, vertical numbers represent internal clades. These lineages represent groupings of the Indo-Malayan/Australasian Regions (M-I, group 1–3), Afrotropical Region (M-II, group 4), Afrotropical/Indo-Malayan/Australasian Regions of 'minute' Meliponini (M-II, group 5) and Neotropical Region (M-III). The regions are further indicated as Afrotropical (AT), Australasian (AA), Indo-Malayan (IM) and Neotropical (NE).

Nevertheless, the knowledge of mtDNA sequences of *Trigona* species is very limited. Therefore, the use of mtDNA as a genetic marker in *Trigona* species appears to be restricted to the conserved gene segment of the 16S rRNA gene (Rasmussen and Cameron, 2007; Costa *et al.*, 2003). The lack of mtDNA sequence information of *Trigona* species is one of the major problems for further studies of *Trigona* species through PCR. More information about mtDNA sequences from *Trigona* species are then required to promote

these research effectively. Thus, the partial sequence of mtDNA genome of *T. pagdeni* was determined using a long PCR technique (Cheng, 1994). Specific primer pairs were developed to amplify the sutible mitochondrial DNA regions, and DNA sequence variation in the amplified regions was detected for population genetic studies of *T. pagdeni* by single strand conformational polymorphism (SSCP) analysis. This method was sensitive and efficient for determining sequence variation inside similar-sized DNA fragments, including convenient, rapid and inexpensive (Weder *et al.*, 2001).

A genetic marker can be used as a marker of genetic diversity among individuals of any organisms (e. g. Klinbunga *et al.*, 2006). In other studies, highly polymorphic molecular markers were also extensively developed and applied in studies of population and conservation genetics. Additionally, molecular markers can be used to study kinship and parentage (e. g. Webster and Reichart, 2005). The PCR technique has been introduced by Mullis and coworkers (1987), with the purpose to make a huge number of copies of particular and specific fragment of DNA sequence. Further, these techniques are being applied at several molecular genetic studies. PCR amplifications use oligonucleotide primers that hold to opposite strands. The primers are located such that extension processes inward across the region between the two primers. Thus, the products of DNA synthesis of one primer are used as the template for the other primer, the PCR procedure comprises three steps; DNA denaturation, annealing of primers, and extension by DNA polymerase results in an exponential increase in the number of copies of the region bounded by the primers (Figure 1.13).

To date, many DNA fingerprinting techniques have been developed and used to illustrate the genetic polymorphism among individuals, population or species, such as PCR-restriction fragment length polymorphism (PCR-RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and single strand conformational polymorphism (SSCP), for genetic diversity studies, genetic mapping, marker-trait association studies, and marker assisted selection programs (Jones *et al.*, 1997; Klinbunga *et al.*, 2006).

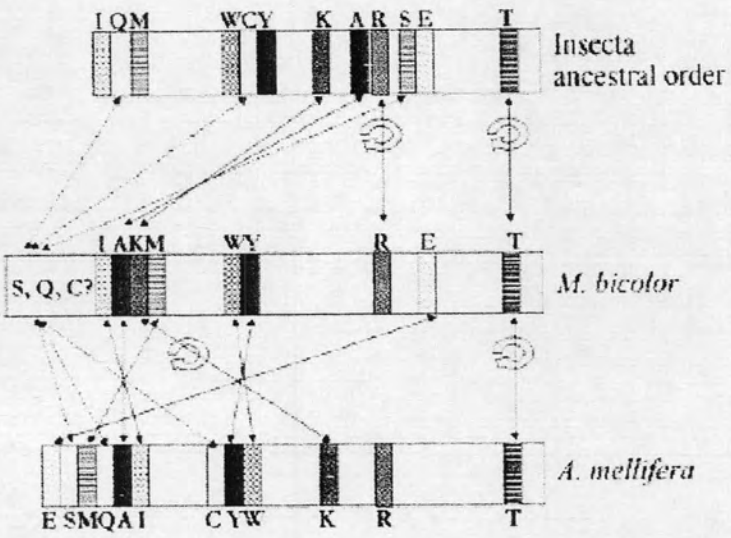

Figure 1.10 Mitochondrial tRNA gene order between *Apis mellifera* and *Melipona bicolor*. The putative ancestral gene order of Insecta (Boore, 1999) is used for comparison. Each tRNA gene is given by the letters of the amino acid code. The arrows indicate translocations, and the circular arrows present strand inversions (Silvestre and Arias, 2006).

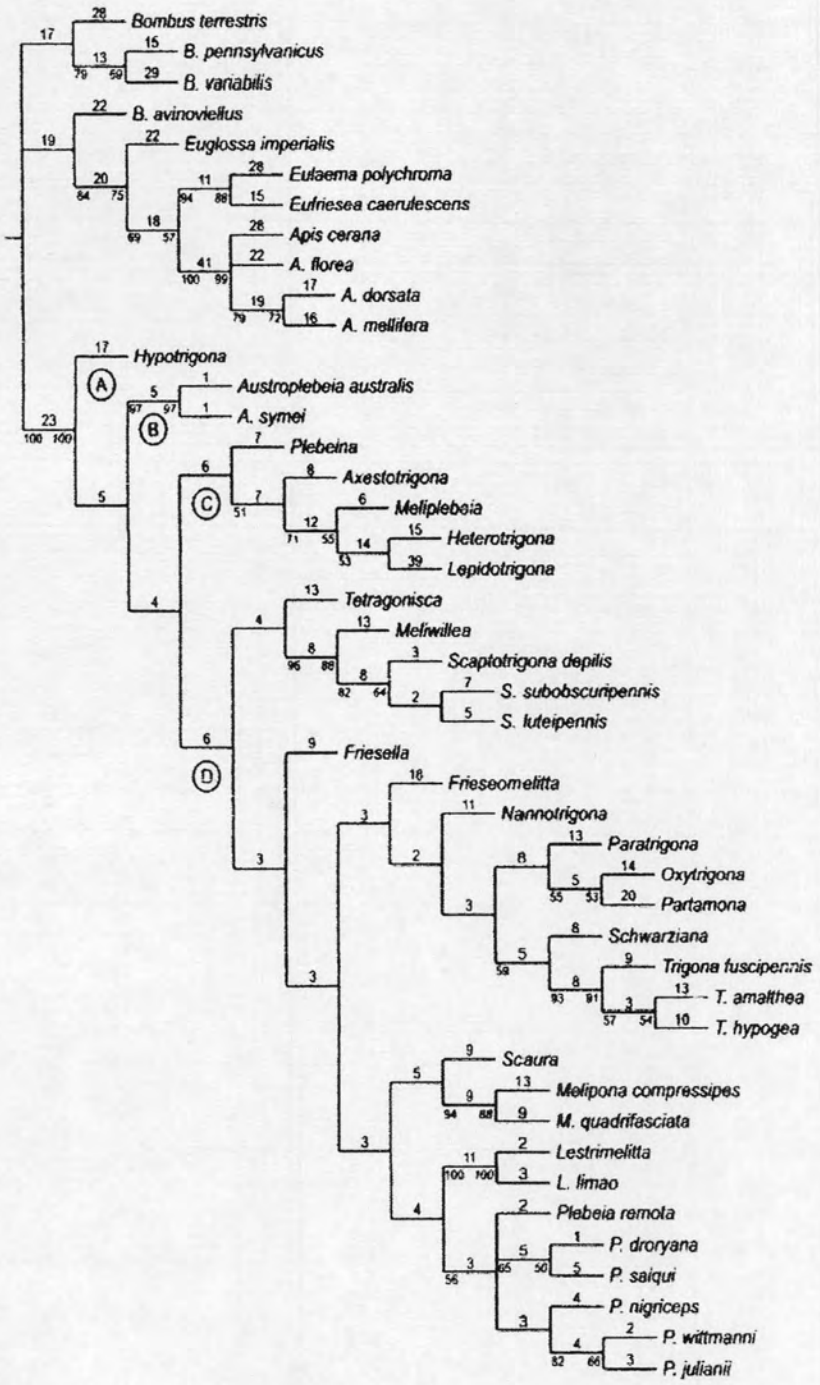

Figure 1.11 Meliponini phylogeny (strict consensus of 6 trees) estimated from parsimony consensus analysis of the 16S rRNA gene based on sequence data.

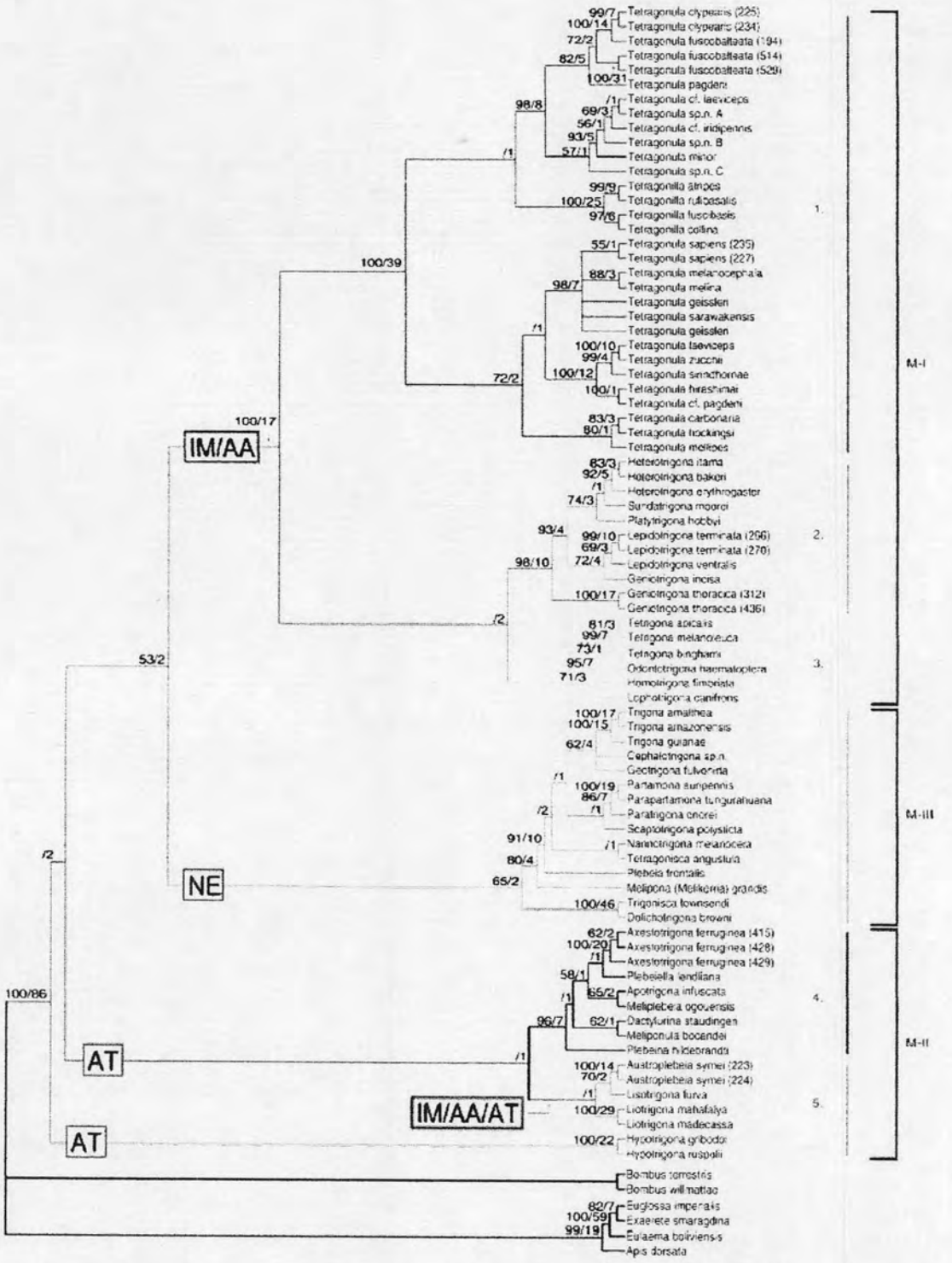

Figure 1.12 Meliponini phylogeny (strict consensus of 12 trees) estimated from maximum parsimony (MP) analysis of four gene fragment sequences (Rasmussen and Cameron, 2007).

Amplified fragment length polymorphisms (AFLP)

Amplified Fragment Length Polymorphism (AFLP) is the DNA fingerprinting approach that combines adventages from both RFLP (cutting of genomic DNA with restriction endonucleases) and RAPD (the amplification of particular DNA sequences using arbitrary primers techniques). It has the potential to screen many different DNA regions randomly distributed throughout the entire genome without the need for knowledge of sequences of the genome under investigation (Blears, 1998; Vos *et al.*, 1995). AFLP provided more polymorphic results than RAPD (random amplified polymorphic DNA). The main disadvantages of AFLP is the difficulty in identifying homolocus markers (alleles), rendering this method less useful for studies that require precise assignment of allelic states. However, because of the rapidity and ease with which reliable, reproducible, high-resolution markers can be generated. According to Vos and coworkers (1995), AFLP analysis involves restriction digestion of genomic DNA with a combination of rare cutter (e.g. *Eco*RI or *Pst*I) and frequent cutter (e.g. *Mse*I or *Taq*I) restriction enzymes (Figure 1.14). Then, double-stranded oligonucleotide adaptors are ligated to both ends of the restriction fragments to provide templates for PCR amplification.

The PCR amplification was occurred by the primers containing the sequences that are able to anneal to the sequences of the adapters and the additional bases which complementary to the restriction sites. The PCR product was then diluted and subjected to the second PCR amplification under higher stringent condition with primers complementary to the adaptors, along with one to three selective bases added at their 3' ends (Figure 1.14). The selective bases of one, two or three bases are useful to reduce a large number of amplified bands by factors of 4, 16 and 64, respectively that make it easy to detect polymorphisms (Vos *et al.*, 1995). According to Matthes and coworkers (1998), polyacrylamide gel electrophoresis (PAGE) was used to detect polymorphisms at the different length of the amplified fragments. Furthermore, AFLP fragments are visualized on polyacrylamide gel with silver straining.

Three enzyme fragment length polymorphism (TE-AFLP)

Three enzyme fragment length polymorphism (TE-AFLP) is developed from the typical AFLP technique. This technique uses three restriction enzymes in stead of two to reduce the number of restriction fragments and provides discrimination at restriction sites of the third endonuclease, but still uses two primers with selective nucleotides like AFLP (van der Wurff *et al.*, 2000). The digestion and ligation reaction were processed by adding three restriction endonucleases together with only two sets of adapters in a single reaction. This method can simplify the two-step amplification to one-step amplification in fingerprinting complex genomes. Therefore, TE-AFLP technique is one of the most common techniques used for genetic variation analysis or marker detection. In recent years, this method was used to reveal population structure in insect (van der Wurff *et al.*, 2003).

PCR-Single strand conformational polymorphism (PCR-SSCP)

The need to accurately detect mutations and identify DNA polymorphisms have increased with the rapid growth in characterize important genes. PCR single-strand conformational polymorphism (PCR-SSCP) is one of popular techniques extensively used to identify a sequence variation or a polymorphism in a known gene. SSCP is based on the principle that the double strand DNA loses their double stranded conformation, folded into single stranded secondary structures. SSCP are of high sensitivity in detection of mutations because a single base change of the sequence can influence to the radical change in nucleic acid migration, these base changes are detected by differential mobility on a non-denaturing gel (Orita *et al.*, 1989) (Figure 1.15). SSCP bands can be visualized by using autoradiograms (radioactive detection), or silver staining or fluorescent. Because of its high sensitivity, SSCP experimental conditions can be optimized by alteration of the temperature or the degree of cross-liking or the adding of glycerol or sucrose, to maximize differential migration among fragments. Thus, the SSCP technique is considered to be a method that reveals inexpensive cost, convenience, highly efficiency and sensitivity for detecting mutation or sequence variation (Iwahana *et al.*, 1992; Sheffield *et al.*, 1993).

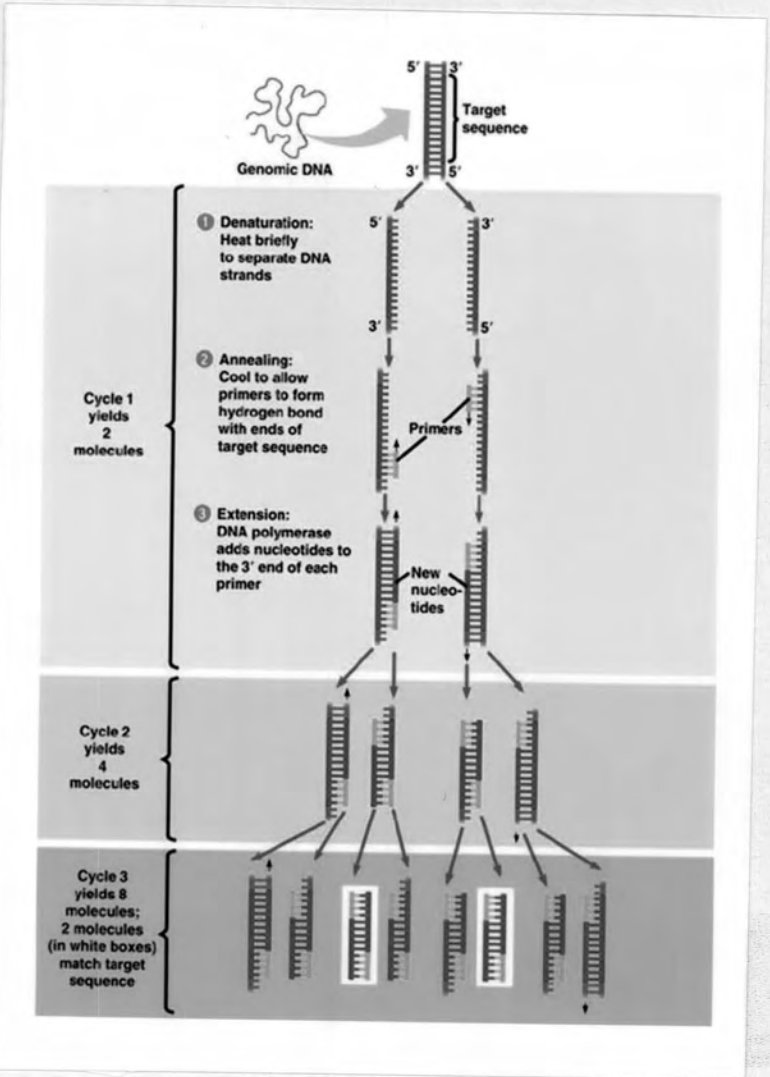

Figure 1.13 Steps of the polymerase chain reaction for DNA amplification (http://fig.cox.miami.edu/~cmallery/150/gene/c7.20.7.pcr.jpg)

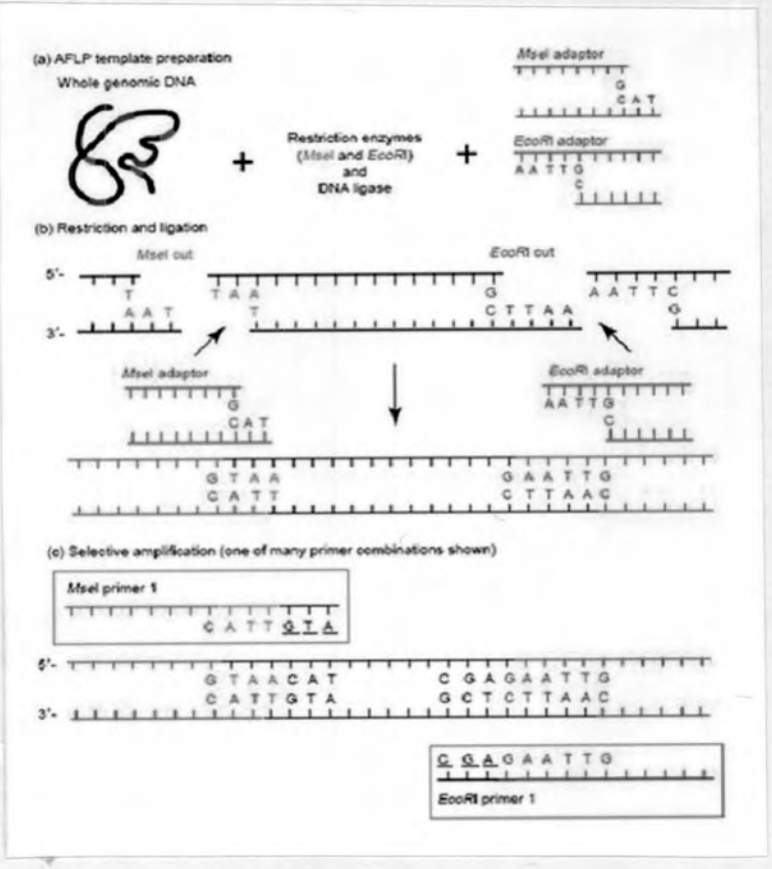

Figure 1.14 AFLP procedures: a). Genomic DNA is digested with two restriction enzymes, and b). adaptors are ligated to the end of restriction fragments. The adaptor sequence was marked in red and the remaining part of the restriction sequence was marked in blue and green. c). For PCR amplifications, the primers extending selective base into the unknown part of the fragments (in black and underlined base pairs) were needed. The first PCR amplification is performed with a 1-bp extension, followed by a more selective primer with a 3-bp extension.

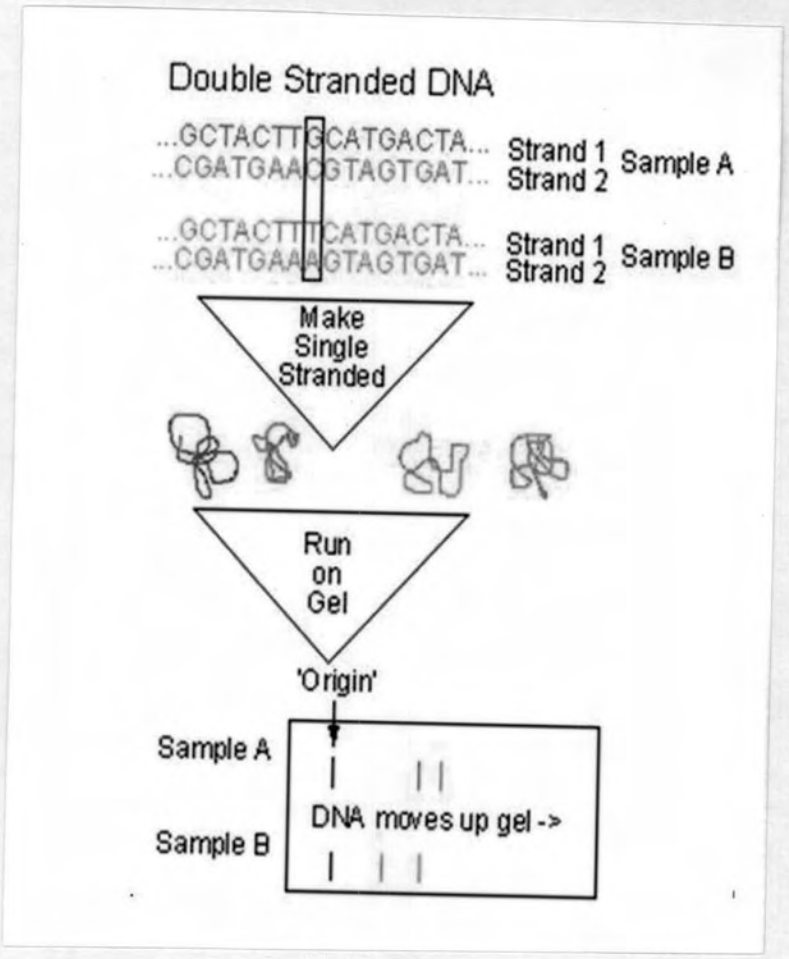

Figure 1.15 Diagram shows the principal diagram of SSCP analysis (http://www.austmus.gov.au/evolutionary_biology/images/sscp.gif)

1.7 OBJECTIVES OF THIS DISSERTATION:

1.7.1 To establish reliable species diagnostic markers to distinguish *T. pagdeni* from other morphologically similar stingless bees in Thailand.

1.7.2 To estimate genetic diversity and population structure within *T. pagdeni*.

1.7.3 To determine the partial sequences of the mtDNA genome of *T. pagdeni*.