CHAPER II

LITERATURE REVIEW

2.1 Evolution of stingless bees (Meliponinae)

The subfamily Meliponinae (stingless bees) is considered first because it is believed to have been the earliest to branch off from less social ancestors and develop highly social behavior (Winston and Michener, 1977). These bees are confined to tropical regions.

In the 1950s a fossil stingless bee from the Miocene (25 million years ago) was found in Mexico, preserved in solidified tree resin known as amber (Roth, 1958); it is closely related to living species and was named *Trigona (Nogueirapis) silacea*.

More recently a fossil female stingless bee was found in amber from coniferous resin 80 million years old, i.e. from the late Cretaceous. This bee is the oldest known specimen of social bees, and its discovery doubles their previously established antiquity. It was named *Trigona prisca*, and it has characteristics known in a living species (Engel, 2000; Grimaldi, 1988; Michener and Grimaldi, 1988).

2.2 Morphological characters of five species of stingless bees

The taxonomic hierarchy of five species of *Trigona* has been recognized as follows (Sakagami *et al.*, 1983; Schwarz, 1939):

Kingdom Animalia
Phylum Arthropoda
Class Insecta
Order Hymenoptera
Family Apidae
Subfamily Meliponinae
Genus *Trigona*
Species *Trigona collina* Smith, 1857
T. fuscobalteata Cameron, 1908
T. laeviceps Smith, 1857
T. terminata Smith, 1878
T. thoracica Smith, 1857

T. collina Smith, 1857

Its nesting sites are usually found in a cavity of underground and termite mounds. The nest entrance tube length and diameter are about 10-15 cm. and 1.5-2.0 cm., respectively. Morphological characters of this species are as followed; body and wing length are each 6.0 mm., body predominantly dark, fore wings basally distinctly darker and contrasting to milky white apical half, veins basally dark brown and apically pale orange (Sakagami, 1985) (Figure 2.4).

T. fuscobalteata **Cameron, 1908**

It is the smallest species in Thailand. Its nesting site is usually found in a cavity of human architecture. The nest entrance tube length and diameter are about 2.0-2.5 cm. and 0.7 cm., respectively. Morphological characters of this species are as followed; body and wing length are each 3.5 mm., light brown abdomen, fore wings rather uniformly transparent or slightly infuscate (Sakagami, 1985) (Figure 2.5).

T. laeviceps **Smith, 1857**

It is the most common species in Thailand. Its nesting site is usually found in a cavity of human architecture. Its nest entrance diameter is about 1.0 cm. Morphological characters of this species are as followed; body and wing length are each 3.5-4.0 mm., body predominantly dark, fore wings rather uniformly transparent or slightly infuscate (Sakagami, 1985) (Figure 2.6).

T. terminata **Smith, 1878**

Its nesting site is usually found in a cavity of trees. The nest entrance tube length and diameter are about 10 cm. and 2.0 cm., respectively. Morphological characters of this species are as followed; body and wing length are each 5.5-6.0 mm., fore wings rather uniformly transparent or slightly infuscate, mesoscutal tomenta usually bright yellow brown and extending to mesoscutellum (Sakagami, 1985) (Figure 2.7).

T. thoracica **Smith, 1857**

It is the largest species in Southeast Asia. Nesting site of this species is usually found in a cavity of tree. Its nest entrance diameter is about 3.5-4.0 cm. Morphological characters of this species are as followed; body and wing length are each 9.0 mm. or more, body extensively reddish, fore wings rather uniformly transparent or slightly infuscate (Sakagami, 1985) (Figure 2.8).

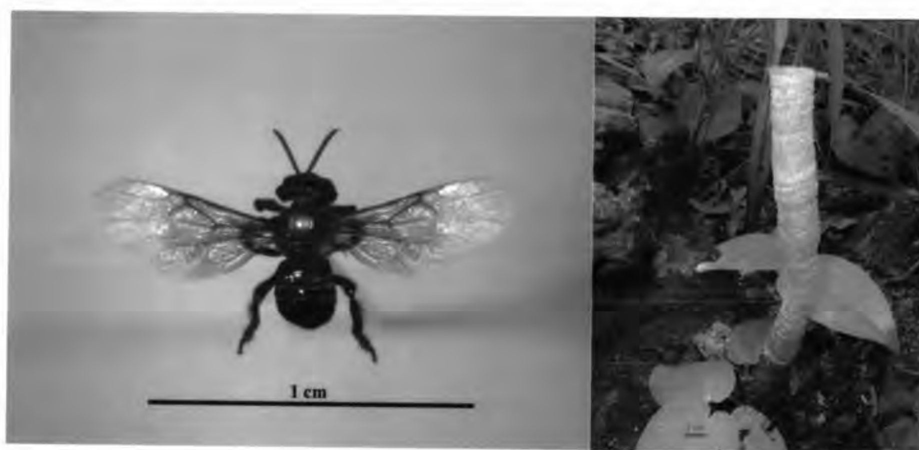

Figure 2.1 *T. collina* and its nest entrance tube.

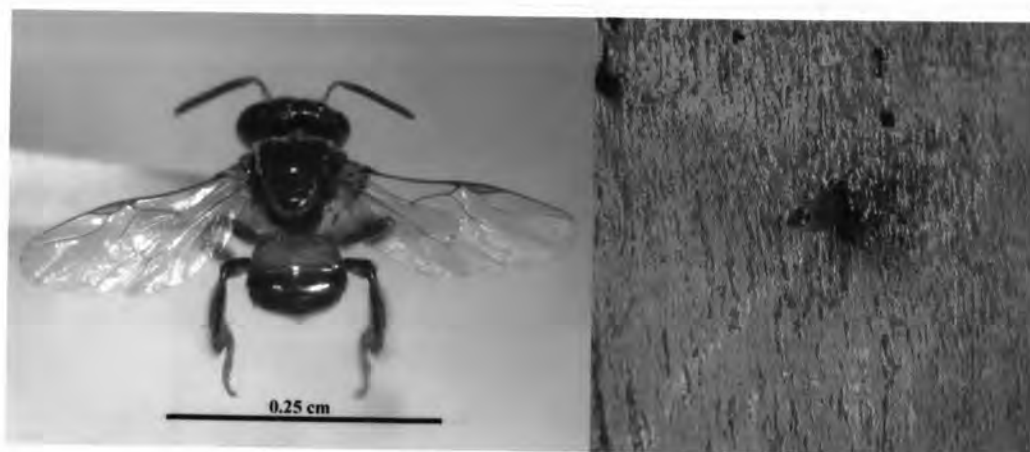

Figure 2.2 *T. fuscobalteata* and its nest entrance tube.

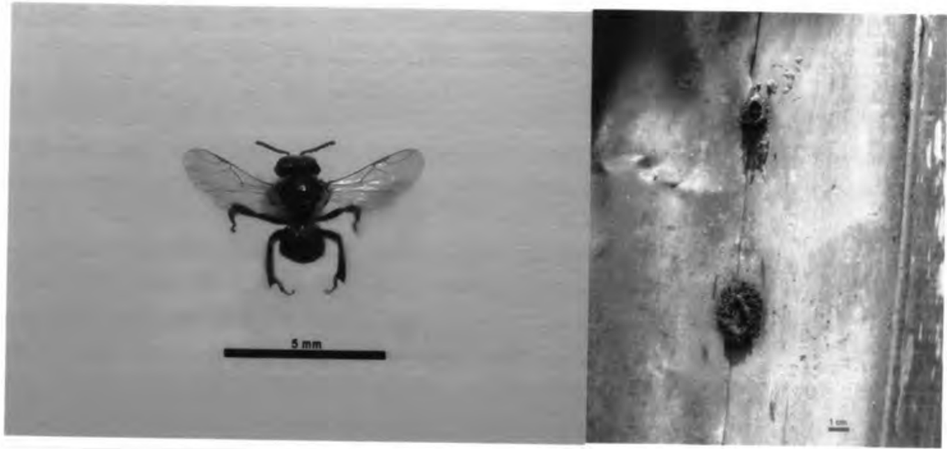

Figure 2.3 *T. laeviceps* and its nest entrance tube.

Figure 2.4 *T. terminata* and its nest entrance tube.

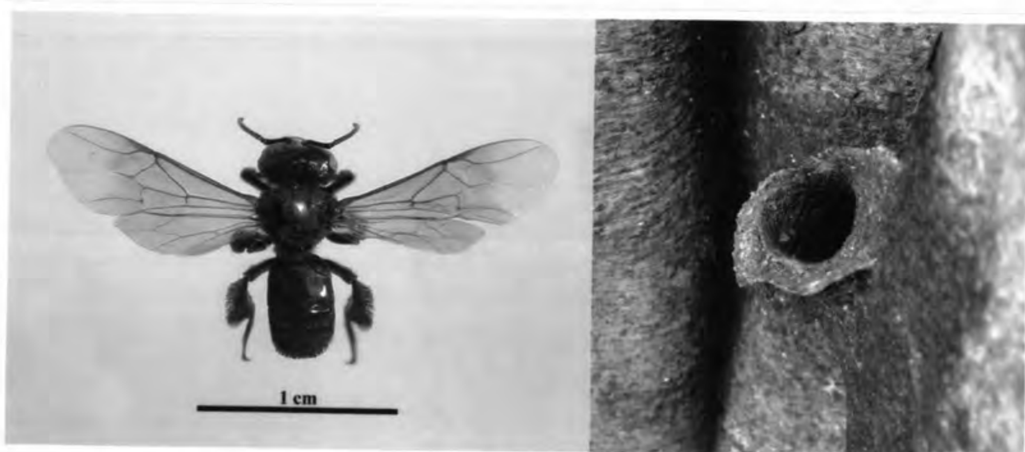

Figure 2.5 *T. thoracica* and its nest entrance tube.

2.3 Biology of stingless bees

Each colony has a single queen, many workers, and drones. Depending on the species, nest populations range in number from 300 to 180,000 (Daly *et al.*, 1998). All stingless bees nests are in a cavity, often underground or in a tree or termite nest (Crane, 1999). The nest is usually made of 5 parts; brood comb, involucrum, store pots, batumen, and an entrance (Figure 2.2). The comb consists of brood cells, in each of which a single young is reared, and surrounded by a sheath of cerumen. Therefore, the cavity where the brood cells are present is called a brood chamber. Cerumen is made of a mixture of wax secreted from the glands on the abdomen of workers and propolis which derived from resins collected from plants. Honey and pollen are stored in the pots quite different from the brood cells. These storage pots are made of cerumen and usually placed above and below the involucrum. The extra space in the cavity is sealed by batumen plates usually made of cerumen and mud. The entrance of the nest is a simple hole and it often extends from the nest as a tube and also continues inside the nest cavity. There are pillars and connectives inside the nest to support all the other structures within the batumen plates (Michener, 1961).

Hymenopterans have a peculiar sex differentiation system where females hatch from diploid, fertilized eggs while males are produced by haploid, unfertilized eggs (Roubik, 1989; Toth *et al.*, 2002).

Reproductive swarming and mating of stingless bees do not proceed in the same way as honey bees, although the timing is similar. When colonies are populous, and drones are present, following is likely to be true in general. Before the issue of a swarm, workers from the parent colony rear new queens; they also select a new nest site for the swarm and transport wax,

propolis, and cerumen to it, and start building the nest. The nest building finished in several weeks. They also carry pollen mixed with honey, in their honey sacs. The queen heading the parent colony tolerates the young queens although the workers treat them as queens. The swarm that issues consists of many young workers and one of the young virgin queen, perhaps the one most attractive to them. When it arrives at the new nest a congregation of drones is waiting, and the queen mates – not necessarily in flight (Carmago, 1972). Meanwhile the young workers of the swarm have started to construct brood cells, and the queen starts to lay eggs in them very soon after mating (Darchen, 1977; Engels and Imperatriz-Fonseca, 1990).

A single queen of honey bees mates many times, while a stingless bees queen mates just once (Contel and Kerr, 1976; Palmer *et al.*, 2002; Peters *et al.*, 1999; Silva *et al.*, 1972).

Figure 2.6 Stingless bee, *T. collina*.

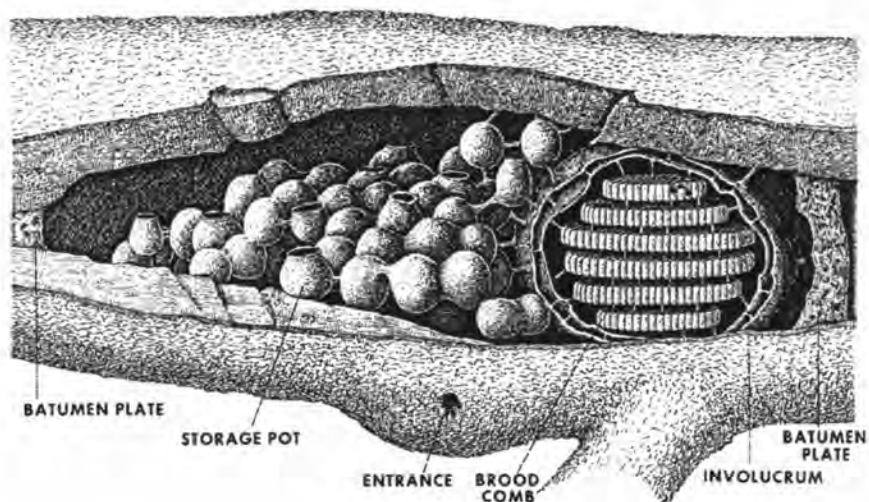

Figure 2.7 Nest of stingless bee, *M. interrupta* (Camargo, 1970).

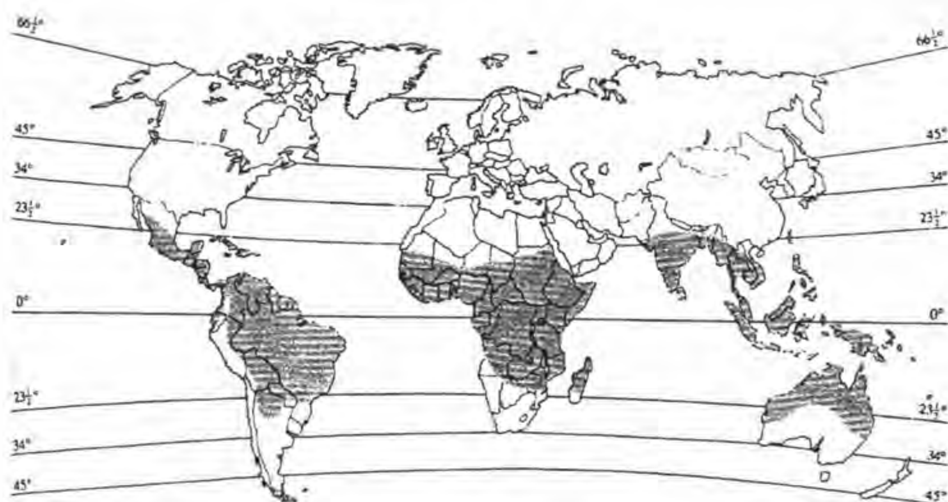

Figure 2.8 Distribution map of stingless bees, Meliponinae (Crane, 1990).

2.4 Mitochondrial DNA polymorphism in stingless bees

DNA analysis is the direct approach to determine genetic variation and relatedness among populations.

Within eukaryotic cells, DNA which found inside nucleus is nuclear DNA and the extranuclear DNA can be found in mitochondria and chloroplast are called mitochondrial DNA and chloroplast DNA, respectively (Hoy, 1994). Nuclear DNA, the combination of paternal genotypes can be used to study genetic divergence in population, and within population structure (Cornuet and Garnery, 1991; Hepburn and Radloff, 1998; Moritz *et al.*, 1986).

In most animals, including stingless bees, mtDNA is maternally inherited without recombination (Singh *et al.*, 1995; Smith and Brown, 1988). It is a small, circular, double stranded DNA molecule, generally about 16,000 bp long. The mitochondrial genome contains 13 protein coding genes, the genes for 22 tRNAs, 2 ribosomal RNAs genes and noncoding region, containing the origin of replication (Crozier and Crozier, 1993). The gene content is highly conserved (Moritz, 1994). There are many identical copies in each cell, while the nuclear genome is usually present in just two copies per cell (one from each parent in a diploid organism) (Moritz *et al.*, 1986; Smith, 1991).

The complete sequence of the honey bee (*Apis mellifera ligustica*) mtDNA reported by Crozier and Crozier (1993), is 16,343 bp long. The gene order for this molecule is shown in Figure 2.9, relative to their positions in the *Drosophila yakuba* map, 11 of 22 tRNA genes are in different positions, but the gene-coding protiens, control region and rRNAs are in the same

relative position. The base composition is 84.9 % A-T, whereas in *D. yakuba* is 78.6 % A-T.

Figure 2.9 Map of circular mitochondrial genome of honey bee *A. mellifera ligustica* (Crozier and Crozier, 1993).

One of the useful properties of mtDNA, is its exclusively maternal inheritance, meaning that all offspring of a single queen will have an identical mtDNA molecule (Hepburn and Radloff, 1998). The mtDNA is not sensitive to environmental selection pressure (Avise, 1994; Franck *et al.*, 2000), so it makes mtDNA an excellent marker for analysis of phylogeographic variation and relationships among populations of bees (Avise, 1994, Franck *et al.*, 1998, 2000; Garnery *et al.*, 1991, 1993; Smith, 1991, 1999). Different portions of mitochondrial genome evolve at different rates. The non-coding region of mtDNA evolves rapidly, while genes (coding regions) evolve very slowly (Smith, 1991), thus different regions can provide the level of genetic variation appropriate to the question.

The Polymerase Chain Reaction (PCR) is a powerful technique that can amplify large amounts of DNA from the small amounts of DNA template (Cornuet and Garnery, 1991). The PCR involves combining a DNA template with oligonucleotide primers, deoxynucleotide triphosphases (dNTPs), and a DNA polymerase, usually *Taq* polymerase, in buffer. Its reactions contain three important steps: denaturation of double stranded DNA template at high temperature, annealing to allow primers to form hybrid molecules at optimal temperature, and extension at working temperature of DNA polymerase (Hoy, 1994). PCR-amplified DNA can be used to detect variation in length (as in microsatellites), restriction site (Hall and Smith, 1991) or sequence (Cornuet *et al.*, 1991; Garnery *et al.*, 1991).

Restriction Fragment Length Polymorphisms (RFLPs) are variations in the length of DNA fragments generated by specific restriction endonuclease. These variations are caused by mutations that create or eliminate recognition sites for the restriction enzymes (Hoy, 1994). MtDNA-RFLP is a popular technique for evaluating levels of genetic variation between species and among and between various species. RFLP analysis provided information about the differences between sequences in mtDNA. It can be used effectively and economically to analyse variation in populations.

RFLP and PCR-RFLP methods have been used to investigate interspecific and intraspecific variation in honey bees. Sylvester and Wongsiri (1993) used nuclear DNA probes from European honey bee (*A. mellifera*) to identify genetic variation in Asian species of *Apis* (*A. cerana*, *A. dorsata*, *A. florea*, and *A. andreniformis*) and suggested that there were interspecies differences among these honey bees. They found that it should be possible to obtain single probe which will simultaneously be useful for

detecting variation within colonies, among populations or races and among species.

Moritz *et al.* (1986) studied on mtDNA polymorphism in *A. mellifera.* They isolated mtDNA from larvae of workers, and digested with restriction enzymes (*Bgl* II). The digestion results showed a mtDNA different among *A. mellifera carnica*, *A. mellifera ligustica*, and *A. mellifera caucasica.*

Cornuet and Garnery (1991) had been reported 3 major lineages of *A. mellifera* from the mtDNA variability investigation, 1) African colonies (lineage A) including *A. mellifera intermissa*, *A. mellifera adansonii*, *A. mellifera scutellata*, *A. mellifera capensis*, and *A. mellifera monticola*, 2) *A. mellifera* colonies (lineage M); *A. mellifera ligustica* and *A. mellifera carnica*, and 3) *A. mellifera caucasica* colonies (lineage C).

Amplification of intergenic CO I-CO II region of mtDNA and followed by digestion with *Dra* I had been reported by Garnery *et al.*, (1993). Twenty one different haplotypes had been found and were used to study interspecific variation in *A. mellifera*. Twelve subspecies of *A. mellifera*; *A. mellifera mellifera*, *A. mellifera iberica*, *A. mellifera sicula*, *A. mellifera intermissa*, *A. mellifera scutellata*, *A. mellifera monticola*, *A. mellifera adansonii*, *A. mellifera caucasica*, *A. mellifera ligustica*, *A. mellifera meda*, *A. mellifera carnica*, and *A. mellifera anatoliaca* were distinguished. This approach is a useful way to study genetic structure and evolution of the large fraction of the species.

Meixner *et al.* (1993) used a set of restriction enzymes; *Acc* I, *Bcl* I, *Bgl* II, *Eco*R I, and *Xba* I to determine variation in mtDNA of *A. mellifera carnica* and *A. mellifera ligustica.* They had found that only *Xba* I showed polymorphism with an asymmetrical distribution between the subspecies.

Deowanish *et al.*, (1996) studied on mtDNA variation of *A. cerana* from Japan, Korea, Taiwan, Vietnam, Thailand, Nepal, and the Philippines using RFLP analysis. Total DNA was digested using ten restriction enzymes. After digestion, DNA fragments on the gel were transferred to nylon membrane and hybridized with labeled probe from $tRNA^{Leu}$ to CO II region. It was found that *A. cerana* from these localities could be discriminated into six groups as: 1) Japan, 2) Nepal, Vietnam and north to central Thailand, 3) Korea-Tsushima, 4) Taiwan, 5) south of Thailand, and 6) the Philippines.

Yong (1986) studied on allozymes variation in stingless bee *T. fuscobalteata* from Peninsular Malaysia. Analysis of genetic variation at 9 gene-enzyme systems comprising 13 loci was carried out. Two gene-enzyme systems were polymorphic in the 20 colonies of studied samples. The number of loci for several enzyme systems appeared to be different from that reported for the Australian stingless bees.

Rocha and Pompolo (1998) studied karyotypes of different *Melipona* species (Apidae, Meliponini) and divided the species into two groups based on chromatin and composition and on heterochromatin content Group I species composes of *M. bicolor*, *M. quadrifasciata*, *M. asilvai*, *M. marginata*, and *M. subnitida* and group II species composes of *M. capixaba*, *M. compressipes*, *M. crinita*, *M. seminigra*, and *M. scutelaris*.

Francisco *et al.* (2001) characterized the mtDNA of five *Plebeia* bee species (*P. droryana*, *P. emerina*, *P. remota*, *P. saiqui*, and *P.* sp.) by RFLP. The mtDNA of each species was analyzed using 17 restriction enzymes and restriction maps were constructed. The enzymes *Bam*H I and *Xba* I cut the mtDNA only once and were species specific of *P. droryana* and *P. remota*, respectively. High level of interspecific variability was detected and estimated of total mtDNA length was about 18,500 bp.

Green (2001) characterized the microsatellite loci of *T. carbonaria*, a endemic stingless bee of Australia. Nine of 10 microsatellite loci were found to be polymorphic amongst 20 unrelated *T. carbonaria* workers, with observed heterozygosity estimated ranging from 0.168 to 0.800. Several primers, distinct polymorphic products were also amplified when DNA from a selection of other bee species was used as the template.

Costa *et al.* (2003) sequenced the 16S rDNA of 34 species from 22 genera of stingless bees. Phylogenetic trees were built using parsimony and maximum likelihood methods. 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.

Weinlich *et al.* (2004) characterized the mtDNA of seven species of *Melipona* (*M. bicolor*, *M. compressipes*, *M. marginata*, *M. melanoventer*, *M. quadrifasciata*, *M. rufiventris*, and *M. subnitida*). The maps were obtained through RFLP and PCR-RFLP using 15 restriction enzymes. The total number of sites mapped ranged from 12 to 19, indicated a high level of genetic diversity among these species.