CHAPTER I

INTRODUCTION

1.1 Stingless bee biology

1.1.1 Division of labor

Social life in the stingless bees involves division of labor which is similar to that in honeybees: very young bees start to work with wax and cerumen, away from the brood nest, then some of them shifts towards building and provisioning brood cells, during which period eggs may be laid. From brood care they go to foraging: first they receive incoming nectar and dehydrate it before becoming actual foragers (Bassindale, 1955; Velthuis, 1997). Only the females are divided into castes which are queens and workers. They are morphologically very different. Stingless bees are highly eusocial. The queen is unable to live alone nor do workers alone form viable colonies because the queen never forages and workers can not mate and produce female off-spring (Michener, 2007). The different activities among workers are related to ages. The sequence of tasks in workers is divided into five stages as follows: (1) self-grooming (during the first hours after emergence from the pupae); (2) incubation and repairs in the brood chamber; (3) construction and provisioning of cells, cleaning of the nest, and feeding young adults and the queen; (4) further cleaning of the nest, reconstruction of the involucrum, reception of nectar, and guard duty at the entrance of nest; (5) collection of pollen, nectar, and propolis. The duration of of each stage depends on the species and on the condition of the colony (Wille, 1983).

1.1.2 Nest architecture of stingless bees

The nests of stingless bees are more elaborate and complex than those of most other bees. Their nesting sites are diverse. Nests of most species are built within protective cavities such as hollow trees or in the ground. Some species establish nests within nests of termites or ants. Few species build their nests in exposed positions (Roubik, 1979; Sakagami et al., 1983; Sakagami et al., 1989; Sommeijer, 1999). In general term, stingless bees may build solid batumen plates to shield and protect the colony. Nests are then constructed using wax in a mixture with resins or gum, mud,

feces, or other materials collected by the bees. The nest entrance provides access into the nest where the brood is located. A mixture of wax with resins, which is called cerumen, is used to build involucrum sheaths as a protective layer or sheath around the brood chamber, called brood involucrum, or around the whole colony, called external involucrum, including the storage vessels for honey and pollen (Figure 1.1). In principle, there are two cell types in nest of stingless bees: brood cell and storage pots (Figure 1.2). Brood cells can be clustered or arranged in combs that are usually positioned in a horizontal plane. Outside the brood involucrum, small pots with food provisions are built in clusters. All of these nest characteristics are variable across the stingless bees (Michener, 1961; Wille and Michener, 1973; Sakagami, 1982; Wille, 1983; Roubik, 2006).

1.1.3 Foundation of new colonies and mating

Stingless bees produce swarms which are different process from that of honeybees (Kerr, 1951; Kerr et al., 1962). The process of swarming starts with transferring nest material and food 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 (Velthuis et al., 2005). Afterward, the new queens fly out for a single mating, followed by hundreds of males (Peters et al., 1999). Then, brood cell construction and oviposition are started in the new nest (Moure et al., 1958, Sakagami 1982, Inoue et al., 1984). The recent report reveals that males are produced by workers (Tóth et al., 2004). Subsequently, the bond between the mother and daughter colony slowly degrades (Wille, 1983).

1.2 Distribution of stingless bees

Stingless bees belong to the tribe Meliponini in the family Apidae along with honeybees, carpenter bees, orchid bees, and bumblebees. Relevant evidence suggests that the stingless bees have a center of origin and dispersion in Africa and migrated to tropical and subtropical regions around the world, such as Southeast Asia, Australia, parst of Mexico and Brazil (Michener, 1974; Sakagami, 1982; Michener, 1990; 2007) (Figure 1.3). Stingless bees are the most diverse in morphology and behavior of the eusocial bees. They live in permanent colonies and multiply through a process of

swarming. Colony size is diverse and ranges from a few dozen to 100,000 or more individuals (Michener, 2007).

Today, over 600 species in 56 named genera live in tropical and subtropical areas of the world (Cortopassi-Laurino et al., 2006). *Melipona* and *Trigona* are the most important genera. *Melipona*, including approximately 50 species restricted to the neotropic regions, has more complex communication systems (Nieh and Roubik, 1995). It is able to buzz pollinate (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 more than 120 species in ten subgenera from the Indo-Malayan/Australasian and Neotropical regions (Michener, 2007). Recently, Rasmussen (2008) provides a catalog of Indo-Malayan/Australasian stingless bees that is an index to previous studies in taxonomy, behavioral research, and pollination ecology. This study divides the stingless bees in *Trigona* into several genera. Following this catalog, the former subgenera of *Trigona* are raised to genera (e.g. *Trigona collina* changed to *Tetragonilla collina*).

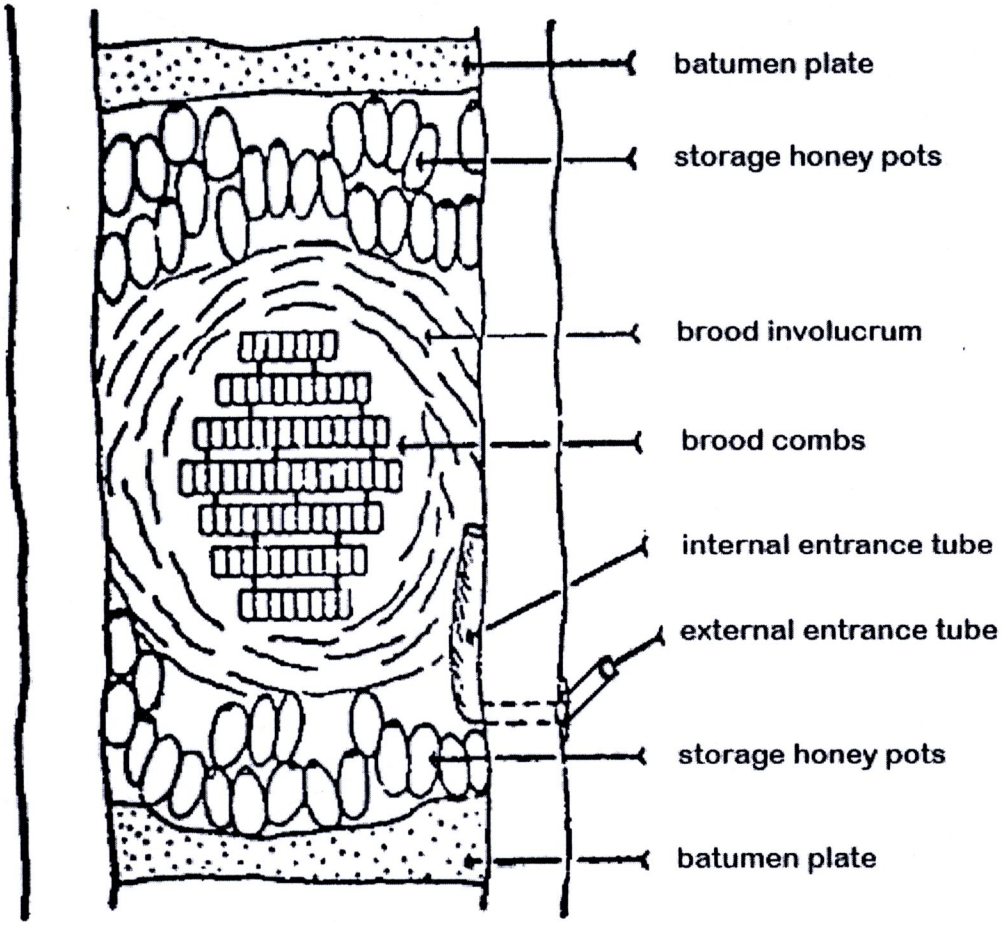
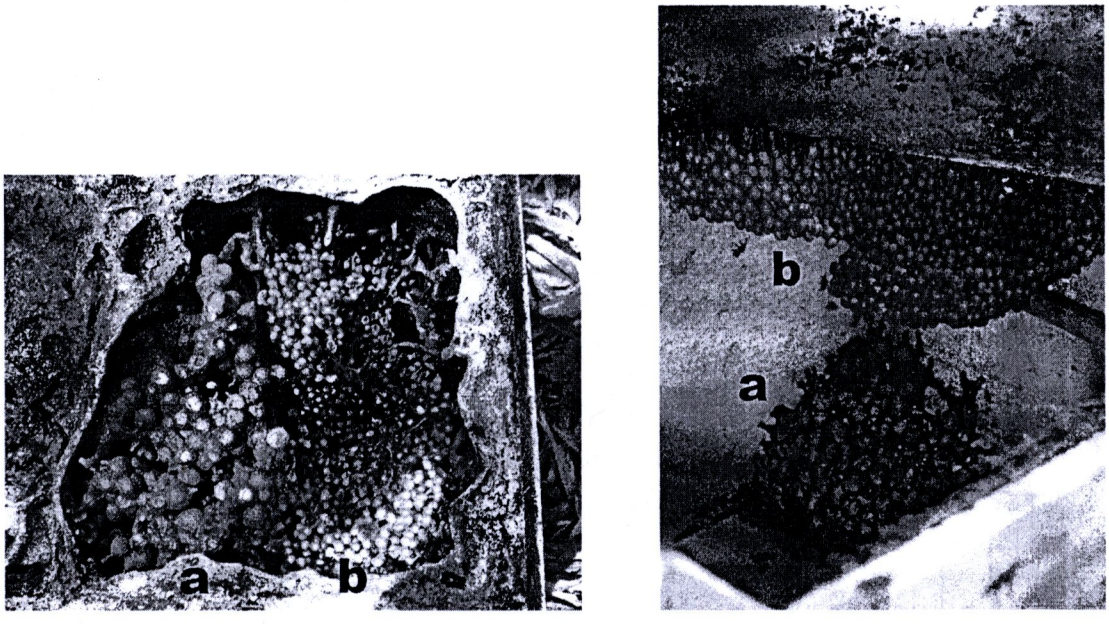


Figure 1.1 Diagram of stingless bees nest with the structure labeled (modified from Wille, 1983).



(A) (B)

Figure 1.2 Nest of stingless bees; *Tetragonilla collina* (A), *Tetragonula laeviceps* (B). There are two cell types: storage honey pots (a) and brood cell (b) in the nest of stingless bees.

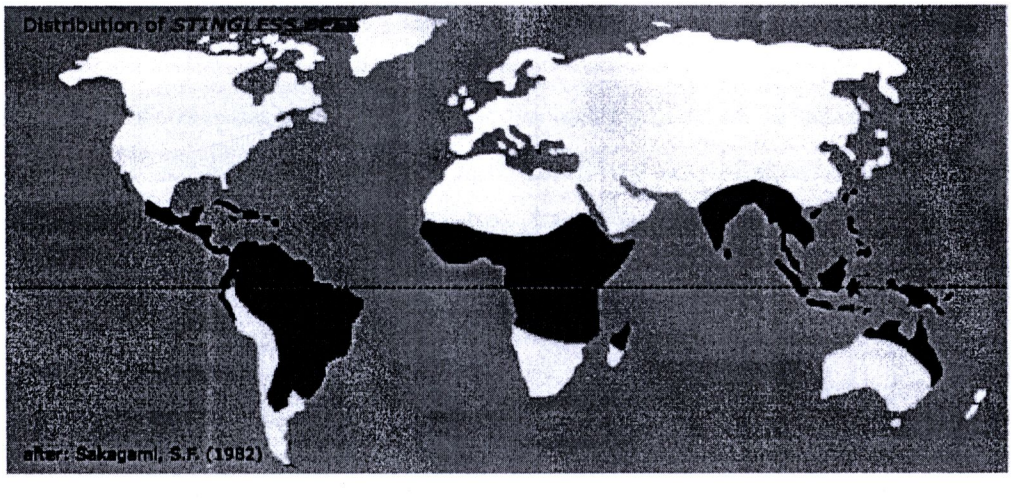


Figure 1.3 Distribution of stingless bees (marked in black).
(http://www.blab.at/Bilderchens/Distribution-of-stingless-bees.jpg)

1.3 Exploitation of native species

Stingless bees play an important ecological role as pollinators of many wild plant species and seem good candidates for future alternatives in commercial pollination (Slaa et al., 2006). They are true generalists, collecting nectar and pollen from a vast array of plants (Heithaus, 1979). The fact that they lack a functional sting makes them especially suitable for pollination in enclosures (Slaa et al., 2000). Stingless bees are used widely as crop pollinators under greenhouse conditions for the following reasons: they are harmless to beekeepers and greenhouse workers, visit a wide range of crops (polylecty), are tolerant of high temperatures, are active throughout the year, can be transported easily (Amano, 2004). They also show some characteristics that support the ability as pollinators; for example, workers usually visit only one plant species on a single trip (Ramalho et al., 1994), workers collect food beyond immediate needs and store in nests allowing colonies to survive long periods (Roubik et al., 1986), and workers can help nest mates by providing information on the position of those floral resources. Moreover, many species of stingless bees can be managed in hives boxes for using as crop pollinators and for commercial production (Slaa et al., 2006). *Tetragonula laeviceps* is one of the commonest for using as tropical fruit pollinators in Thailand (Oldroyd and Wongsiri, 2006).

Stingless bee beekeeping is known as meliponiculture. This activity has local characteristics according to regional and traditional knowledge. Honey, wax, and resin are the traditional products of stingless bees and they are an important income source for the stingless bee beekeeper. The extraction of honey is often the income for keepers of stingless bees, followed by cerumen and resin (Cortopassi-Laurino et al., 2006). Propolis is made from plant exudates that workers forage and accumulate for construction, protection, and adaptation of their nests (Velikova et al., 2000). Subsequently, the study of Meliponinae propolis provides information on the chemical composition of stingless bee propolis, as well as the plants which they use as a resource for collecting propolis to support the medicinal properties of stingless bee propolis (Bankova and Popova, 2007).

1.4 Stingless bees in Thailand

Several genera of stingless bees have been recognized in Thailand, where 32 species have been recorded (Schwarz, 1939; Sakagami et al., 1983; Michener and Boongird, 2004; Klakasikorn et al., 2005; Rasmussen, 2008) (Table 1.1). Of these, the stingless bee *Tetragonilla collina* Smith, which is one of the commonest and most widespread species in Southeast Asia, is distributed throughout Thailand (Sakagami, 1975; Jongjitvimol et al., 2005). Like most stingless bee species, the nests of *T. collina* are usually constructed in small to large hollows in trees or in cavities in the soil (Velthuis, 1997; Michener, 2007). Colonies of *T. collina* have an elongate entrance tube leading to an underground nest (Figure 1.4). Their nests are often built in the roots of large trees and they have an aggregated distribution, with many colonies nesting in close proximity (i.e., nesting under the same tree) with an average of 2 nests per occupied nest tree (Eltz et al., 2002 and 2003).

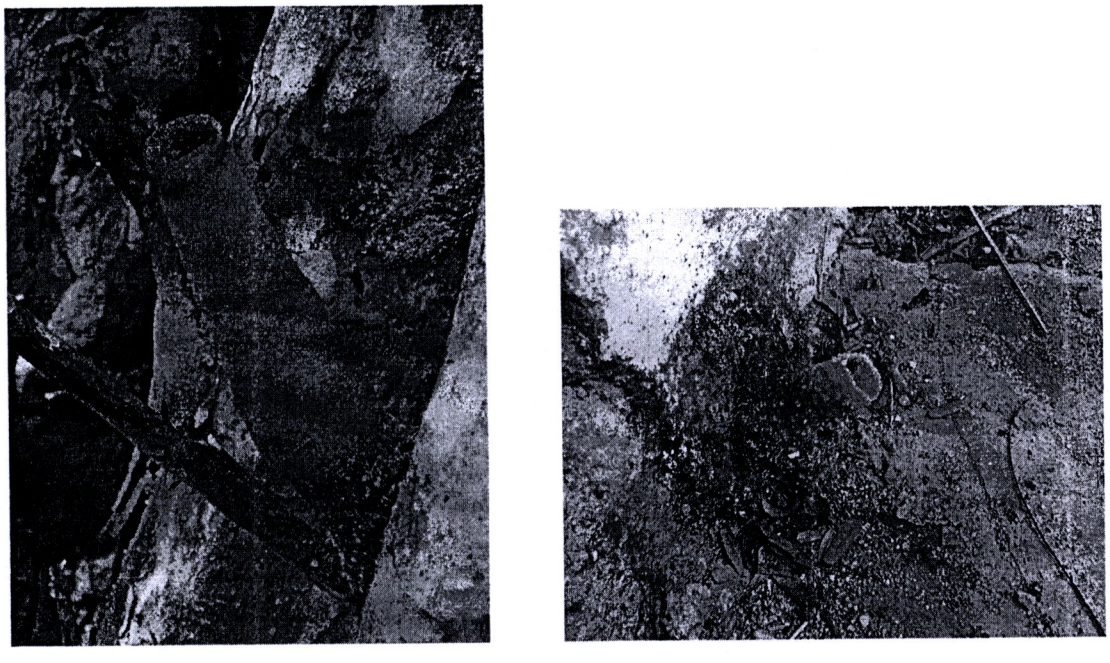


Figure 1.4 Pictures of nest entrances of stingless bees *T. collina*

Table 1.1 Stingless bee species found in Thailand (modified Klakasikorn et al., 2005)

Stingless bee species	Klakasikorn et al. (2005)	Michener and Boongird (2004)	Rajitparinya et al. (2000)	Sakagami et al. (1985)	Schwarz (1939)
Tetragonula sirindhornae Michener and Boongird, 2004		*			
Tetragonilla collina Smith, 1857	*		*	*	*
Lepidotrigona terminata Smith, 1878	*		*	*	*
Tetrigona apicalis Smith, 1857	*		*	*	*
Lepidotrigona doipaensis Schwarz, 1939	*				*
Tetragonula laeviceps Smith, 1857	*		*	*	
Tetragonula minor Sakagami, 1978	*				
Geniotrigona thoracica Smith, 1857	*			*	*
Tetrigona binghami Schwarz, 1939	*				
Homotrigona fimbriata Smith, 1857	*		*	*	
Tetragonula fuscobalteata Cameron, 1908	*			*	*
Heterotrigona itama Cockerell, 1918				*	*
Tetrigona melanoleuca Cockerell, 1929			*	*	*
Tetrigona peninsularis Cockerell, 1927				*	*
Lophotrigona canifrons Smith, 1857				*	*
Homotrigona aliceae Cockerell, 1929					*
Homotrigona lutea Bingham, 1897					*
Tetragonula pagdeni Schwarz, 1939				*	*
Tetragonula geissleri Cockerell, 1918				*	*
Tetragonula iridipennis Smith, 1854					*
Tetragonula valdezi Cockerell, 1918					*
Tetragonula melina Gribodo, 1893				*	*
Tetragonula sarawakesis Schwarz, 1937					*
Lepidotrigona flavibasis Cockerell, 1929					*
Lepidotrigona ventralis Smith, 1857			*	*	*
Lisotrigona cacciae Nurse, 1907			*	*	*
Lepidotrigona nitidiventris Smith, 1857				*	
Tetragonilla atripes Smith, 1857				*	
Tetragonilla fuscibasis Cockerell, 1920				*	
Tetragonula hirashimai Sakagami, 1978				*	
Tetragonula pagdeniformis Sakagami, 1978				*	
Tetragonula reepeni Friese, 1918				*	

1.5 *Tetragonilla collina* Smith in Thailand

The taxonomy of *T. collina* Smith was identified according to Michener (2007) and Rasmussen (2008).

Kingdom: Animalia

Phylum: Arthropoda

Class: Insecta

Order: Hymenoptera

Superfamily: Apoidae

Tribe: Meliponini

Genus: Tetragonilla

Subgenus: Tetragonilla

Scientific name: *Tetragonilla collina* (Smith, 1857)

Common name: Stingless bee

Morphological structures of stingless bees are shown in Figure 1.5 and 1.6. *Tetragonilla collina* Smith, an indigenous stingless bee, is distributed covering vast geographic locations in Thailand. Body coloration of *T. collina* ranges from blackish to dark brown. Clypeus varies from nearly as dark as the face above to distinctly pale. The tegulae are dark brown to black. Fore wing basal is distinctly darker, contrasting to milky white apical half and veins are basally dark brown while apically pale. The northward increase of body size is detected from Malaya and Southern Thailand to Northern Thailand (Sakagami, 1975; Sakagami et al., 1985).

Jongjitvimol and Wattanachaiyingcharoen (2007) report the distribution, nesting sites and nest structures of *T. collina* in Thailand. Most nests of *T. collina* are found in mixed deciduous forests. Their nesting sites are divided into 4 groups; cavities in tree trunks, cavities in termite mounds, underground cavities, and cavities in buildings. Moreover, the first record of an assassin bug, *Pahabengkakia piliceps* is reported as a specialized predator of the stingless bee *T. collina* (Wattanachaiyingcharoen and Jongjitvimol, 2007).

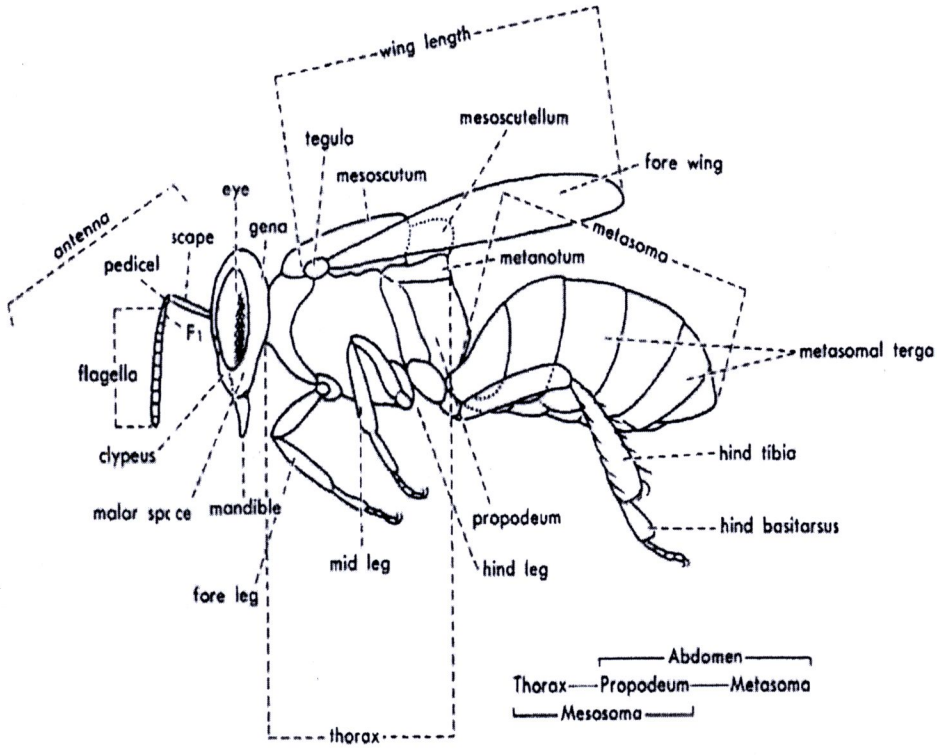


Figure 1.5 Morphological structures of stingless bees (Sakagami et al., 1985)

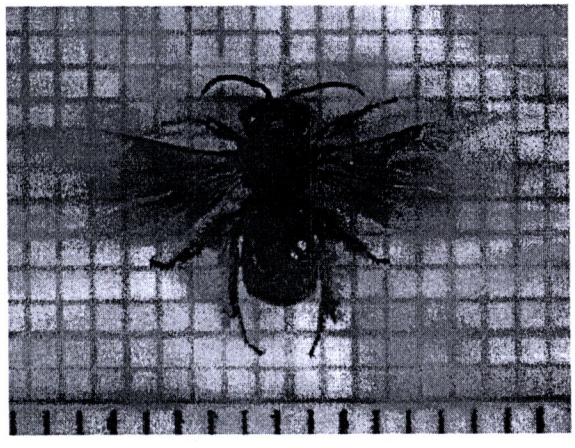


Figure 1.6 Morphological structure of *T. collina* (Wattanachaiyingcharoen et al., 2004)

1.5.1 Identification of *T. collina*

Today, the stingless bees are exploited as pollinators in agriculture and their products are also valuable. Therefore, the knowledge of stingless bees is continually increased and identification of stingless bees has also been reported over the years (Michener, 1961; Sakagami, 1978; Dollin et al., 1997). They are classified based on morphology and nest architecture. However, taxonomic identification of stingless bees remains unclear and requires experienced scientists.

Morphology is the most commonly used method of classification but many stingless bee species are sympatric species (e.g. between *T. pagdeni* and *T. fuscobalteata*; Sakagami 1978) and can not be preliminary distinguished based on geographic distribution. In addition, species recognition of stingless bees is more complicated by the existence of cryptic species (e.g. between *T. carbonaria* and *T. hockingsi* and between *T. iridipennis* and *T. laviceps*; Starr and Sakagami 1987). The external characteristics are thus unstable and not reproducible because of a variety of habitats and environmental conditions. Nevertheless, nest architecture characters are usually relevant but they are reported that they are not sufficient criteria for authenticating species origins of Australian stingless bees (i.e. *T. hockingsi* and *T. davenporti*; Franck et al. 2004).

The use of specimens with correct species origin is one of main factors that affect further molecular genetic studies of stingless bees. Therefore, species-diagnostic markers for reliable differentiation of abundantly distributed species such as *T. collina* are a prerequisite for eliminating confusion of similar species in genetic diversity and population structure analyses of this species. The development of molecular biology techniques, such as DNA-based 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 has been widely used to study polymorphism among populations and species (Blears et al., 1998; Mueller and Wolfenbarger, 1999) and to identify species-diagnostic markers in

various taxa (Liu and Cordes, 2004; Klinbunga et al., 2007). AFLP can generate high-resolution markers that exhibit such a purpose, where no data in stingless bees are reported at present.

Recently, a species-diagnostic AFLP-derived marker for identification of Thai *T. pagdeni* is successfully developed. The CUTp1 marker can discriminate *T. pagdeni* from 10 stingless bee species in Thailand. The further analysis (SSCP analysis) then differentiates *T. pagdeni* from two more species (*T. fuscobalteata* and *T. collina*) while the remaining 2 species (*T. laeviceps* and *T. fimbriata*) can be clearly identified by morphology (Thummajitsakul et al., 2010).

After 3 years of this study, the catalog of published literature on stingless bees from the Indo-Malayan/Australasian region is recompiled (Rasmussen, 2008). The subgenus of Indo-Malayan stingless bees in *Trigona* genus is treated as genus. All collected samples in this study are in *Trigona* genus. Therefore, they are separated to several genera such as *Geniotrigona* (*T. thoracica*), *Heterotrigona* (*T. itama*), *Homotrigona* (*T. fimbriata*), *Lepidatrigona* (*T. terminata* and *T. doipaensis*), *Lophotrigona* (*T. canifrons*), *Tetrigona* (*T. apicalis* and *T. melanoleuca*), *Tetragonilla* (*T. collina*) and *Tetragonula* including *T. laeviceps*, *T. pagdeni*, *T. melina*, *T. minor*, and *T. fuscobalteata*.

1.5.2 Genetic diversity of *T. collina* in Thaialnd

Sustainable conservation and the construction of effective genetic management of important natural resource species require basic knowledge of the genetic population structure of that species (Avise, 1994). 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. These may provide the evolution of new characteristics within a single species for survival and adaptation in their environment. Genetic diversity has been studied in several social insects. These studies reveal the useful knowledge from the insects for further studies; for example, genetic diversity can help to 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 polyandrous queen 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 insects (Sánchez and Keena, 2009). A DNA marker is also used as a marker of genetic diversity within and among individuals of any organisms such as in Thai honey bee, *Apis cerana* (Sitttipraneed et al., 2001) and Thai stingless bee, *T. pagdeni* (Thummajitsakul et al., 2008). In Thailand, Cameron et al. (2004) showed that colonies of *T. collina* within nest aggregations are not genetically related-that is, queens in a nest aggregation are not related as sisters or mother and daughter-and suggested that new nests are established in an unrelated nest aggregation. However, population genetic structure of *T. collina* at larger scales has not been reported. 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 Molecular marker

Molecular markers can reveal genetic variation (polymorphism) at the protein level (protein marker) or at the DNA level (DNA marker) without environmental factors. At the DNA level, types of genetic variation include: base substitutions, commonly referred to as single nucleotide polymorphisms (SNPs), insertions or deletions of nucleotide sequences (indels) within a locus, inversion of a segment of DNA within a locus, and rearrangement of DNA segments around a locus of interest. Through long evolutionary accumulation, many different instances of each types of mutation should exist in any interest species and the number and degree of the various types of mutations define the genetic variation within species. DNA marker can be applied to reveal these mutations (Liu et al., 2004).

Numerous molecular markers have been characterized e.g. allozyme, restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), microsatellite, etc. These molecular markers are classified into 2 groups: markers that require prior

molecular information (e.g. allozyme, RFLP, microsatellite) and markers that no need prior molecular information (e.g. RAPD, AFLP).

Fernandes-Salomao et al. (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 RNA Intergenic Transcribed Spacer 1 sequences. Likewise, Franck et al. (2004) studied genetic diversity of the *cabonaria* species (*Trigona carbonaria, T. hockingsi,* and *T. davenporti*) from eastern Australia using 13 microsatellite loci. These reports investigated polymorphisms of stingless bees that have already been reported the molecular information. Recently, Thummajitsakul et al. (2008, 2010) revealed the studies of Thai *T. pagdeni* that lack their molecular information. They developed a species-diagnostic AFLP-derived marker and also investigated genetic diversity of this species in Thailand based on three enzymes amplified fragment length polymorphism (TE-AFLP).

Mitochondrial DNA (mtDNA) is also widely employed as a molecular marker in systematic, species characterization, population structure, and phylogenetic studies. Animal mtDNA is a circular molecule and maternally inherited in most animals without recombination, so the whole set of genes is inherited as one unit. MtDNA is used in study of honey bees, *A. cerana* for example, to study the genetic polymorphisms of *A. cerana* based on PCR-RFLP method (Sittipraneed et al., 2001; Songram et al., 2006; Warrit et al., 2006).

Amplified fragment length polymorphism (AFLP)

Amplified Fragment Length Polymorphism (AFLP) is a DNA fingerprinting approach that combines advantages from both RFLP (cutting of genomic DNA with restriction endonucleases) and RAPD (the amplification of particular DNA sequences using arbitrary primers). 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). The main disadvantages of AFLP is the difficulty in identifying homologous markers (alleles), rendering this method less useful for studies that require precise assignment of allelic states. However, it is advantageous because of the rapidity and ease with

which reliable, reproducible, high-resolution markers can be generated. According to Vos et al. (1995), AFLP analysis involves the digestion of genomic DNA with a combination of rare cutter and frequent cutter restriction enzymes. Then, double-stranded oligonucleotide adaptors are ligated to both sides of the restriction fragments to provide templates for PCR amplification. The PCR amplification is twice performed by the primers containing the sequences that are able to anneal to the sequences of the adapters and one additional base at the 3' ends which complementary to the restriction sites for preamplification while the primers with two or three additional bases at the 3' ends are used for the selective amplification (Figure 1.7).

Three enzyme amplified fragment length polymorphism (TE-AFLP)

Three enzyme amplified fragment length polymorphism (TE-AFLP) is a type of fingerprinting technique based on AFLP. This technique is the use of three endonucleases instead of two enzymes as in AFLP. The use of three enzymes provides highly discriminating fingerprinting because the addition of third endonuclease reduces the number of bands amplified. According to 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.

PCR-Single strand conformational polymorphism (PCR-SSCP)

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 the electrophoretic separation of single strand DNA (ssDNA) on non-denaturing polyacrylamide gel. The mobility of ssDNA depends on the secondary structure of ssDNA. The differences in DNA sequence result in a different secondary structure and mobility of DNA although they are the same size. SSCP has high sensitivity in detection of mutations because a single base change of the sequence can cause a radical change in nucleic acid migration (Orita et al., 1989) (Figure 1.8). SSCP bands can be visualized by using autoradiograms

(radioactive detection), or silver staining or fluorescent labels. Because of its high sensitivity, SSCP experimental conditions can be optimized by alteration of the gel temperature or the degree of cross-liking or by the addition 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 (Sheffield et al., 1993).

1.7 Objectives of this dissertation

The aims of this dissertation are to develop species diagnostic markers to distinguish and the first time according to Michener (2007) identified *T. collina* from other stingless bee species in Thailand based on AFLP analysis, to estimate genetic variation of *T. collina* in Thailand using nuclear DNA marker analysis; TE-AFLP analysis and TE-AFLP derived markers, and to estimate mitochondrial DNA diversity of *T. collina* in Thailand using PCR-SSCP analysis

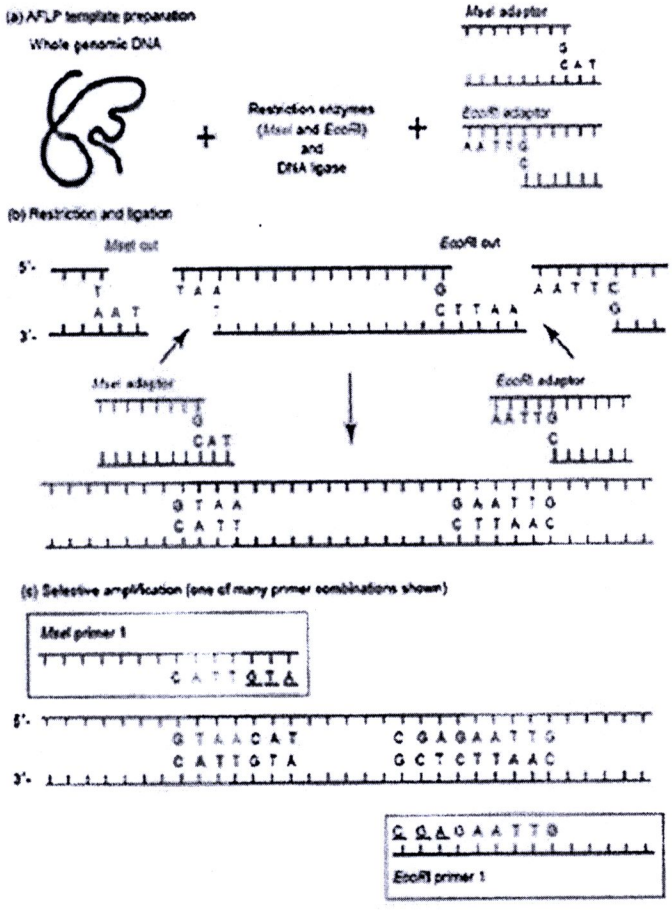


Figure 1.7 AFLP procedures: Genomic DNA is digested with two restriction enzymes and adaptors are ligated to the end of restriction fragments. The adaptor sequence was marked in red and the remaining part of the restriction sequence was marked in blue and green. For PCR amplifications, the primers extending 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.

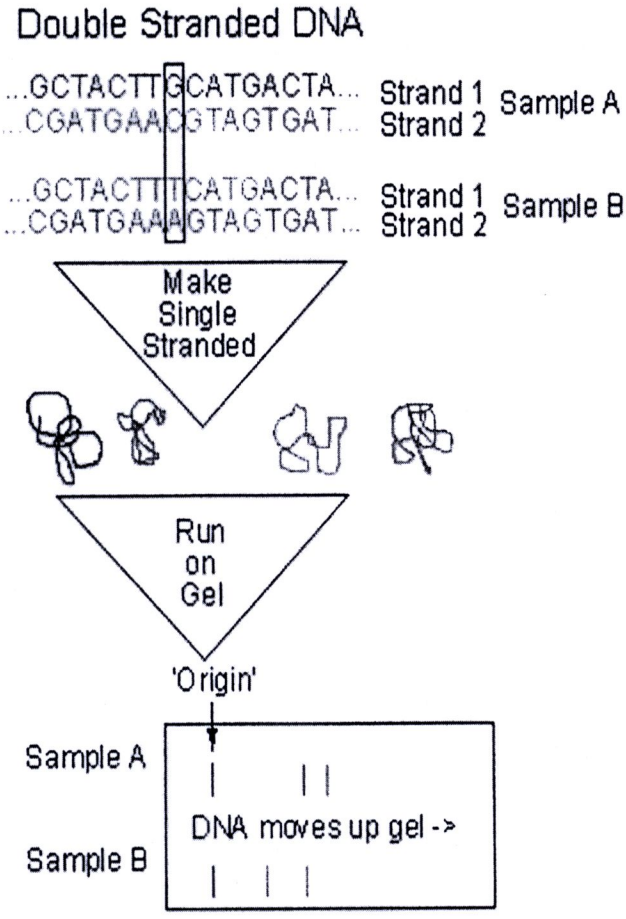


Figure 1.8 Diagram shows the principal diagram of SSCP analysis

(http://www.austmus.gov.au/evolutionary_biology/images/sscp.gif)