การตรวจสอบประชากรและโครงสร้างทางพันธุกรรมในผึ้งหลวง *Apis dorsata* Fabricius ในประเทศไทย

นายอัศเลข รัตนวรรณี

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INVESTIGATING POPULATION AND GENETIC STRUCTURE IN GIANT HONEY BEE *Apis dorsata* Fabricius IN THAILAND

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Thesis Title	INVESTIGATING POPULATION AND GENETIC STRUCTURE IN GIANT HONEY BEE *Apis dorsata* Fabricius IN THAILAND
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อัศเลข รัตนวรรณี : การตรวจสอบประชากรและโครงสร้างทางพันธุกรรมในผึ้งหลวง*Apis dorsata* Fabricius ในประเทศไทย. (INVESTIGATING POPULATION AND GENETIC STRUCTURE IN GIANT HONEY BEE *Apis dorsata* Fabricius IN THAILAND) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ. ดร. จันทร์เพ็ญ จันทร์เจ้า, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : Prof. Benjamin P. Oldroyd, Ph.D., ศ. ดร. สิริวัฒน์ วงษ์ศิริ, 151 หน้า.

ผึ้งหลวง *Apis dorsata* เป็นแมลงผสมเกสรที่สำคัญของป่าลุ่มต่ำในแถบภูมิภาคเอเชีย ผึ้งชนิดนี้ ได้รับผลกระทบอย่างหนักจากการล่าและการรบกวนถิ่นอาศัยตลอดแนวของการกระจายตัว ดังนั้น ในวิทยานิพนธ์นี้ได้ทำการตรวจสอบถึงปัจจัยดังที่ได้กล่าวข้างต้นมีผลต่อความเชื่อมโยงและการแปรผันของประชากรผึ้งหลวงในประเทศไทยได้อย่างไร ในบทที่ 3 ทำการบรรยายการวิเคราะห์ทางมอร์โฟเมตรีของรูปร่างของปีกคู่หน้า ซึ่งสามารถนำมาจัดจำแนกผึ้ง 4 ชนิดของทั้งเพศผู้และเพศเมียที่พบในประเทศไทยได้อย่างถูกต้อง ดังนั้น มอร์โฟเมตรีแบบจีโอเมตริกของปีกเพียงอย่างเดียวสามารถใช้จัดจำแนกชนิดของผึ้งเอเชียได้ในทุกสถานการณ์ ส่วนในบทที่ 4 ได้ประยุกต์วิธีดังกล่าวเพื่อใช้ในการจัดกลุ่มของตัวอย่างผึ้งหลวง 73 รัง จาก 31 พื้นที่ ในประเทศไทย การวิเคราะห์พหุตัวแปร (MANOVA) แสดงให้เห็นว่า ไม่มีความแตกต่างอย่างมีนัยสำคัญระหว่างผึ้งที่เก็บตัวอย่างมาจากทั้ง 5 เขตภูมิศาสตร์ ดังนั้น สิ่งนี้เสนอแนะว่า ประชากรผึ้งหลวงที่พบในประเทศไทยเป็นประชากรกลุ่มเดียวกัน ในบทที่ 5 ทำการตรวจสอบโครงสร้างทางพันธุกรรมและความใกล้ชิดกันของรังของประชากรผึ้งหลวง โดยอาศัยการวิเคราะห์ด้วยเครื่องหมายดีเอ็นเอแบบไมโครแซทเทลไลต์ของตัวอย่างผึ้ง 54 รัง จาก 3 กลุ่มรัง ข้าพเจ้าแสดงให้เห็นว่าประชากรมีระดับเฮตเทอโรไซโกซิตีสูง และค่า F_{ST} ระหว่างกลุ่มรังไม่มีความแตกต่างอย่างมีนัยสำคัญจากศูนย์ ($P > 0.05$) การวิเคราะห์นี้ยังแสดงให้เห็นว่าไม่มีรังใดเลยที่มีความสัมพันธ์ในรูปแบบ แม่-ลูก ดังนั้น หากมีการสืบพันธุ์ของผึ้งหลวงเกิดขึ้นภายในพื้นที่ศึกษา รังลูกที่เกิดขึ้นใหม่จะแยกตัวออกไปจากกลุ่มรังเดิม สิ่งนี้เสนอแนะว่าการเพิ่มจำนวนรังของผึ้งหลวงขึ้นอย่างรวดเร็วในช่วงการบานของดอกไม้ มีแนวโน้มสูงสุดที่จะเกิดโดยการอพยพเข้ามาของผึ้งหลวงจากบริเวณอื่นมากกว่าเกิดจากการสืบพันธุ์ในพื้นที่ ในบทที่ 6 ทำการเปรียบเทียบความถี่ของการผสมพันธุ์ของนางพญาผึ้งหลวงและความหลากหลายทางอัลลีลระหว่างรังผึ้งหลวงที่เก็บตัวอย่างมาจากพื้นที่ถูกรบกวนและพื้นที่ไม่ถูกรบกวนในประเทศไทย การวิเคราะห์ด้วยเครื่องหมายดีเอ็นเอแบบไมโครแซทเทลไลต์ของผึ้ง 18 รัง จาก 6 กลุ่มรัง แสดงให้เห็นว่าไม่มีความแตกต่างอย่างมีนัยสำคัญในความถี่ของการผสมพันธุ์ของผึ้งนางพญาทั้งในพื้นที่ถูกรบกวนและพื้นที่ไม่ถูกรบกวน สิ่งนี้เสนอแนะว่าพฤติกรรมการผสมพันธุ์ของผึ้งหลวงสามารถทนทานต่อการเปลี่ยนแปลงของพื้นที่ที่ถูกรบกวนโดยมนุษย์ได้

จึงสรุปว่าทั้ง ๆ ที่มีความกดดันจากการถูกมนุษย์เข้าไปรบกวนภายในพื้นที่อย่างยากเกินป้องกัน แต่ประชากรผึ้งหลวงก็สามารถดำรงอยู่ได้ ผึ้งชนิดนี้ยังคงมีขนาดประชากรที่มีประสิทธิภาพขนาดใหญ่และมีการปฏิสัมพันธ์ระหว่างกลุ่มประชากรของผึ้งหลวงได้อย่างต่อเนื่อง นอกจากนี้ การค้นพบนี้ชี้ให้เห็นว่า การรบกวนถิ่นอาศัยของผึ้งหลวงไม่มีผลต่อความความถี่ของการผสมพันธุ์หรือความหลากหลายทางพันธุกรรม จึงสรุปว่า ผึ้งหลวงในขณะนี้สามารถทนทานต่อถิ่นอาศัยที่ถูกแบ่งเป็นพื้นที่เล็กๆ และการเก็บเกี่ยวน้ำผึ้งตามฤดูกาลได้

สาขาวิชา วิทยาศาสตร์ชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2554 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

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ATSALEK RATTANAWANNEE: INVESTIGATING POPULATION AND GENETIC STRUCTURE IN GIANT HONEY BEE *Apis dorsata* Fabricius IN THAILAND. ADVISOR: ASSOC. PROF. CHANPEN CHANCHAO, Ph.D., CO-ADVISOR: PROF. BENJAMIN P. OLDROYD, Ph.D., PROF. SIRIWAT WONGSIRI, Ph.D. 151 pp.

The giant honey bee (*Apis dorsata*) is an important pollinator of Asian lowland forests. Across its range, the species is impacted by heavy hunting and habitat disturbance. In this thesis, it was investigated how these pressures impact the connectivity and viability of the *A. dorsata* population of Thailand. In Chapter III, a morphometric analysis of forewing shape that can accurately identify any of the four species of honey bee present in Thailand was described, regardless of sex. Thus, geometric morphometry of the wing alone can be used to identify Asian honey bee species in most circumstances. In Chapter IV, the procedure to characterize 73 *A. dorsata* colonies collected from 31 different localities in Thailand was applied. Multivariate analysis of variance (MANOVA) demonstrated no significant differences between the bees sampled from five geographic regions. Therefore, this suggests that the *A. dorsata* populations of mainland Thailand are a single population. In Chapter VI, the genetic structure and colony relatedness of *A. dorsata* populations based on microsatellite analysis of 54 nests in 3 aggregations was examined. Also, it was shown that the population has high levels of heterozygosity and that F_{ST} values between aggregations were not significantly different from zero ($P > 0.05$). The analysis also showed that no colonies were related as mother-daughter. Thus, if reproduction occurred at the study site, daughter colonies dispersed. This suggests that rapid increases in *A. dorsata* colony numbers during general flowering events most likely occur by swarms arriving from other areas rather than by *in situ* reproduction. In Chapter VII, queen mating frequency and allelic diversity between colonies sampled in disturbed and undisturbed areas in Thailand was compared. Microsatellite analysis of 18 colonies in 6 aggregations showed no significant difference in queen mating frequency at disturbed and undisturbed habitats. This suggests that the mating behaviour of *A. dorsata* is robust to anthropogenic changes to the landscape.

It could be concluded that despite the formidable anthropogenic pressures that the *A. dorsata* population endures in Thailand, the species continues to enjoy a large effective population size and has high connectedness. Furthermore, this finding suggests that habitat disturbance has no effect on mating frequency or genetic diversity. It was concluded that *A. dorsata* is currently able to tolerate habitat fragmentation and annual harvesting.

Field of Study : Biological Sciences Student's Signature : .....

Academic Year : 2011 Advisor's Signature : .....

Co-advisor's Signature : .....

Co-advisor's Signature : .....

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CHAPTER I

INTRODUCTION

Deforestation is a particularly severe issue in Southeast Asia, with natural habitats such as lowland rain forests being destroyed at relative rate of 0.71% per year and a degradation rate of 0.42%. This value is higher than those of other tropical regions (Achard *et al.*, 2002). If present levels of deforestation continue unabated, Southeast Asia will lose almost three-quarters of its original forest cover by the turn of the next century (Achard *et al.*, 2002; Sodhi *et al.*, 2004), resulting in massive species declines and extinctions (Brooks *et al.*, 2002). An estimated of 100,000 of every million species could be extinct by 2050 because of this habitat loss (Pimm and Raven, 2000).

Pollinators play an important role in many terrestrial ecosystems as they play a vital role in the maintenance of both wild plant communities and agricultural productivity (Ashman *et al.*, 2004; Klein *et al.*, 2007; Potts *et al.*, 2010). Insects, particularly bees, are the primary pollinators of many agricultural crops and wild plants (Potts *et al.*, 2010; Winfree *et al.*, 2008). Several factors have been proposed to explain the decline of insect pollinators. Habitat destruction and fragmentation are the thought to be the primary causes of insect pollinator declines (Carvalheiro *et al.*, 2010). The broad-scale conversion of primary forests for the production of short-cycle forestry practices, rubber and oil palm plantations, increasing of urban and agricultural areas as well as the use of pesticides is of particular concern for the continued conservation of wild honeybee populations (Kevan and Viana, 2003; Sodhi *et al.*, 2004). All these activities affect wild bee populations by decreasing the number

of mature trees suitable for nesting and food resources (Blanche *et al.*, 2006), and has been causally related to declines of populations and genetic variability in wild bees (Blanche *et al.*, 2006; Oldroyd and Nanork, 2009).

Because of these observed declines of insect pollinators in natural habitats, the development of tools that facilitate the identification of different species in the field has become increasingly important in order to investigate biodiversity thoroughly (Biesmeijer *et al.*, 2006; Francoy *et al.*, 2009a). Because of its high practicability and low cost, morphometric analysis has recently become the most widely used authoritative method for identifying honeybee subspecies and populations (Francoy *et al.*, 2008; Rattanawannee *et al.*, 2010). Morphometric methods involve measuring multiple parts of the bodies of many individuals within a population. This set of characters is known as the standard morphometry (Tofilski, 2008). Recent advances in statistical analysis and image recognition software have made morphometric analysis more precise and practical for discriminating between subspecies and at the population level (Francoy *et al.*, 2008).

Another more recent morphometric method that shows promise is geometric morphometrics, which is based on the description of features being measured in Cartesian coordinates (Slice, 2007). Geometric morphometrics is used in a range of fields, such as evolutionary biology, physical anthropology, paleontology and systematic (Pretorius, 2005; Villemant *et al.*, 2007). This technique has been shown to be sufficiently powerful to solve species level taxonomic problems (Gumiel *et al.*, 2003). Instead of distances and angles, geometric morphometrics uses the coordinates of points, known as landmarks. The selected landmarks are then superimposed by

translation, scaling and rotation. Since after this treatment the landmark configurations differ only in their shape, they can be analyzed by multivariate statistical methods (Rattanawannee *et al.*, 2010; Slice, 2007; Villemant *et al.*, 2007).

The giant honeybee (*Apis dorsata Fabricius*, 1793), keystone pollinator within Asian lowland forest, is distributed over vast geographic areas in South and Southeast Asia, and is found throughout Thailand (Ruttner, 1988). Unlike the single comb of the dwarf honeybees (*A. florea* and *A. andreniformis*) that the crown of the comb always encircles the support, the massive single comb colony of *A. dorsata* is always attached under the surface of a stout tree branch or an overhang of a rock face, and nowadays also sometimes to the eves of buildings or other urban structures (Oldroyd and Wongsiri, 2006; Paar *et al.*, 2004a; Rattanawannee and Chanchao, 2011). The species is heavily hunted throughout its range for honey, wax and brood, providing an important source of household income (Lahjie and Seibert, 1990; Nath *et al.*, 1994; Soman and Kshirsagar, 1991 ; Strickland, 1982). Unfortunately, harvesting is often done at night, resulting in the death of the harvested colony (Oldroyd and Wongsiri, 2006). Additionally, habitat lost and fragmentation is likely to have a significant impact on population viability (Oldroyd and Nanork, 2009; Oldroyd and Wongsiri, 2006; Rattanawannee *et al.*, 2012).

A. dorsata differs significantly in behavior and ecology from other *Apis* species (Moritz *et al.*, 1995; Oldroyd and Wongsiri, 2006). For example, *A. dorsata* colonies are found in dense aggregations (Kastberger and Sharma, 2000; Oldroyd and Wongsiri, 2006; Ruttner, 1988), in which more than 150 colonies may occur on a single tree (Oldroyd *et al.*, 2000), often only separated by only a few centimeters

(Paar *et al.*, 2004a). Colonies often undergo seasonal migration between alternate nesting sites, occupying them for 3–4 months intervals (Paar *et al.*, 2004a). Toward the end of this period, brood rearing ceases and the honey and pollen stores are depleted (Oldroyd and Wongsiri, 2006; Paar *et al.*, 2004a; Ruttner, 1988). Ultimately, the colonies abscond to an alternative nesting site that may be up 200 km distant (Koeniger and Koeniger, 1980). The proximate cause of migration may be related to available food sources, as *A. dorsata* swarms have been observed to travel between habitats with different blooming seasons (Crane *et al.*, 1993; Dyer and Seeley, 1994; Itioka *et al.*, 2001; Koeniger and Koeniger, 1980; Liu *et al.*, 2007; Sheikh and Chetry, 2000; Underwood, 1990). Absconding may also help control levels of the parasitic mite, *Tropilaelaps clareae*, which needs brood in order to reproduce (Paar *et al.*, 2004a). Thus a colony may reduce infestation by this parasite with a period of broodless migration (Kavinseksan *et al.*, 2003; Rinderer *et al.*, 1994). Nesting sites are often reoccupied annually for decades (Oldroyd *et al.*, 2000; Oldroyd and Wongsiri, 2006). Interestingly, queens often return to the same nest site even after an absence of up to 18 months (Neumann *et al.*, 2000; Paar *et al.*, 2000).

All *Apis* species have been shown to have a high but incredibly variable levels of polyandry (Oldroyd *et al.*, 1998; Strassmann, 2001). Virgin queens leave the nest for nuptial flight, mate in flight with several drones (males) at a drone congregation area (DCA) and return to the nest with a semen load. As in other honeybees, in *A. dorsata* mating of virgin queens and drones takes place in the air at some distance from the colony. Virgin queens and drones leave their colonies at dusk, fly to well-defined perennial drone congregation areas (DCAs), and return 15 - 30 min later (Koeniger *et al.*, 1994; Rinderer *et al.*, 1993; Tan *et al.*, 1999). Using microsatellite

DNA markers m it has been shown that *A. dorsata* has the highest observed mating frequency of all the *Apis* species (Moritz *et al.*, 1995; Oldroyd *et al.*, 1996; Wattanachaiyingcharoen *et al.*, 2003). *A. dorsata* queens are known to mate with up to 100 drones over two to three successive mating flights (Paar *et al.*, 2004a; Wattanachaiyingcharoen *et al.*, 2003).

Population structuring can arise via processes such as genetic drift and restricted gene flow, resulting in a heterogenous distribution of genetic variation within and among populations (Frankham, 1995; Frankham *et al.*, 2010). When gene flow is restricted, isolated populations can diverge genetically and suffer loss of heterozygosity and inbreeding depression. In most endangered species, habitat loss and degradation are the main causes of population isolation, decline, or extinction (Foin *et al.*, 1998).

A. dorsata colonies are hunted relentlessly across most if not all of Thailand. Thailand has also suffered significant deforestation (Sodhi *et al.*, 2004) and the remaining forest is often degraded by frequent forest fires that are lit by humans. Widespread use of pesticides and proliferation of street lighting (which attracts bees, often resulting in their death) are likely to have significant impacts on population viability. Therefore, this study aims to determine the geometric morphometry, genetic structure, genetic variation and the level of polyandry within *A. dorsata* colonies in Thailand. We used geometry of forewing venation and microsatellite DNA markers to assess whether habitat disturbance had an effect on population genetic variability and mating frequency in this species.

Objectives

1. To discriminate honeybee species based on their wing venation pattern information.

2. To verify if the *A. dorsata* population in Thailand is composed of distinct geographical subpopulations using geometric morphometric based analysis.

3. To investigate genetic structure and the relatedness of queens within and between aggregated colonies in *A. dorsata.*

4. To determine the genetic variation and level of polyandry within *A. dorsata* colonies in both disturbed and undisturbed areas in Thailand.

Significance

The many anthropogenic influences upon *Apis dorsata* within it‟s range in Thailand is likely to have a significant impact on population viability. Investigation of geometric morphometric variation, genetic structure, genetic diversity, genetic relatedness, and level of polyandry of aggregated colonies in *A. dorsata* in both disturbed and undisturbed areas will indicate whether populations are beginning to show signs of inbreeding or fragmentation, which suggests a prelude to local extinctions. In addition, the results obtained will provide information on basic biology, biodiversity, geographic variation, and genetic relationships among *A. dorsata* populations in Thailand.

CHAPTER II

LITERATURE REVIEW

2.1 The biodiversity of Southeast Asia

According to Mittermeier *et al.* (2004), there are currently 34 „biodiversity hotspots" in the world. Biodiversity hotspots are defined as areas containing high concentrations of endemic species and undergoing immense habitat loss. Southeast Asia overlaps with four of these recognised biodiversity hotspots (i.e. Indo-Burma, Sundaland, Wallacea and the Philippines) (Koh, 2007), each of which has a unique and complex geological history that has contributed to its rich and often unique biota (Sodhi *et al.*, 2004).

The International Union for the Conservation of Nature and Natural Resources Red List lists four vascular plants, one fish, one bird and five mammal species as „extinct" or „extinct in the wild" in Southeast Asia (IUCN, 2006). Moreover, 47 insect species are listed as threatened on the IUCN Red List, which comprises the threatened categories of; critically endangered (CR), endangered (EN) and, vulnerable (VU); (IUCN, 2006). Due to the high proportion of endemic species in Southeast Asia, the loss of species habitats in the area would result in a large number of species extinctions (Koh, 2007). For example, 43% of Sulawesi butterfly all species are endemic (Fermon *et al.*, 2005).

Between 1880 and 1980, Southeast Asia experienced an average loss of 0.3% of its primary forest cover (Billington *et al.*, 1996; Flint, 1994; Koh, 2007). This has

been caused primarily by agricultural expansion and commercial logging (Flint, 1994). Over the past 15 years, the loss of natural forest in the region has continued at an annual rate of 1.3% between 1990 and 2000, and 1.5% between 2000 and 2005 [excluding Singapore and Borneo (< 0.2% of total land area in Southeast Asia)] (Koh, 2007). These deforestation rates are higher than that of other tropical regions, such as Latin America and the Caribbean (1990–2005: 0.5%) and sub-Saharan Africa (1990–2005: 0.7%). In 2005, less than half (42.8%) of the original forests remain intact in the South-east Asia region (Koh, 2007).

2.2 Threats to wild honeybees and consequences of pollinator decline in Southeast Asia

2.2.1 Deforestation and destruction of nesting sites

In study reported of Flint (1994), Southeast Asia showed an average forest loss of 0.3% primarily due to agricultural expansion and commercial logging between 1880 and 1980. Achard *et al*. (2002) showed that Southeast Asian forests are being destroyed at relative net forest loss rate of 0.71% and degradation rate of 0.42%. This is higher than those of other tropical regions. Achard *et al.* (2002) also suggest that if present levels of deforestation continue unabated, Southeast Asia will lose almost three-quarters of its original forest cover by the turn of the next century. Particularly, the island state of Singapore has largely been urbanized except for small forested areas totalling about 20 km^2 or 3% of Singapore"s total land area (Liow *et al.*, 2001). There have also been intensive land-use changes in Peninsular Malaysia since the 1970s and now the total lowland evergreen broadleaf forest (including disturbed natural forests) stands at 31.7%, of which only 9.0% (2.9% of the total land area of Peninsular Malaysia) is protected (Iremonger *et al.*, 1997). In Johor, Peninsular

Malaysia, most of the original Dipterocarp forests have been logged for timber or cleared for plantations (Liow *et al.*, 2001). Sukaimi et al. (1993) reported that palm oil plantations accounted for more than 520,000 ha or 26.3% of the total land area in Johor in 1990.

Little is known about how deforestation will affect native honeybees, including the giant honeybee, *A. dorsata*. Liow *et al.* (2001) revealed that the proportion of stingless bees and honeybees (Hymenoptera: Apidae) is very low in oil palm plantation areas and very high in undisturbed area. This implied that oil palm plantations do not provide a suitable habitat for pollinators such as honeybee species. The palm trees do not produce nectar and the dense leaves render them unsuitable for nest building by *A. dorsata* (Oldroyd and Nanork, 2009). Therefore, deforestation may not only cause decrease of food sources but also destruction of suitable nesting sites for the giant honeybees.

The removal of trees suitable for *A. dorsata* nesting is the one of issues identified to be of high concern for honeybee conservation (Oldroyd and Nanork, 2009). The giant honeybee tends to build the nests in aggregations, sometimes with more than 150 colonies on a single tree (Oldroyd *et al.*, 2000; Oldroyd and Wongsiri, 2006). In addition, *A. dorsata* colonies are often migrate long distances, but return to the previous nesting site year after year (Dyer and Seeley, 1994; Itioka *et al.*, 2001; Koeniger and Koeniger, 1980; Neumann *et al.*, 2000; Oldroyd *et al.*, 2000; Paar *et al.*, 2004a; Paar *et al.*, 2000). Therefore, the felling of major bee trees may have a significant impact on *A. dorsata* populations.

2.2.2 Brood and honey hunting

Honey hunting is the general term given to the collection of honey from wild honeybee colonies (Oldroyd and Wongsiri, 2006). Traditional honey hunting is an important aspect of the life of many Asian people. Honeybees have been hunted by humans for more than 40,000 years (Crane, 1999) and still remains widely practiced throughout the region (Oldroyd and Nanork, 2009). The existing method of honey hunting giant honeybees is similar across Asia. Hunting the two giant honeybee species, *A. dorsata* and *A. laboliosa* is ruthless destructive process, and usually involves burning the bees with a smouldering torch of tightly-bound brush (Crane, 1999; Lahjie and Seibert, 1990; Oldroyd and Nanork, 2009; Tsing, 2003). In traditional honey hunting practices, moonless night is preferred by many hunters. The smoking is considered crucial to disorientate the bees and reduce the number of stings received. After smoking off the bees from the comb, most honey hunters cut down the whole comb, destroying all the brood and food stores (Oldroyd and Wongsiri, 2006). A large number of young bees, some hundreds of adult bees and drones are also killed while hunting honey (Joshi and Gurung, 2005; Oldroyd and Nanork, 2009; Tsing, 2003). Many queens must be lost during these harvest methods, and their colonies perish along with them(Oldroyd and Nanork, 2009). Therefore, honey hunting may kill a large proportion of the colonies within colony an aggregation in a single night (Oldroyd and Nanork, 2009; Oldroyd and Wongsiri, 2006).

As honey hunting is a traditional activity that has been practiced for thousands of years, it would appear that populations of Asian honeybees are capable of sustaining a certain level of human predation (Oldroyd and Wongsiri, 2006). But as

human populations increase, and additional pressures such as deforestation, habitat fragmentation and the commercialization of honey hunting grow ever stronger, it is now unclear whether rates of honey hunting will remain sustainable into the future. Efforts are therefore needed to develop a non-destructive method of honey hunting for conservation of wild honeybee populations. Additionally, raising awareness among local communities, government and non-government institutions about the role and importance of wild honeybees is equally vital for conserving these species (Joshi and Gurung, 2005).

2.2.3 Competition with the introduced European honeybee, *A. mellifera*

European honeybee, *A. mellifera*, is endemic to Europe, Africa and Western Asia with a large number of well identified regional subspecies (Franck *et al.*, 1998; Ruttner, 1988). The species have been introduced worldwide for an apicultural industry (Dietemann *et al.*, 2009). Furthermore, *A. mellifera* has been wildly used as a model organism for study in social behavior (Solignac *et al.*, 2007).

Since *A. mellifera*, is not native to Asia, it is unlikely that it is capable of displacing native Asian honeybee species within their natural ranges (Oldroyd and Wongsiri, 2006). Oldroyd and Nanork (2009) reviewed some reasons why *A. mellifera* has not colonised tropical regions. *A. mellifera* has difficulty regulating their rate of brood production in tropical regions due to the comparatively small variation in day length between the different seasons. Thus, they rarely reach swarming strength (Rinderer, 1988). Additionally, feral *A. mellifera* is commonly infested and killed by the parasitic mites *Tropilaelaps clareae* and *Varroa destructor* (Oldroyd and Nanork, 2009; Rinderer *et al.*, 1994). In spite of this, direct competition between

European honeybees and their Asian counterparts for floral resources and the European bee‟s potential as a vector for diseases may have an effect on *A. dorsata* populations in the wild.

2.2.4 Honeybee diseases and parasites

Honeybee colonies are often targeted by numerous pathogens (viruses, bacteria, fungi and protozoa), and parasitic insects and mites (Le Conte and Navajas, 2008; Morse and Nowogrodzki, 1990.; Oldroyd and Wongsiri, 2006). Normally, honeybee populations are not threatened by the parasites and pathogens with which they co-evolved (Oldroyd and Nanork, 2009). However, the introduction of novel parasites and pathogens by introduced honeybee vectors can have an effect on honeybee populations. Allen *et al.* (1990) reported that *A. laboriosa* populations in Nepal were infected by European foulbrood (*Mellisococcus pluton*), which they attributed to environmental stress due to deforestation. Moreover, *A. mellifera* colonies have been introduced into many countries in Southeast Asia. Thus, the anthropogenic movement of honeybee population between countries increasingly exposes wild populations to novel pathogens and parasites that they have no resistance (Oldroyd and Nanork, 2009).

The *Tropilaelaps* mite is a external parasite of the honeybees. Its primary host is the giant honeybee, *A. dorsata* (Laigo and Morse, 1968) and is found throughout the entire distribution range of *A. dorsata* (Matheson, 1996). It is also associated with other Asian honeybees, including *A. laboriosa*, *A. cerana* and *A. florea* (Delfinado-Baker *et al.*, 1989; Delfinado-Baker *et al.*, 1985). Parasitism of colonies by these mites can cause abnormal brood development as well as death of both brood and bees,

leading to colony decline and collapse. In response to an infestation, whole colonies of bees will often abscond from the hive (Fries *et al.*, 2006). The introduction of *A. mellifera* into the distribution range of *A. dorsata* has provided the mite with a new host and increased the mite population in the wild (Kavinseksan *et al.*, 2003; Oldroyd and Nanork, 2009; Oldroyd and Wongsiri, 2006).

The greater wax moth, *Galleria mellonella*, is the most serious pest of honeybee colonies worldwide. Its larvae cause considerable damage to bee colonies by feeding on its wax combs and cells containing brood, honey and pollen. Wax moth larvae destroy the comb structure by forming tunnels inside the comb. Jyothi *et al.* (1990) reported that old and weak colonies of *A. dorsata* showed varying degree infestation of *G. mellonella*, but young colonies were relatively free from wax moth attack. They also suggested that a high rate of infestation by *G. mellonella* during July and August may result in premature migration of *A. dorsata* colonies in the Bangalore area, India. Moreover, the deserted combs with a high incidence of infestation may serve as a source of infection for new colonies (Jyothi *et al.*, 1990; Oldroyd and Nanork, 2009).

Tingek *et al.* (2004) reported that a Conopid fly, *Physocephala parralleliventris* Kröber (Diptera: Conopidae) parasitizes *A. dorsata*, *A. cerana*, and *A. koschevnikovi* in Borneo. This fly grasps foraging bees in flight and deposits a larva on the integument. Then, the larva penetrates the bee cuticle and consumes the bee from the inside. Oldroyd and Nanork (2009) also suspected that this fly or close relate species occurs in Thailand, because they have seen fly larvae in the abdomens of *A. florea* workers.

2.2.5 Pesticides

Many commercial fruit crops, such as longan (*Dimocarpus longan*), litchi (*Litchi chinensis*) and citrus are major sources of nectar and therefore highly attractive to honeybees (Oldroyd and Nanork, 2009; Oldroyd and Wongsiri, 2006). Sun flower (*Helianthus annuus*) is a crop, which is heavily produced throughout Thailand and provides a rich potential source of pollen and nectar. However, these commercial crops are regularly sprayed with insecticides, especially during the flowering period. Oil palm (*Elaeis* spp.) orchards are also regularly exposed to insecticides, and this may be a contributing factor for the low honeybee numbers within oil palm crops (Oldroyd and Nanork, 2009). Regulation of pesticide use is lax in some Southeast Asia countries, and can increase the possibility of honeybee pesticide exposure (Oldroyd and Wongsiri, 2006).

2.2.6 Impact of climate change

Climate influences flower development as well as nectar and pollen production, both of which are directly linked with a colonies‟ foraging activity and development (Winston, 1987). A major potential effect of climate change on honeybee populations stems from changes in the distribution of flower species on which they depend on for food (Thuiller *et al.*, 2005). Rain can also have an effect on nectar collection. For example, when acacia (*Acacia* spp.) flowers are washed by rain, they are no longer attractive to honeybees as their nectar stores become too diluted (Conte and Navajas, 2008). Likewise, an overly dry climate can reduce the production of flower nectar available for honeybees to harvest, since many plant flowers produce no nectar when the weather is too dry.

According to the Intergovernmental Panel on Climate Change Fourth Assessment Report (2007), since 1906, there has been a 70% increase in greenhouse gas emissions worldwide. There has been an average global temperature increase of 0.74 °C, and a decreased level of precipitation in the Southeast Asia region. Wild fires and drought are anticipated to occur more frequently in Southeast Asia with increased global warming (Duncan *et al.*, 2003). Extreme wild fire events combined with deliberate fire setting associated with burn agriculture (Brown, 1998) are changing the structure of plant communities across Asia (Taylor *et al.*, 1999).

Another way climate change effects plant communities is through changes in the flowering period (Conte and Navajas, 2008; Oldroyd and Nanork, 2009). This change could destabilize relationships between flowers and pollinators. In the tropical region, climates may evolve towards more distinct seasons with dry periods (Conte and Navajas, 2008). In this case, Asian honeybees would need to rapidly step up their honey-harvesting strategy to amass sufficient stores to survive periods without flowers. Or else they could develop a migration strategy, as has *A. dorsata*. This honeybee species readily migrates in response to the changing seasons, flowering patterns or disruption. Therefore, if the flowering period is changed, it will impact the timing and movement patterns of *A. dorsata* populations in Southeast Asia.

Some known pathogens of honeybee have been distributed worldwide by anthropogenic movement. They include: *Varroa destructor* in of *A. mellifera* and *A. cerana*; bacteria that cause American and European foulbrood (*Nosema apis* and *N. Cerana*); and numerous viruses affecting honeybees. These pathogens tend to have different haplotypes of varying virulence. Climate change combined with extreme

environments can encourage the transfer of these haplotypes to wild honeybee populations including *A. dorsata* (Oldroyd and Wongsiri, 2006).

2.3 Conservation genetics

According to the report of the International Union for Conservation of Nature (IUCN) (in Frankham, 1995), there are three levels of biodiversity need to conserve: genetic diversity, species diversity, and ecosystem diversity. Genetics is directly involved in the first two levels (Frankham, 1995). Conservation genetics is the use of genetic theories and methods to aid conservation and minimize the risk of extinction in threatened species (Frankham *et al.*, 2010).

There are several major genetics issues that aids conservation biology (Frankham, 2003; Frankham *et al.*, 2010):

(a) Fragmentation and restriction of gene flow: the fragmentation of natural habitat is generally considered to be a major threat to many species (Kajtoch, 2011). Habitat fragmentation has the potential to impede dispersal of animals and plants, thereby decreasing gene flow and colonization (Frankham, 2003; Frankham *et al.*, 2010; Keyghobadi, 2007; Leidner and Haddad, 2011). Therefore, the information regarding the extent of gene flow among populations is critical to determine whether a species requires prevention for inbreeding and loss of genetic diversity (Frankham *et al.*, 2010).

(b) Increasing inbreeding and loss of genetic diversity: low genetic variation and inbreeding in population minimizes the ability of species to adapt in response to

environmental change (Frankham, 2003; Haig, 1998). In particular, these effects depress the reproductive fitness in small population (Hedrick, 2001).

(c) Understandings in species biology: the basic species biology are important in conservation (Frankham *et al.*, 2010) such as reproductive systems, dispersal and migration. These are often difficult to determine directly in rare species. Many researches show that the using genetic markers can be resolved for conservation biology (Frankham, 1995; Frankham, 2003; Keyghobadi, 2007; Leidner and Haddad, 2011).

2.4 Standard morphometric and geometric morphometric analysis

Morphometric methods are based on multiple measurements of various body parts across many individuals within population. For morphometric study in honeybees, there are 2 criteria which must be considered: (1) Means of colony characters are used as variable parameters in statistical analysis but not characters of individual bees; (2) Numeric data, resulting from measurements and analyzed with statistical method, are used for classification (Ruttner, 1988).

Daly *et al.* (1982) reported the first successful use of digital measurements to examine honeybee morphometry, which significantly reduced the time required for the accurate measuring of the characters. This set of characters is now known as the standard morphometry (Tofilski, 2008). Furthermore, the recent advances in statistical analysis and image recognition software have made morphometric analysis more precise and practical for discriminating between subspecies and at the population level (Francoy *et al.*, 2008).

Crewe *et al.* (1994) showed that 10 morphological characters were enough to discriminate *A. mellifera capensis* and *A. m. scutellata.* Furthermore, Tilde *et al.* (2000) determined the morphometric variation of *A. cerana* in the Philippines using 39 morphometric characters. They collected honeybee samples throughout the Philippine archipelago. They found that bees from Palawan were unequivocally distinct and were separated from the others. Also, bees from the Philippine Islands still showed a high degree of variation. Bees from Luzon were obviously differed from those from Visayas and Mindanao. Moreover, among bees within Luzon, the bees from the highland were obviously different from those from the lowland. They have now been placed into separate groups. The result was supported by Hepburn *et al.* (2001) that they measured 54 quantitative morphological characters of 3,704 *A. cerana* workers from 279 colonies that randomly collected from 64 localities in southern Himalayan. They reported that among 4 morphoclusters, 2 morphoclusters are further subdivided into 3 biometric subgroups. In addition, they found that bees from the west to the east decrease in size but bees from higher altitude are bigger in size.

In Thailand, Limbipichai (1990) successfully used standard morphometry to verify the geographic subpopulations of *A. cerana* in Thailand. He showed that these subpopulations come into contact at the Isthmus of Kra, which is a biogeographic transition area (12° N latitude). Moreover, single morphocluster group of the dwarf honeybee *A. florea* (Chaiyawong *et al.*, 2004) and *A. andreniformis* (Rattanawannee *et al.*, 2007) in Thailand were reported using standard morphometric analysis. These morphormetric results were supported by mitochondrial DNA sequence based analysis (Deowanish *et al.*, 1996; Hepburn *et al.*, 2001; Nanork, 2001; Rattanawannee *et al.*, 2007; Smith *et al.*, 2000).

Recently, a new morphometric method that base on the description of the shape in Cartesian coordinates is geometric morphometrics (Slice, 2007). Geometric morphometric approaches have been shown to be sufficiently powerful to solve species and population level taxonomic problems (Gumiel *et al.*, 2003). Unlike standard morphometric analysis that uses distances and angles, geometric morphometrics base on the description of shape in Cartesian coordinates (Francoy *et al.*, 2008), known as landmarks. The selected landmarks are then superimposed by translation, scaling and rotation (Tofilski, 2008). After superposition the landmark configurations differ only in shape, and can be analyzed by multivariate statistical methods (Tofilski, 2008; Zelditch *et al.*, 2004).

Several studies have demonstrated that geometric morphometric analysis of forewing can be used to identify some bee species, including within bumble bees (Aytekin *et al.*, 2007), stingless bees (Francisco *et al.*, 2008; Francoy *et al.*, 2009a) and honeybees (Rattanawannee *et al.*, 2007). Francoy *et al.* (2006) showed that the geometric morphometric of a single wing cell can be used to discriminate three racial groups of *A. mellifera* (Africanized, Italian and Carniolan) with a fidelity level of nearly 99% of the individuals. Tofilski (2008) demonstrated that geometric morphometrics of forewing is marginally more reliable than standard morphometry for the discrimination of honeybee subspecies.

2.5 Microsatellites DNA markers

Among the molecular techniques available, microsatellites have been widely used in studies of the population genetics of various groups of animals including honeybees (Pamilo *et al.*, 1997). Microsatellites are tandem sequence repeats of

motifs with 1–6 bases randomly distributed along the euchromatic regions (Arias *et al.*, 2006). Microsatellite loci are considered codominant, selectively neutral, highly polymorphic, and show Mendelian inheritance (Moritz *et al.*, 2003). Due to these characteristics they have been extremely useful in analyses of relatedness, parentage, intraspecific variation, species hybridization, population dynamics, gene mapping and phylogeographic studies (Arias *et al.*, 2006). Microsatellites have been used also to evaluate the impact of reproductive behavior, social structure, and dispersion in endangered populations (Beaumont and Bruford, 1999). At the population level, the use of multiple highly variable loci brings to the precise analysis of the structure of natural populations including the detection of population growth and decline, bottlenecks in the population history, migration and gene flow. Population sizes are however often difficult to determine and are usually estimated indirectly based on allelic diversity. If the family of structured populations is studied, it is possible to infer the number of families from relatedness estimates among the sampled individuals (Kraus *et al.*, 2005b).

In haplodiploid social insect species such as honeybees, microsatellites show high alleles difference and high heterozygosity. This technique therefore is reliable and precise for determining the genetic structure of honeybee both in colony and population levels (Estoup *et al.*, 1994).

2.6 Extreme polyandry and genetic variation in eusocial insect colonies

While many Apinae species are monandrous or have low levels of multiple mating (Palmer *et al.*, 2001), a few have evolved relatively high levels of polyandry (Oldroyd *et al.*, 1998; Palmer and Oldroyd, 2000). In particular, all *Apis* species has

been shown extremely high but variable levels of polyandry (Oldroyd *et al.*, 1998; Strassmann, 2001). Virgin queens leave the nest for nuptial flight, mate in flight with several drones (males) at a drone congregation area (DCA) and return to the nest with a semen load.

Several hypotheses have been proposed to explain the evolution of multiple mating in the genus *Apis*. The ‚genetic variance" (GV) hypothesis is the most plausible explanation of extreme levels of polyandry observed in honeybees (Keller and Reeve, 1994; Oldroyd *et al.*, 1997; Oldroyd *et al.*, 1996; Palmer and Oldroyd, 2000). The GV hypothesis states that queen and colony fitness is increased by the greater intra-colonial genetic diversity that is the consequence of polyandry (Oldroyd *et al.*, 1997; Pamilo, 1993).

Two broad categories of the GV hypotheses can be determined (Palmer and Oldroyd, 2000). The first set suggests that genetic diversity within the worker population leads to greater colony fitness because colonies comprised of particular combinations of worker genotypes are fitter than colonies comprised of just one genotype (Fuchs and Schade, 1994; Oldroyd *et al.*, 1997). There are 4 hypotheses to explain why intra-colonial genetic variance confers selective advantages on queens, colonies, and individuals (Keller and Reeve, 1994; Oldroyd *et al.*, 1996).

(1) Increased expression of caste (Crozier and Page, 1985) or task polymorphism (Oldroyd *et al.*, 1992a; Oldroyd *et al.*, 1993; Oldroyd *et al.*, 1992b).

(2) Increased the range of environments the colony can tolerate (Fewell and Page, 1993; Oldroyd *et al.*, 1997; Oldroyd *et al.*, 1992a; Oldroyd *et al.*, 1993; Oldroyd *et al.*, 1992b; Oldroyd *et al.*, 1996).

(3) Increased colonial resistance to parasites and pathogens (Schmid-Hempel, 1995; Schmid-Hempel and Crozier, 1999).

(4) Increased the frequency of favorable heterotic allelic interaction within individual workers (Palmer and Oldroyd, 2000; Rinderer *et al.*, 1998).

The second set of hypothesis arises from the haplo-diploid reproductive system of Hymenopterans (Palmer and Oldroyd, 2000) and relates to sex determination and sex ratios (Boomsma and Ratnieks, 1996; Moritz *et al.*, 1995; Oldroyd *et al.*, 1997). These hypotheses suggest:

(1) Reduced conflict between queens and workers over preferred sex ratios (Boomsma and Ratnieks, 1996; Pamilo, 1993; Queller, 1993)

(2) Reduced variance in the reproduction of diploid males among colonies (Crozier and Pamilo, 1996; Ratnieks, 1990).

Extremely high levels of polyandry in honeybees have been wildly reported (Palmer and Oldroyd, 2000). Estoup *et al.* (1994) reported that the *A. mellifera* queens mated approximately 7 to 20 times, with an average effective paternity frequency, *m*, of 13.6 ± 2.3 (SE). Observed mating frequency in other cavity nesting species *A. cerana*, *A. koschevnikovi*, and *A. nigrocincta* ranged from 14 to 27 (Oldroyd *et al.*, 1998), 16 to 26 (Rinderer *et al.*, 1998), and 42 to 69 (Palmer *et al.*, 2001), respectively. *A. cerana* and *A. koschevnikovi* appear to be more similar to *A. mellifera*. *A. nigrocincta* queens have a surprisingly high effective mating frequency. The lowest effective mating frequency was reported in dwarf honeybees. In *A. florea*, 13-19 patrilines have been observed (Palmer *et al.*, 2001), while *A. andreniformis*

queens mated approximately 10-23 times (Oldroyd *et al.*, 1997; Takahashi *et al.*, 2008). Like other species in the genus, the mountain giant honeybee *A. laboriosa* shows high mating frequencies, with an effective paternity frequencies varied from 13.63 to 31.57 (Paar *et al.*, 2004b).

The observed mating frequency in *A. dorsata* was first reported by Moritz *et al.* (1995). Three polymorphic microsatellite loci (A14, A76, and A88) were used to estimate the number of patrilines in *A. dorsata* from both aggregated and single colonies. They reported that the mean number of matings was 30.17 ± 5.98, with an average effective paternity frequency of 25.56 ± 11.63. Similarly, Oldroyd *et al.* (1996) showed that *A. dorsata* queens mated approximately 13 to 39 times, with an average effective paternity frequency of 19.96 ± 6.63. Interestingly, Wattanachaiyingcharoen *et al.* (2003) reported that *A. dorsata* queens have the highest number of mating frequency recorded for any social insect. They found the number of patrilines per colony ranged from 47 to 102, with an average effective paternity frequency of 63.0 ± 5.70.

2.7 Colony relatedness and kin structure in honeybee colonies

Following a paternity analysis, there are three parameters that are usually determined to examine the kin structure of the honeybee colony (Boomsma and Ratnieks, 1996; Oldroyd and Wongsiri, 2006; Tarpy and Nielsen, 2002). First, mating frequency, *k*, is the number of copulations by the queen (Oldroyd and Wongsiri, 2006). This parameter can be estimated by examining the number of different patrilines detected in a sample of workers from a particular colony. Second, the effective mating frequency, *m*, is the reciprocal of the sum of squared proportional

paternity (Boomsma and Ratnieks, 1996; Pamilo, 1993). That males contribute equally to the paternity of offspring, hence $m = k$ (Oldroyd and Wongsiri, 2006). Third, the parameter r is the average relatedness of workers, which vary between 0.25 and 0.75 (Oldroyd and Wongsiri, 2006).

The relatedness among individual workers in honeybee colonies varies according to their father. Workers of the same father are more related to each other as super-sister (r = 0.75), while individuals of different father are related as half-sisters (r = 0.25) (Barron *et al.*, 2001; Oldroyd and Osborne, 1999; Oldroyd and Wongsiri, 2006). In polyandrous social insects colonies, kin selection theory (Hamilton, 1964) predicts that reproductive conflicts should arise both among workers and between workers and their queen over the parentage of males (Barron *et al.*, 2001; Oldroyd and Osborne, 1999; Ratnieks and Reeve, 1992). An offspring worker in the species with colonies headed by a single queen mated to more than two males, is more related to her own son (r = 0.5), then to the son of a super-sister (r = 0.375), or to her brothers (the son of her maternal queen) (r = 0.25) and least so to the son of a half- sister (r = 0.125) (Barron *et al.*, 2001; Oldroyd and Osborne, 1999) (Figure 2.1). An average relatedness between pairs of workers approaches r = 0.25 as the number of subfamilies increases within a colony (Crozier and Pamilo, 1996).

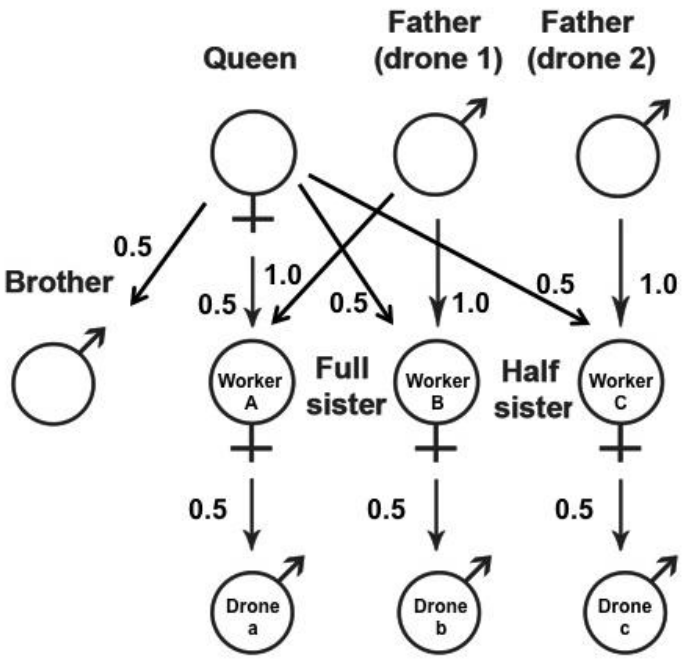

Figure 2.1 Relatedness of the individual member in honeybee colony. The queen relate to her daughter worker by r = 0.5. The relatedness among workers varies according to paternity. Worker A and B are full sister as they are the same father (Drone 1). While, worker C is the half sister to worker A and B as they are the different fathers.

2.8 The giant honeybee, *A. dorsata* Fabricius, 1793

2.8.1 Genetic diversity in *Apis dorsata* and distribution of the species

A magnificent populous nest of a giant honeybee is truly awe-inspiring sight. Thousands of individual are visible at once (Fig. 2.2). The individual workers of *A. dorsata* are at approximately 17 mm long (Fig. 2.3). Unlike the comb of dwarf honeybees (*A. florea* and *A. andreniformis*), in which the crown of the comb always encircles the support, the massive single comb colonies of *A. dorsata* are always attached undersurface of a stout tree branch or an overhang of a rock face, but sometimes to the eves of buildings or other urban structures (Oldroyd and Wongsiri, 2006; Paar *et al.*, 2004a). Where *A. dorsata* nests are found in trees, the diameter of supporting branches varies from 12-30 cm (Morse and Laigo, 1969) or much larger (Oldroyd and Wongsiri, 2006). A slightly sloping branch is preferred (Tan, 2007). The width of *A. dorsata* combs is from 43–162 cm, and the height from 23–90 cm (Fig. 2.2) (Tan, 2007). In the large colonies, the number of individual workers can be over 50,000 (Morse and Laigo, 1969). About 3–4 weeks after nesting, a colony of *A. dorsata* stores on average approx. 4 kg of honey in the comb with the highest recorder being 15.7 kg (Tan, 2007). Honey is stored in the top corner of the comb in an area about 10-20 cm in a large nest (Oldroyd and Wongsiri, 2006).

Apart from their large size, the giant honeybees are distinguished from all other honeybees by their wings, which are fuscous, and quite hairy (Oldroyd and Wongsiri, 2006). The forewing and hind wing length of *A. dorsata* worker are 12.96 and 8.91 mm, respectively (Tan, 2007). Furthermore, Tan (2007) also showed the

development time from egg to adult that is 19.7 days for workers, 23.7 days for drones, and 16.5 days for queen bees.

Neither Ruttner (1988) nor Engels (1999) identified *A. dorsata* as distinct from *A. laboriosa*. However, some authors have reviewed evidence to support designating the two giant honeybees as different species. First, Underwood (1990) reported the mating flights of Nepalese *A. laboriosa* drones between 12:30 and 14:30 hr. Whereas, *A. dorsata* drones mating flights invariably occur just after dusk, between 18:15 and 18:50 hr (Koeniger and Koeniger, 1980; Rinderer *et al.*, 1993). Second, the communication dance performed by *A. dorsata* is strikingly different from that of *A. laboriosa* (Oldroyd and Wongsiri, 2006). Kirchner *et al.* (1996) reported that *A. laboriosa* shows dances silently, whereas *A. dorsata* produces dance sounds. Third, Arias and Sheppard (2005) revealed that DNA sequence divergence between *A. dorsata* and *A. laboriosa* is 10.6-11.5 percent, which strongly supports species status. Finally, Raffiudin and Crozier (2000) showed 100% of Bayesian consensus trees support for grouping *A. dorsata* as a group distinct from *A. laboriosa*, supporting recognition of *A. laboriosa* as a valid species. Moreover, some distinguishing morphological characters are also given in the review of the biology of Asian honeybees of Oldroyd and Wongsiri (2006).

In addition to *A. dorsata* and *A. laboriosa*, another species of giant honeybees have been added by Lo *et al.* (2010). Based on Bayesian and maximum parsimony phylogenetic trees, their analysis supports recognition of the giant Philippines honeybee, *A. breviligula* Maa, 1953, as a separate species from the more broadly distribution lowland *A. dorsata*. *A. breviligula* is found northwest of the Merrill line

in Luzon in the Philippines (Oldroyd and Wongsiri, 2006). This giant is strikingly different from *A. dorsata* owing to black rather than yellow coloration of the abdomen and never forms colony aggregations as do *A. laboriosa* and *A. dorsata* (Lo *et al.*, 2010; Morse and Laigo, 1969; Oldroyd and Wongsiri, 2006). Therefore, three species of giant honeybee in subgenus *Megapis* of genus *Apis* have been recognized.

The distribution of *A. dorsata* is ranged over vast geographic area in South and Southeast Asia (Fig. 2.4). To the west, *A. dorsata* occurs not farther than the Indus River. To the east, the *A. dorsata* areas are all the Philippines, on the other side of the Wallace line (Oldroyd and Wongsiri, 2006; Ruttner, 1988). The giant honeybee is reported to be present in altitudes up to 1000-1700 m, or even up to 2000 m during migration (Ruttner, 1988).

Figure 2.2 A massive single comb nest of *Apis dorsata* attached under the eaves of a building at Mae Fah Luang University, Chiang Rai, Thailand.

Figure 2.3 The size dimorphism between castes of the giant honeybee, *A. dorsata* is smaller pronounced than other *Apis*. (A) A queen (red arrow) is surrounded by her workers. Her thorax is slightly broader than that of workers. (B) Drones have larger eyes (blue arrow) but their abdomen is slightly shorter than workers. Photo by S. Wongvirat.

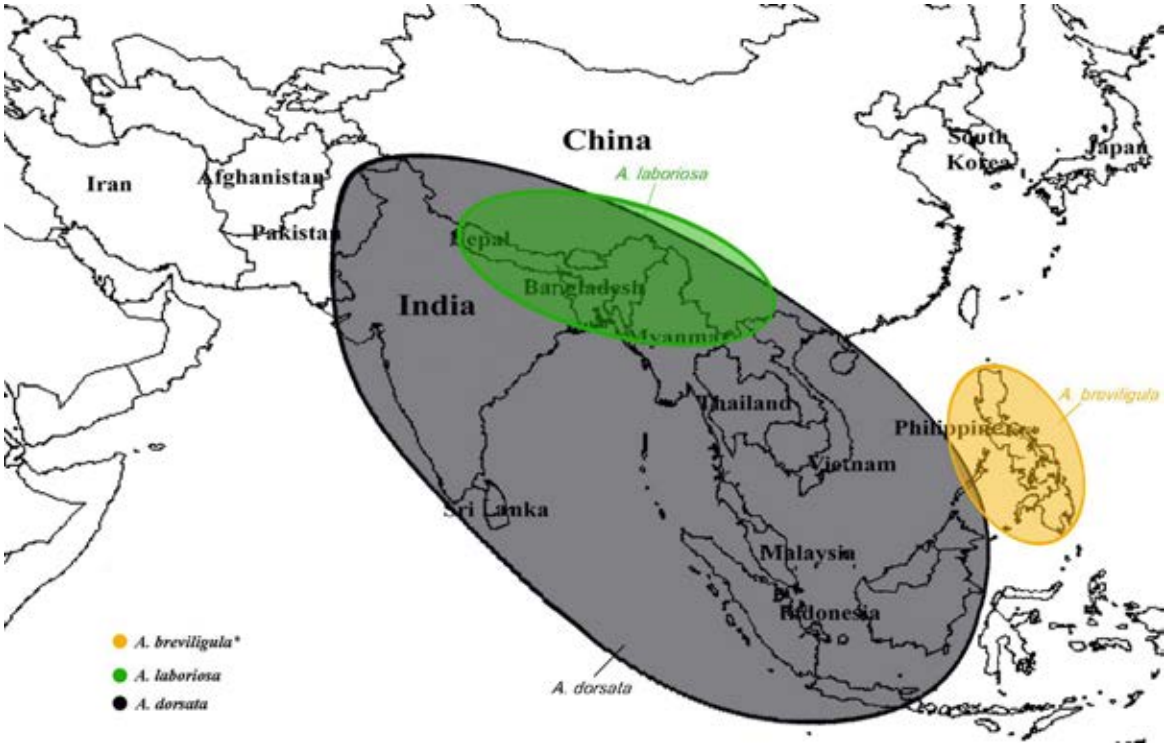

Figure 2.4 Approximate distribution of three giant honeybee species of genus *Apis* (amended in accordance with Ruttner, 1988; Oldroyd and Wongsiri, 2006; Lo *et al*., 2010).

Indeed, this notion is in close agreement to Insuan *et al.* (2007) where PCR-RFLP of mitochondrial DNA combined with nuclear microsatellite DNA markers, which found no significant population differentiation among *A. dorsata* populations throughout Thailand. They demonstrated that one mitochondrial type present at frequencies between 0.92 to 1.00 is found throughout the country and nuclear microsatellites revealed no evidence of population structuring at broad scales (Insuan *et al.*, 2007).

Comparing the degree of genic differentiation between aggregations on disturbed and undisturbed habitats showed no significant difference, implying that there is no barrier to gene flow and geographical obstacles seems to play no role in directing migrating swarms. Rattanawannee *et al.* (2012) found no genetic differentiation in *A. dorsata* populations at local scales in north-western Thailand. These results support the theory that the long distance migratory behavior of *A. dorsata* allows it to tolerate habitat fragmentation (Rattanawannee *et al.*, 2012). Furthermore, there was no genetic differentiation among aggregations, suggesting that matings may occur between aggregations (Rattanawannee *et al.*, 2012), and that migrating swarms that colonize a bee tree come from diverse sources (Paar *et al.*, 2004a).

2.8.2 Behaviour and Ecology of *A. dorsata*

The common giant honeybee (*A. dorsata*) and the closely related species, the mountain giant honeybee (*A. laboriosa*) show substantial differences in behavior and ecology from the other *Apis* species (Morse and Laigo, 1969). First, colonies are found in aggregations (Kastberger and Sharma, 2000; Oldroyd and Wongsiri, 2006; Ruttner, 1988), in which more than 150 colonies may occur on a single tree (Fig. 2.5), under the eves of buildings or rock face (Oldroyd *et al.*, 2000; Paar *et al.*, 2004a; Paar *et al.*, 2000). Second, colonies often undergo seasonal migration between alternate nesting sites. In this population, their nest sites tend to be occupied for 3–4 months. Toward the end of this period, brood rearing stops and the honey and pollen stores are depleted (Fig. 2.6) (Paar *et al.*, 2004a; Ruttner, 1988). Ultimately, the colonies abscond to alternative nest sites that may be 200 km distant (Koeniger and Koeniger,

1980). The proximate cause of migration may be related to available food sources. *A. dorsata* swarms have been observed to travel between habitats with different blooming seasons (Crane *et al.*, 1993; Koeniger and Koeniger, 1980; Mahindre, 2000). Absconding may also help control levels of the parasitic mite, *Tropilaelaps clareae*, which needs brood in order to reproduce (Kavinseksan *et al.*, 2003; Paar *et al.*, 2004a). Thus a colony may reduce infestation by this parasite with a period of broodless migration (Rinderer *et al.*, 1994). Third, Nesting sites are reoccupied year after year over periods of several decades or more (Oldroyd *et al.*, 2000). Interestingly, some returning colonies find their way back to exactly the same nesting site they occupied the previous season (Neumann *et al.*, 2000; Paar *et al.*, 2000).

Figure 2.5 An aggregation of *A. dorsata* colonies on a single tree in Chiang Mai, Thailand. Photo by Wongvirat, S.

Figure 2.6 Abandoned nests within a colony aggregation of *A. dorsata* in Sakonnakorn, Thailand.

2.8.3 Mating behaviour of *A. dorsata*

Mating between honeybee queens and drones takes place mid-flight at a specific area known as the "Drone Congregation Area" (DCA) where many drones from nearby colonies gather (Baudry *et al.*, 1998; Oldroyd and Wongsiri, 2006). When a queen approaches a congregation area, several drones copulate with the queen in quick midair (Gries and Koeniger, 1996), and then die immediately. The DCA‟s persist year after year whether or not a queen is present (Baudry *et al.*, 1998; Gries and Koeniger, 1996). Although DCA‟s and the mating behaviors of queens and

drones have been extensively studied, it is still unclear why drones choose particular areas in which to congregate and how queens locate these areas. Baudry *et al.* (1998) examined the parentage of 142 *A. mellifera* drones collected in a DCA near Oberusel, Germany. They reported that the composition of the DCA contained equal representation from the local colonies, approximately 240 in number. They suggested that most colonies within the recruitment parameter of a DCA delegated equal proportions of males to a DCA. Furthermore, they also found that the relatedness among the drones mated to a common queen is also very low, indicating maximize the genetic diversity among the different patrilines (paternal sub-families) of a colony.

In two Asian honeybees, *A. cerana* and *A. koschevnikovi*, the DCAs occur in the open air close to trees and under cover of trees, respectively (Koeniger and Koeniger, 2000; Oldroyd and Wongsiri, 2006). While, the DCAs of *A. dorsata* are found under the canopy of high emergent trees, approx. 10 – 35 m high above the ground (Koeniger *et al.*, 1994). At present, there is no information available on the drone congregation areas of two dwarf honeybees, *A. florea* and *A. andreniformis*.

Mating time in *Apis* species seem to provide a major behavior barrier increasing reproductive isolation (Koeniger and Koeniger, 2000). Observation of drone flight in *A. dorsata* showed that the mating flight of this species takes place shortly after dusk (Koeniger *et al.*, 1994; Koeniger and Wijayagunesekera, 1976; Rinderer *et al.*, 1993; Tan *et al.*, 1999). Rinderer *et al.* (1993) observed the drone flight time of *A. dorsata* in Thailand, and found that drones take mating flights after sunset between 18.15 h and 18.45 h. A similar time pattern of drone flights in *A. dorsata* was reported in Borneo (Koeniger *et al.*, 1994).

Mating flight of virgin queens of *A. dorsata* are reportedly shorter than that of drones. The queens return from mating flight after 15 - 30 min (Koeniger *et al.*, 1994; Rinderer *et al.*, 1993; Tan *et al.*, 1999). This short mating period is similar to those observed in *A. andreniformis*, *A. florea*, and *A. koschevnikovi* (Koeniger and Koeniger, 2000).

2.8.4 The economic value of *A. dorsata*

2.8.4.1 Pollination services

Up to a third of the food we eat is derived from plants that are either dependent on or benefit from insect pollination (Oldroyd and Nanork, 2009), especially by honeybees (Richards, 2001). The European honeybee, *Apis mellifera*, is the most economically valuable pollinator of agricultural crops worldwide (Conte and Navajas, 2008). However, in most areas of Southeast Asia there is no significant pollination industry. Thus, insect pollinated crops are therefore completely reliant on wild bees, particularly honeybees, for their pollination (Oldroyd and Wongsiri, 2006; Rahman and Rahman, 2000; Rajagopal *et al.*, 1999).

Because of their dance language and wildly foraging length, honeybees can rapidly identify and exploit recourses over a wide range and in a coordinated manner (Beekman *et al.*, 2008; Beekman and Lew, 2008; Dornhaus *et al.*, 2006). Therefore, the honeybees are far better at long-distance dispersal of pollen than solitary arthropods (Oldroyd and Wongsiri, 2006). Circumstantially, honeybees may partially compensate for fragmentation by bridging the gaps between isolated plant communities (Johnson and Steiner, 2000). Corlett (2001) reported that 86% of plant

species in the extremely disturbed area of Hong Kong are visited by *A. cerana*. This honeybee species may not be the traditional pollinator of all these plant species, but it appears to maintain Hong Kong‟s diverse flora in the face of such high levels of habitat degradation.

The lowland forests of Asia are dominated by the trees of the family Dipterocarpaceae. The pollination ecology of this forest type is characterized by infrequent general flowering events that occur every 4-5 years, in which most trees flower simultaneously at a random time of year (Ashton, 1988; Sakai *et al.*, 1999). Most tree species of the canopy layer mass-flower over a several month long period. These forests appear to be adapted for pollination by migratory honeybees that can rapidly increase in population size by both reproductive and migratory swarming (Oldroyd and Nanork, 2009). No other potential pollinators (birds, bats or stingless bees) share the twin characteristics of migration and high rates of reproduction that are necessary for rapid population build up (Oldroyd and Wongsiri, 2006). In addition, because individual trees of each species tend to be widely spaced in Dipterocarp forests, pollen must be transferred over long distances (Itioka *et al.*, 2001). This requires an animal vector that has species fidelity while foraging, a large foraging range, and the tendency to visit multiple trees, either as individual foragers, or via transfer of pollen among foragers in the nest. The giant honeybee has all these characteristics (Oldroyd and Nanork, 2009). In addition, Momose *et al.* (1998b) reported that *A. dorsata* is one of the major pollinators of several dominant components of forest canopy in Southeast Asian lowland Dipterocarp forests. Dipterocarp forests are one of the richest terrestrial ecosystems in the world (Momose *et al.*, 1996; Momose *et al.*, 1998a; Momose *et al.*, 1998b; Sakai *et al.*, 1999).

In southern India, Dyer (1985) reported that *A. dorsata* workers forage all night under a full moon, and are major pollinators of an endangered dry forest tree, *Pterocarpus santalinus* (Fabaceae), whose flowers open at midnight (Rao *et al.*, 2001). However, at Lambir, Sarawak, bees were found foraging on certain Dipterocarps species both before sunrise (05:00–06:00 h) and after sunset (18:00–20:00 h) (Momose *et al.*, 1998b), yet not during the middle of the night The apparent adaptation of nocturnal-flowering canopy trees to a migratory bee species that is usually absent from the site suggests that the relationship between *A. dorsata* migration and mass-flowering episodes in the lowland Dipterocarp forest of Sarawak is an ancient one (Corlett, 2004). In total, *A. dorsata* pollinate at least 15 species of emergent and canopy trees at Lambir (Momose *et al.*, 1998b). It has also been reported as one of the dominant pollinators of the upper strata in rainforest in peninsular Malaysia (Appanah, 1993) and for canopy dipterocarps in Sri Lanka (Dayanandan *et al.*, 1990). Because many large-sized canopy tree species produce a large number of flowers and consequently yield a large amount of nectar and pollen in a general flowering event (Momose *et al.*, 1998b), they would provide *A. dorsata* with abundant food. Therefore, decline in *A. dorsata* populations caused by over hunting or deforestation may lead to significant changes in the pollination ecology of these forests (Itioka *et al.*, 2001; Oldroyd and Nanork, 2009; Oldroyd and Wongsiri, 2006).

2.8.4.2 Harvesting of produce

Due to the large amounts of honey (up to 45 kg; Ruttner, 1988) that can be stored by a colony of *A. dorsata*, wild giant honeybee nests are frequently harvested throughout its range. For many people in Southeast Asia, honey (and

sometimes brood and wax) harvested from *A. dorsata* nests provide an important source of household income (Fig. 2.7) (Joshi and Gurung, 2005; Oldroyd and Nanork, 2009; Paar *et al.*, 2004a).

The honey of *A. dorsata* in Thailand is harvested during the dry season between January and April (Wongsiri *et al.*, 2000). Honey collected during this time of year will keep much longer as the moisture content is lower making fermentation less likely. The bee hunter makes a smoker out of creepers wrapped in leaves. The smoker is wider at one end than the other. Air is blown into the narrower end to keep it smouldering as the bees are smoked off the comb, which is then harvested either in its entirety or for only the honey comb. After loosing its comb, the harvested colony will often abscond within the following few days, migrating back to nearby mountains from which it spent the wet season nesting in (Waring and Jump, 2004).

In Thailand, brood and honey from *A. dorsata* nests is offered for sale in the local markets. The brood is sold for approx. 150 – 200 baht ($US 5.00 – 6.66) per kilogram. Whereas honey is sold for approx. 200 – 250 baht ($US 6.67 – 8.33) per litre. The average honey yield from each colony of *A. dorsata* is approximately five litres (Tan, 2007; Waring and Jump, 2004). Wax from the combs is melted down and sold form approx. 60 baht ($US 2) per kilogram, the bee wax is used for making candles (Oldroyd and Wongsiri, 2006).

Figure 2.7 (A) *A. dorsata* nest and honey for sale in local market in Chantaburi province, Thailand. (B) Honey harvested from *A. dorsata* provides an important source of household income.

CHAPTER III

Gender and Species Identification of Four Native HoneyBees (Apidae: *Apis*) in Thailand Based on Wing Morphometic Analysis

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ABSTRACT

Morphometrics is a relatively powerful analytical tool for the identification of distinct species and subspecies of bees. Typically, within honeybees (Apidae: *Apis*), morphometric analysis has been used to differentiate the groups and species by using multiple body characteristics. However, these procedures are time-consuming for the suitable preparation of the samples and orientating each part into the correct plane for accurate measurement. Here, I was able to discriminate four honeybee species based on their wing venation pattern information only. Geometric morphometric measurements of the right forewing of drones and workers of *Apis andreniformis* Smith, *Apis cerana* F., *Apis dorsata* F., and *Apis florea* F. were analyzed. The results demonstrated that the patterns of forewing venation of native Thai honeybees between sexes in the same species were more closely related to each other than to honeybees of the same sex in another species. The wing venation pattern carried sufficient information to discriminate 99% of the individuals, and so the geometric morphometric analysis of the wing alone could be used to identify Asian honeybee species in most circumstances. In addition, the sex of the individual did not obstruct identification. Therefore, morphometric analysis of a single wing might be a useful tool for biodiversity studies of bees and other insects or fossil records. Many insect fossils are only known from a wing, including several fossil honeybees.

INTRODUCTION

Four honeybees in genus *Apis* are native to Thailand: *Apis andreniformis* Smith, *Apis florea* F., *Apis dorsata* F., and *Apis cerana* F. The first three species are opennesting honeybees, whereas *A. cerana* is a cavitynesting species (Oldroyd and Wongsiri, 2006). Products of these honeybees, such as honey, brood, propolis, and wax, among others, are considered as being of local economic importance (Oldroyd and Wongsiri, 2006). In addition, honeybees play an important role as pollinators for many economic crops and trees in the Southeast Asian lowland forests (Itioka *et al.*, 2001; Oldroyd and Nanork, 2009).

Because of the decline of insect pollinators in natural habitats, the development of tools that facilitate the identification of each species in a field study is important to investigate their biodiversity (Biesmeijer *et al.*, 2006; Francoy *et al.*, 2009a), as long as such tools are accurate. In honeybees, such a comparison of the noninvasive morphometrics with the frequently invasive molecular and thorough morphometric based analysis is available allowing an assessment of its likely validity. Thus, in honeybees, not only morphometry but also genetic analyses have been used to determine the variation within and between populations and species (Francoy *et al.*, 2009b). Considering mitochondrial sequence based phylogenetic analyses, *A. andreniformis* in Thailand forms a single group, a result that is also supported by the morphometric analyses of 24 characters (Rattanawannee *et al.*, 2007). In addition, by similar morphometric and mitochondrial sequence phylogenetic based analyses, no genetic population differentiation was found in *A. dorsata* (Insuan *et al.*, 2007),

whereas, in contrast, three different geographic populations of *A. cerana* were genetically differentiated (Songram *et al.*, 2006).

Although mitochondrial DNA has been widely used in determining the genetic diversity of honeybees, it also has been reported that nuclear DNA sequences, and especially intron sequences, may be informative. When three major clusters of honeybees (giant bees, dwarf bees, and cavity-nesting bees) were subject to nuclear and mitochondrial DNA sequence based phylogenetic analyses, the groupings were still in accord with the morphometric analysis (Arias and Sheppard, 2005).

Typically, morphometric characters are derived from various to all parts of the insect body, and this can lead to time-consuming specimen preparation and measurement procedures to ensure each diverse character is accurately and consistently measured. Within honeybees, Daly et al. (1982) successfully used digital measurements to investigate honeybee morphometry, a method that significantly reduced the time required for measuring and analyzing the data.

It is possible that for some genera wing morphology alone could be used to identify insects to the species or even subspecies level. Thus, several researches have focused on wing information to discriminate insect species or even subspecies and populations. In bees, it has been found that wing pattern morphometrics can give good identification rates (Francoy *et al.*, 2008; Mendes *et al.*, 2007).

The geometric morphometric analysis of forewings is a new methodology that has been applied to the identification of stingless bees (Francoy *et al.*, 2009a), to resolve taxonomic problems in bumble bees (Aytekin *et al.*, 2007), and to identify honeybee subspecies (Francoy *et al.*, 2006). In addition, relative warp analysis of the forewing of the stingless bee *Plebeia remota* (Meliponini) showed no evidence of

gene flow between two different populations collected from various regions of Brazil (Francoy *et al.*, 2008), whereas it was also sufficient to determine differentiating subpopulations of the stingless bee *Nannotrigona testaceicornis* Rondani from a single locality (Mendes *et al.*, 2007). Indeed, Francisco et al. (2008) showed that geometric morphometric based analysis of the forewings was more efficient than traditional morphometrics in assessing the variability within different populations of the stingless bee *P. remota.*

It is widely known that drones (males, typically haploid) and workers (diploid females) of honeybees have different behaviors and flight activities during their life span. This could lead to differentiation in the patterns of wing venation. The objective of this study was to examine the morphology of the forewing of drones and workers of four native honeybee species in Thailand so as to ascertain whether this information was sufficient to and reliable enough in the discrimination between species and between sexes within a species.

MATERIALS AND METHODS

Sample Collection and Measurement

Adult drones and workers of *A. andreniformis* (May 2009), *A. florea* (February 2009), *A. dorsata* (May 2009) and *A. cerana* (January 2009) were sampled from different locations in Thailand (Table 3.1). Thirty drones and 30 workers from each of three colonies of the four species were collected, one colony per locality. The right forewing of each bee sample was dissected, mounted on a microscope slide and photographed by a digital camera attached to a stereomicroscope. Fifteen drones and

15 workers from each colony were analyzed, giving a total of 90 bees and 45 members of each sex per species being analyzed. Fifteen landmarks were plotted at the junctions of the forewing venation (Fig. 3.1) based upon published methods (2009a; Francoy *et al.*, 2006). Measurements of angles between the landmarks, cell area, continuous curvature and arc length were made by using the tpsDig2 version 2.04 software (Rolhf, 2005a). The Cartesian coordinates of the landmarks were then aligned and a partial warps analysis was performed using the tpsRelw version 1.42 software (Rolhf, 2005b). These software packages are available at http://life.bio.sunysb.edu/morph/.

Table 3.1 Sampling locations of four honeybee species in Thailand

Sampling locations	**Coordinated**	***A. andreniformis***	***A. florae***	***A. dorsata***	***A. cerana***
Chiang Rai	20º 17.16′ N 99º 48.88′ E			X[a]	
Sakon Nakhon	16º 52.23′ N 103º 56.28′ E				X
Maha Sarakham	16º 10.39′ N 103º 18.15′ E				X
Bangkok	13º 44.10′ N 100º 31.51′ E		X		
Samut Songkhram	13º 22.56′ N 99º 57.37′ E		X	X	X
Chanthaburi	12º 36.33′ N 102º 09.59′ E	X		X	
Kanchanaburi	14º 13.32′ N 98º 54.40′ E	X	X		
Phetchaburi	12º 47.87′ N 99º 27.46′ E	X			

[a]X, collected from a colony within that province.

Data Analysis

A principal component analysis (PCA) of the Cartesian coordinates of drones and workers of four honeybee species was conducted following previously published methods (Amssalu *et al.*, 2004; Francoy *et al.*, 2006; Rattanawannee *et al.*, 2007). A stepwise analysis was carried to determine the classification functions. Then, canonical analysis and a cross-validation test were calculated to check the accuracy of the equations in identifying the sample groups (Francoy *et al.*, 2009a). In the cross-validation nalysis, each case (bee) was classified by the functions derived from all cases other than that case. Furthermore, Mahalonobis square distances between the bee sample groups (drones and workers of four species) were calculated as reported previously (Francoy *et al.*, 2009a). After extraction of the measurements, all statistical analyses were performed using the SPSS version 10.0 (SPSS, Chicago, IL).

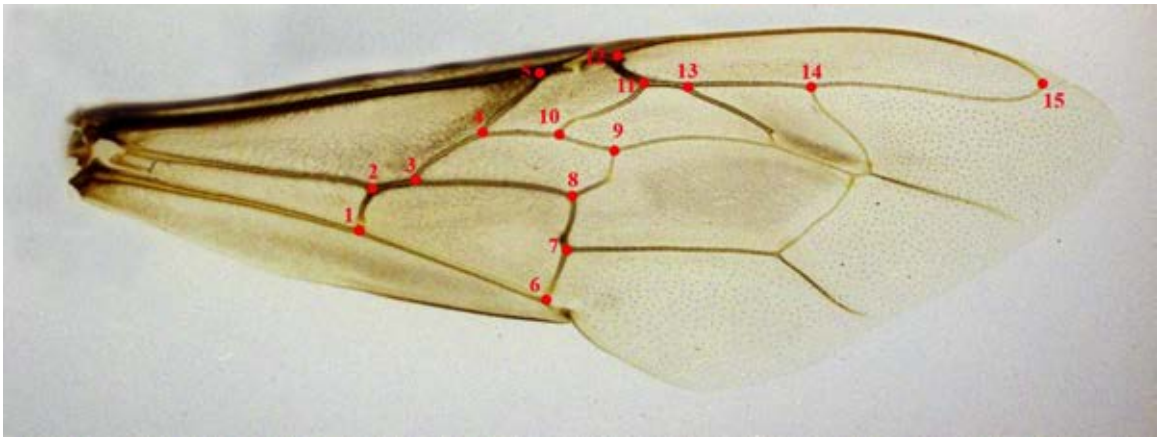

Figure 3.1 Right forewing from a (haploid) drone *A. dorsata*. The circles indicate the respective position of each of the plotted landmarks.

RESULTS

The PCA of the Cartesian coordinates obtained from the right forewing of drones and workers of four honeybee species gave five eigenvalues. More than one value could explain 74.5% of the variation among the groups. The variable 4y mostly influenced the first factor and explained 30.0% of the variability among the groups. In contrast, the variable 2x mostly influenced the second factor and explained 16.7% of the variation.

Based on the positions of the groups in the PCA plots of factor 1 (30.0%) and factor 2 (16.7%), the gender and the species were fairly well distinguished (Fig. 3.2). Considering the drones, each species was well resolved except for the proximity between the boundaries of *A. andreniformis* and *A. florea* drones. Likewise for the workers, all four species were well separated except for one *A. florea* worker that was close to the *A. andreniformis* workers (and within the drone domain). Thus, reasonably good species separation with each sex was available. However, in contrast, confusion (overlap) among the groups was found between drones and workers of the same species, and they overlapped between the different sexes of different species. Therefore, this morphometric approach could separate the species of four Thai native honeybees relatively well and was further improved if the gender (drone or worker) was known as well.

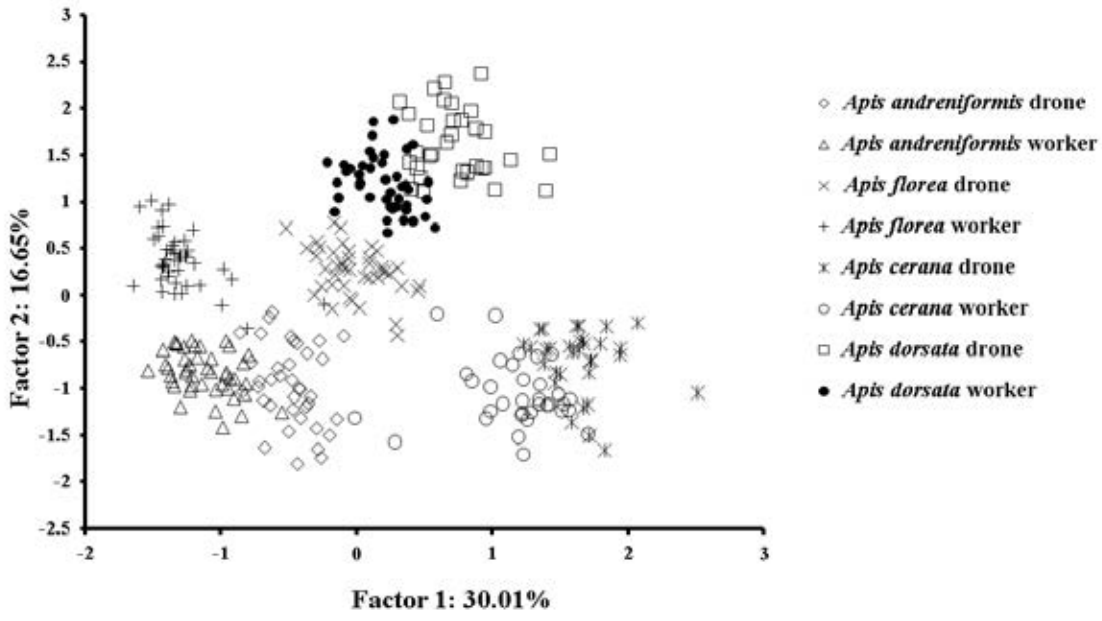

Figure 3.2 Scatterplot of the PCA of drones and workers of four native honeybee species.

All the partial warps of the Cartesian coordinates extracted from the wing were significant ($\alpha = 0.05$) for the eight honeybee groups (drones and workers of each of the four species). Multivariate analysis of variance (MANOVA) revealed that the honeybee groups were significantly different (Wilk‟s $\lambda = 0.0001$; $P < 0.0001$). The cross-validation test correctly identified 99.0% of the individuals to each respective group. Therefore, the misidentifications were not found among the species groups.

There was a significant discrimination of workers among the four bee species when only workers were analyzed ($\alpha = 0.05$), a notion that is supported by the MANOVA analysis that demonstrated that the worker groups of these bee species were significantly different (Wilk‟s $\lambda = 0.001$; $P < 0.0001$). In addition, the cross-validation test correctly classified 98.8% of the individuals in each respective group by using the equations generated in the discriminant analysis. The probability of correctly identifying workers to species ranged from 97.1 to 100%.

The graphical distribution of the worker groups of the four honeybee species showed that the species were fairly well separated, except for the close bordering between *A. florea* and *A. andreniformis,* and indeed one *A. florea* nested within the *A. andreniformis* cluster (Fig. 3.3). Although, as expected, *A. andreniformis* and *A. florea* were placed closely to each other, they were significantly isolated from each other in most cases, and analysis of the Mahalanobis distances between the centroids of the groups revealed that each group was significantly different from the other groups (Table 3.2).

Table 3.2 Mahalanobis distances (D^2) between the centroid of the honeybee group distributions[a]

	A. andreniformis	*A. cerana*	*A. dorsata*	*A. florea*
A. andreniformis	-	473.573	852.090	128.433
A. cerana	580.265	-	416.332	564.247
A. dorsata	746.632	328.141	-	673.550
A. florea	109.168	335.608	272.642	-

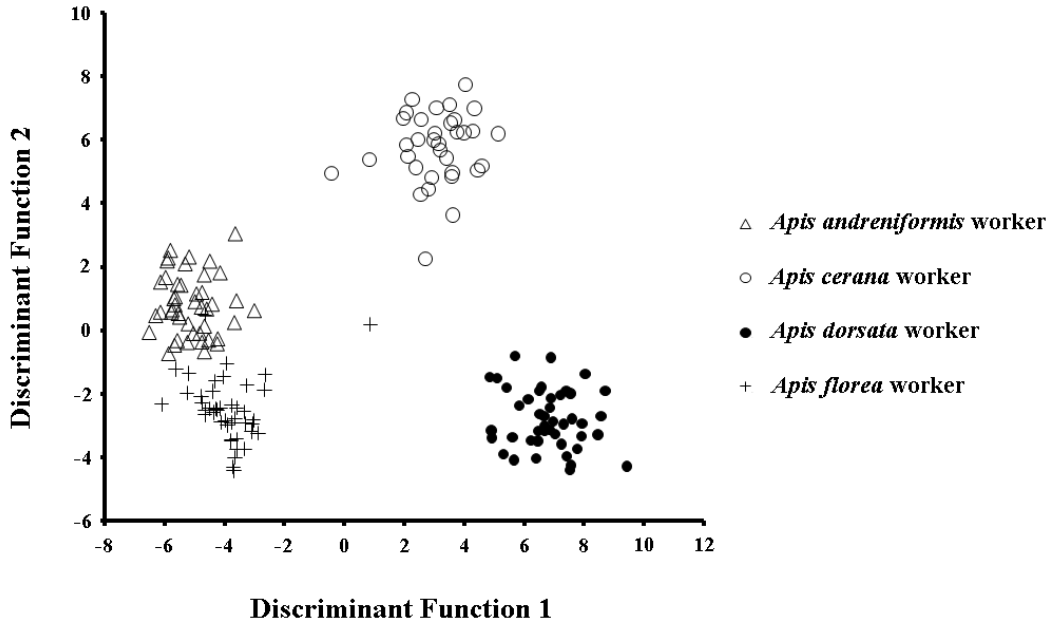

Figure 3.3 Discriminant analysis of the partial warps extracted from the right forewing of workers of four native honeybee species in Thailand.

With respect to the analysis of drones only, all the partial warps of the Cartesian coordinates contributed to the significant difference in the division of the four bee species groups ($\alpha = 0.05$), supported by the results of the MANOVA analysis that revealed that the drone groups were significantly different (Wilk‟s $\lambda = 0.005$; $P < 0.0001$). In addition, the drone cross validation test was capable of correctly classifying 99.3% of the individuals to each respective group. All individual drones were correctly identified with a probability of between 97.4 and 100% of belonging to each respective group. The graphical distribution of the drone groups showed that the species were well separated and somewhat similar to that for workers (Fig. 3.4). Again, *A. andreniformis* and *A. florea* were still close and not unequivocally resolved, but in all cases the Mahalanobis distances between the centroids of the groups for workers were greater than the groups for drones (Table 3.2).

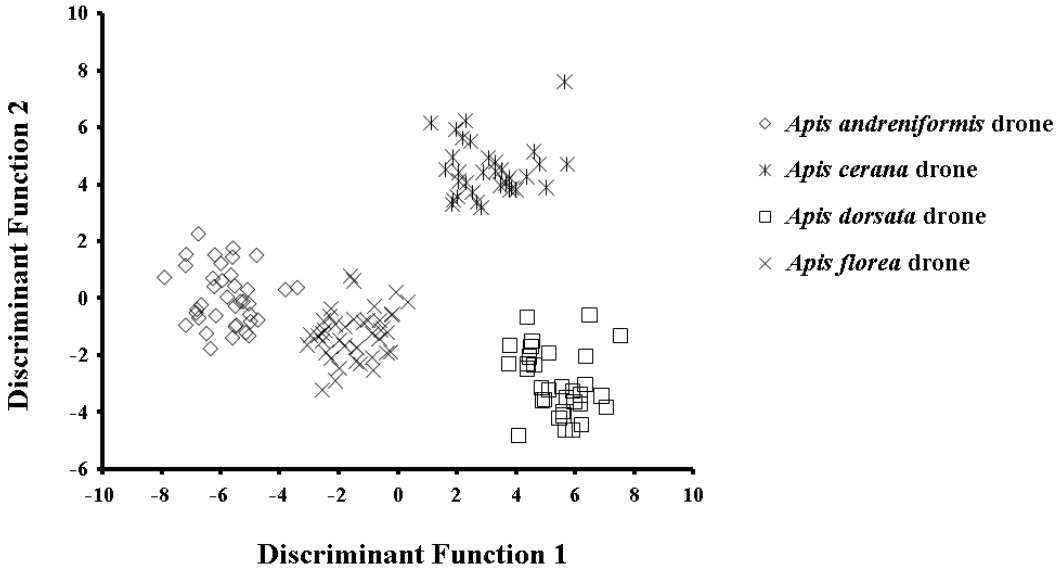

Figure 3.4 Discriminant analysis of the partial warps extracted from the right forewing of drones of four native honeybee species in Thailand.

DISCUSSION

Several studies have shown that wing morphometry alone can be used to identify some bee species, including bumble bees (Aytekin *et al.*, 2007), stingless bees (Francisco *et al.*, 2008; Francoy *et al.*, 2009a) and honeybees. For example, Francoy et al. (2006) demonstrated that a single wing cell carried enough information to discriminate three racial groups of *A. mellifera* (Africanized, Italian, and Carniolan), with a fidelity level of nearly 99% of the individuals, whereas Francisco et al. (2008) also reported that the information of wing morphology, corroborated with molecular analysis, could confirm the discovery and classification of a new species of stingless bee in the genus *Plebeia.* Moreover, outside of bees, Villemant et al. (2007) showed that the application of geometric morphometrics to wing venations could discriminate a complex case of four sibling parasitoids species in the parasitic wasp genus *Eubazus* (Hymenoptera, Braconidae).

The results of the PCA in this research demonstrated that the forewing venation patterns between the sexes in the same species of native Thai honeybees were more closely related to each other than to samples of the same sex in other species (Fig. 3.2). Wing venation patterns and morphometrics were significantly different between species but not between sexes in a given species due to mean configuration of the plotted 15 landmarks between the sexes of four native honeybee species in Thailand (data not shown). The MANOVA analysis revealed that for both workers and drones, they were significantly different between each of the four species, although within a species workers and drones varied also. Also, the cross-validation test correctly classified 98.8 and 99.3% of the individuals in each

respective group by using the equations generated in the discriminant analysis. Thus, the patterns of both workers and drones, when analyzed alone, were sufficiently different to distinguish each of these four *Apis* species at >97% accuracy (Figs. 3.3 and 3.4), suggesting that this kind of analysis can be used for identification of Asian honeybee species and that the sex of the individual does not obstruct identification.

The results obtained in this research largely agree with those of Francoy et al. (2009a), who revealed that geometric morphometric analysis of the forewing venation patterns of males and workers alone were sufficient to classify five species of stingless bee (*Nannotrigona testaceicornis* Lepeletier, *Melipona quadrifasciata* Lepeletier, *Frieseomelitta varia* Lepeletier, *Scaptotrigona* aff. *depilis* Moure, and *Plebeia remota* Holmberg), and that although the patterns of males and workers from the same species were different, they were more similar to each other than the patterns of individuals of the same sex from different species.

Morphometric techniques also have been widely used to discriminate intraspecific groups of honeybees, such as populations (Hepburn *et al.*, 2005; Radloff *et al.*, 2005), subspecies (Amssalu *et al.*, 2004), and ecotypes (Andere *et al.*, 2008). Most attempts to differentiate honeybee groups based on morphological data have used multiple body characteristics, including the body size; antenna length; proboscis length; hair length; metatarsus length and width; and wing angle, length, and width (Amssalu *et al.*, 2004; Andere *et al.*, 2008; Hepburn *et al.*, 2005; Radloff *et al.*, 2005; Rattanawannee *et al.*, 2007). However, these procedures are time-consuming for the preparation and accurate measurement of the various body parts. Here, we were able to discriminate four honeybee species by wing morphology alone, which will greatly facilitate a speedy analysis, especially because we used digitalized wing images

(Fig. 3.1). I conclude that measurements of a small part of the entire bee body, in this case forewing veination patterns, could be sufficient to discriminate among honeybee species. This methodology is simple and can be extended to finer identifications among species of bees with the addition of future landmarks. Moreover, computer program-assisted morphometric analysis of the wing might be a useful implement for biodiversity and conservation studies.

CHAPTER IV

Geometric Morphometric Analysis of Giant Honeybee (*Apis dorsata* Fabricius, 1793) Populations in Thailand

A version of this chapter was accepted to published in Journal of Asia-Pacific Entomology

ABSTRACT

The application of geometric morphometry was used to characterize 73 *Apis dorsata* colonies collected from 31 different localities in five major geographic regions of mainland Thailand. I measured 19 easily identified landmarks from the digitalized images of the right forewing of 10 worker bees from each colony (730 bees in total), and thus avoided confounding variation from haploid or diploid males. After plotting the factor scores, *A. dorsata* from (mainland) Thailand were found to belong to a single group, a notion further supported by a hierarchical cluster analysis generated dendrogram. Multivariate analysis of variance (MANOVA, $\alpha = 0.05$) demonstrated no significant differences between the five geographic groups of *A. dorsata* in Thailand, giving a low degree of accuracy (31.2%) in the identification of the geographic region from which any individual bee originated. In addition, when the bee samples were classified into two groups, those north and south of the Isthmus of Kra, no significant difference between the two groups (MANOVA, $\alpha = 0.05$), and a low rate of correct classification in a cross-validation test (65% correct), were found. Therefore, this geometric morphometric based analysis of the worker bee‟s wing venation pattern found no evidence to support that *A. dorsata* populations in mainland Thailand are not panmictic.

INTRODUCTION

The common giant honeybee (*Apis dorsata* Fabricius, 1793) is distributed over vast geographic areas in Southeast Asia and is found throughout Thailand. Many local people, in Thailand at least, believe that the honey from *A. dorsata* is superior in quality to that from other honeybee species, making the products of this honeybee, such as honey and brood, of considerable local cultural and economic importance (Oldroyd and Wongsiri, 2006). In addition, this species plays an important role as a pollinator for many economic crops and tree species of the Southeast Asian lowland forests (Itioka *et al.*, 2001; Oldroyd and Nanork, 2009).

Various methods have been developed to discriminate between bee species, subspecies, races and populations, including the analysis of mitochondrial and nuclear DNA sequence polymorphisms (Francisco *et al.*, 2001; Whitfield *et al.*, 2006). However, these molecular methods, like the biochemical methods (allozymes or cuticular hydrocarbon analysis), require relatively expensive laboratory equipment and reagents (Francoy *et al.*, 2008).

Because of its high practicability and low costs, morphometric analysis has recently become the most widely used authoritative methodology for identifying honeybee subspecies and populations (Francisco *et al.*, 2008). Morphometric methods are based on multiple measurements of various parts that are performed across many individuals. In earlier studies, Ruttner *et al.* (1978) used 42 characters of *A. mellifera* workers for the analysis of their geographic variability. Then, Daly *et al.* (1982) reported the first successfully used digital measurements to investigate honeybee morphometry, which significantly reduced the time required for the accurate

measurement of the characters. This set of characters is now known as the standard morphometry set (Tofilski, 2008). In addition, recent advances in statistical analysis and image recognition software have made morphometric analysis more precise and practical for discriminating between subspecies and at the population level (Francoy *et al.*, 2008).

For example, (Limbipichai, 1990) successfully used standard morphometry to verify the geographic subpopulations of *A. cerana* in Thailand. He reported that these subpopulations come into contact at the Isthmus of Kra (12° N latitude), which is a biogeographic transition area (Warrit *et al.*, 2006). This morphometric result was supported by mitochondrial DNA sequence based analysis (Deowanish *et al.*, 1996; Hepburn *et al.*, 2001; Smith *et al.*, 2000). In addition, the classification of a single genetic population of *A. andreniformis* (Rattanawannee *et al.*, 2007) and *A. florea* (Chaiyawong *et al.*, 2004; Nanork, 2001) throughout Thailand based upon standard morphometrics was supported by the analysis of mitochondrial DNA sequence polymorphisms. However, the standard morphometric analysis requires time-consuming specimen preparation and measurement procedures (Francoy *et al.*, 2006).

Another more recent morphometric method that appears to be promising is geometric morphometrics, which is based on the description of the shape in Cartesian coordinates (Slice, 2007). Geometric morphometric approaches have been used successfully in evolutionary biology, physical anthropology, paleontology and systematics (Pretorius, 2005; Villemant *et al.*, 2007). This technique has been shown to be sufficiently powerful to solve species level taxonomic problems (Gumiel *et al.*, 2003). Instead of distances and angles, geometric morphometrics uses the coordinates of points, known as landmarks. The selected landmarks are then superimposed by

scaling, translation and rotation. Since after this treatment the landmark configurations differ only in their shape, they can be analyzed by multivariate statistical methods. For example, Tofilski (2008) reported that geometric morphometrics is marginally more reliable than standard morphometry for the discrimination of honeybee subspecies.

Since the 1970ʻs, insect wings have been increasingly used in morphological-based studies in systematics and phylogenies (Aytekin *et al.*, 2007; Gumiel *et al.*, 2003). Because insect wings are rigidly articulated or solid structures they have also become very useful tools for geometric morphometric studies (Aytekin *et al.*, 2007). In bees, wing morphology based analyses have provided a good identification at the species, subspecies and even at the population level (Francoy *et al.*, 2008; Mendes *et al.*, 2007).

Several studies have demonstrated that wing morphometry alone can be used to identify some bee species, including within bumble bees (Aytekin *et al.*, 2007), stingless bees (Francisco *et al.*, 2008; Francoy *et al.*, 2009a) and honeybees (Rattanawannee *et al.*, 2010). For instance, Francoy *et al.* (2006) demonstrated that a single wing cell carried enough information to discriminate three racial groups of *A. mellifera* (Africanized, Italian and Carniolan) with a fidelity level of nearly 99% of the individuals, whilst Rattanawannee *et al.* (2010) reported that the geometric morphometric analysis of the wing alone could be used to identify four Asian honeybee species in Thailand and that, importantly, the sex of the individual does not impede identification. In stingless bees, Francisco *et al.* (2008) also reported that the information of wing morphology, corroborated with molecular analysis, could confirm the discovery and classification of a new species of stingless bee in the genus

Plebeia. Moreover, outside of bees, Villemant *et al.* (2007) showed that the application of geometric morphometrics to wing venation patterns could discriminate a complex case of four sibling parasitoids species in the parasitic wasp genus *Eubazus* (Hymenoptera, Braconidae).

Thus, geometric morphometrics analysis of wings is potentially sufficiently powerful enough to discriminate among bee species, subspecies (Francoy *et al.*, 2006; Tofilski, 2008) and even subpopulations (Mendes *et al.*, 2007). Therefore, the aim of this study was to verify if *A. dorsata* in Thailand is composed of distinct geographical subpopulations using geometric morphometric based analysis.

MATERIALS AND METHODS

Sample collection and measurement

Adult workers of *A. dorsata* were sampled from 31 different geographic locations throughout Thailand and including from within each of the six regions (Fig 4.1 and Table 4.1). We collected samples of 10 workers (and so all females) per colony from 73 colonies of *A. dorsata* (Table 4.1), giving a total of 730 bees. The right forewing of each bee sample was dissected, mounted flat on a microscope slide and photographed by a digital camera attached to a stereomicroscope. Nineteen homologous landmarks were manually plotted at the forewing vein intersections (Fig. 4.2) using the software tpsDig2, version 2.04 (Rolhf, 2005a). The Cartesian coordinates of the landmarks were then aligned and partial warps analysis was performed using the software tpsRelw, version 1.42 (Rolhf, 2005b). In these analyses we set $\alpha = 0$, thus the same weight was given to all the variables. This procedure is the most suitable for

exploratory and taxonomic studies (Mendes *et al.*, 2007). The software is available from the internet (http://life.bio.sunysb.edu/morph/).

Data analysis

The significant univariate F values ($\alpha = 0.05$) were used to identify those wing parameters that contributed the most to the discrimination between the groups (Francoy *et al.*, 2006; Mendes *et al.*, 2007). These measurements were then used to compare the different geographic groups of honeybee samples (Fig. 4.1). Note, however, that the Central and Eastern Thailand samples were combined into one group (Central-Eastern Thailand) and thus five and not six different geographical regions were analyzed. A principal component analysis (PCA) of the Cartesian coordinates that contributed the most to the group‟s discrimination was conducted (Francoy *et al*. 2006, 2009). Then, cluster analysis was used to investigate the relationship between groups of bee samples in Thailand. A stepwise analysis was performed to determine the classification functions, followed by a canonical analysis. The cross-validation test, using 10% of the randomly selected individuals as unknown (Francisco *et al*. 2008), was then performed to check the accuracy of the equations in identifying the colonies (Francoy *et al.*, 2009a). Furthermore, Mahalonobis square distances between the groups of bees were calculated (Francoy *et al.*, 2009a). After extraction of the measurements, all statistical analyses were performed using the SPSS Version 10.0 (1999; SPSS, Chicago, IL, USA) software.

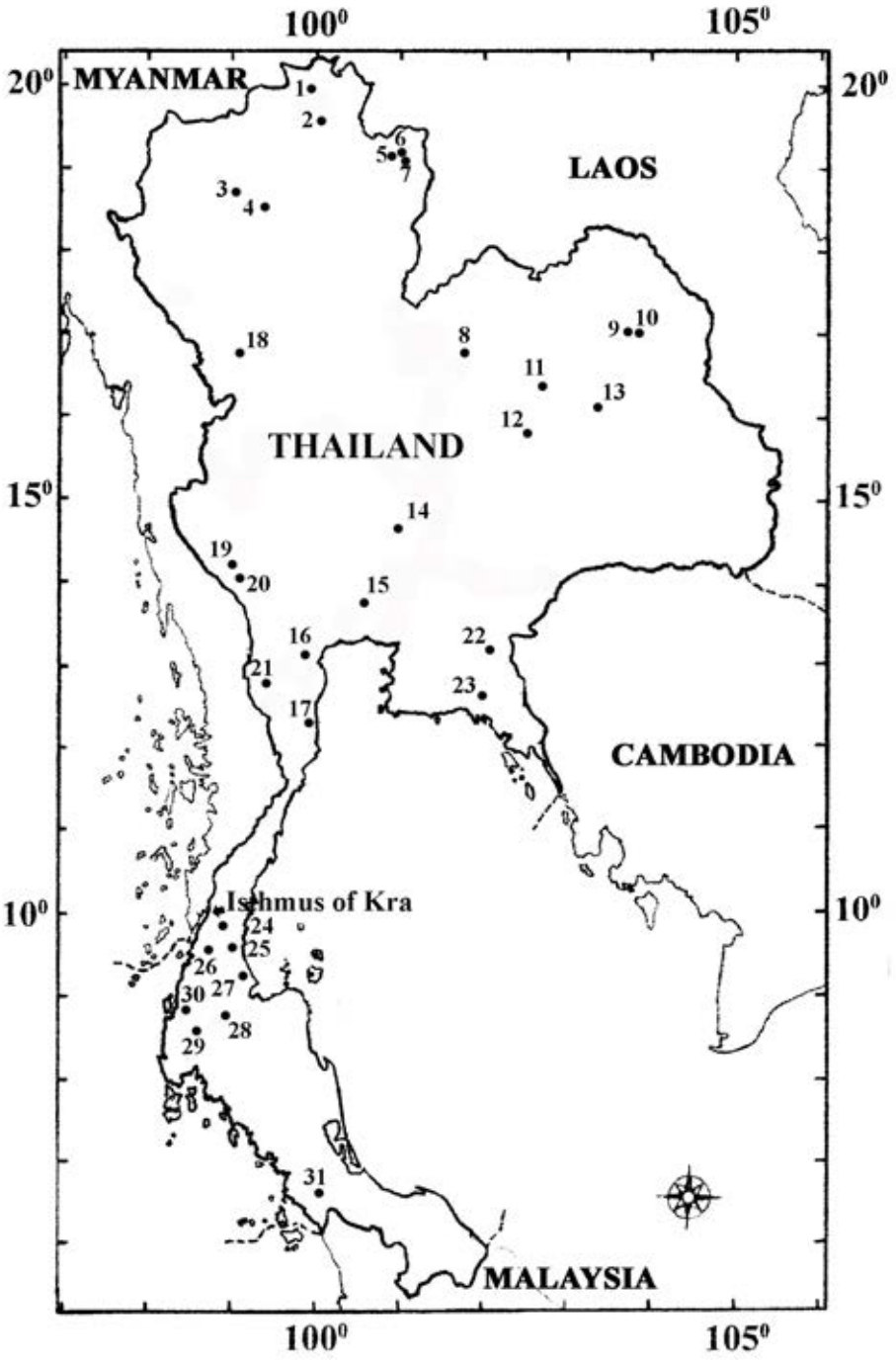

Figure 4.1 *Apis dorsata* collection sites in Thailand. Numbers correspond to those in Table 4.1, where the number of colonies sampled per site is also indicated. Sites 1 – 23 are north of the Kra of Isthmus, and further divided as North (1 – 7), Northeast (8 – 13), Central (14 – 16) West (17 – 21) and the East (22 & 23), whilst sites 24 – 31 are south of the Kra of Isthmus and defined as the South.

Table 4.1 *Apis dorsata* collections in Thailand. Locality numbers (No.) correspond to those in Figure 4.1. Latitude and longitude measurements were determined using a Garmin Model *GPS Map76s*.

No.	Geographic region	Locality	Coordinate	Sample Code
1	North	Mueang, Chiang Rai	20° 17′ N 99° 48′ E	N-Chiang Rai 1 - 9
2	North	Mae Fah Luang, Chiang Rai	20° 02′ N 99° 53′ E	N-Chiang Rai 10
3	North	Mae Rim, Chiang Mai	18° 53′ N 98° 51′ E	N-Chiang Mai 1 - 2
4	North	Sansai, Chiang Mai	18° 53′ N 99° 00′ E	N-Chiang Mai 3
5	North	Pua, Nan	19° 10′ N 100° 55′ E	N-Nan 1 - 4
6	North	Pua (Sathan), Nan	19° 12′ N 100° 58′ E	N-Nan 5 - 7
7	North	Pua (Phu Kha), Nan	19° 15′ N 101° 02′ E	N-Nan 8 - 10
8	Northeast	Nam Nao, Phetchabun	16° 44′ N 101° 33′ E	NE-Phetchabun 1
9	Northeast	Phu Pan (Hongsim), Sakon Nakhon	16° 54′ N 103° 57′ E	NE- Sakon Nakhon 1 - 5
10	Northeast	Phu Pan (Chomphupan), Sakon Nakhon	16° 52′ N 103° 56′ E	NE- Sakon Nakhon 6
11	Northeast	Mueang, Khonkaen	16° 26′ N 102° 53′ E	NE- Khonkaen 1
12	Northeast	Ban Phai, Khonkaen	16° 04′ N 102° 53′ E	NE- Khonkaen 2
13	Northeast	Mueang, Maha Sarakham	16° 12′ N 103° 16′ E	NE- Maha Sarakham 1
14	Central	Phatthana Nikhom, Lop Buri	14° 47′ N 100° 54′ E	C- Lop Buri 1
15	Central	Phathumwan, Bangkok	13° 44′ N 100° 31′ E	C- Bangkok 1 - 3
16	Central	Mueang, Samut Songkhram	13° 23′ N 99° 57′ E	C- Samut Songkhram 1
17	West	Hua Hin, Prachuap Khiri Khan	12° 34′ N 99° 56′ E	W-Prachuap Khiri Khan 1
18	West	Ban Tak, Tak	17° 02′ N 98° 57′ E	W-Tak 1 - 11
19	West	Sai Yok (Wang Khamen), Kanchanaburi	14° 20′ N 98° 56′ E	W- Kanchanaburi 1 - 2
20	West	Sai Yok (Wang Kajae), Kanchanaburi	14° 10′ N 99° 03′ E	W- Kanchanaburi 3 - 6
21	West	Kaeng Krachan, Phetchaburi	12° 52′ N 99° 38′ E	W- Phetchaburi 1
22	East	Wang Sombun, Sa Kaeo	13° 19′ N 102° 10′ E	E-Sa Kaeo 1
23	East	Khlung, Chanthaburi	12° 30′ N 102° 10′ E	E-Chanthaburi 1 - 2

Table 4.1 (Continued)

24	South	Sawi, Chumphon	10° 19′ N 99° 05′ E	S-Chumphon 1 - 3
25	South	Lang Suan, Chumphon	09° 58′ N 99° 04′ E	S-Chumphon 4 - 5
26	South	La-un, Ranong	10° 09′ N 98° 42′ E	S-Ranong 1
27	South	Chaiya, Surat Thani	09° 26′ N 99° 09′ E	S-Surat Thani 1
28	South	Phanom, Surat Thani	08° 50′ N 98° 44′ E	S-Surat Thani 2
29	South	Kapong, Phang-nga	08° 54′ N 98° 31′ E	S-Phang-nga 1
30	South	Takua Pa, Phang-nga	08° 51′ N 98° 22′ E	S-Phang-nga 2 - 3
31	South	Khuan Don, Satun	06° 42′ N 100° 09′ E	S- Satun 1 - 2
Total				**73 Colonies**

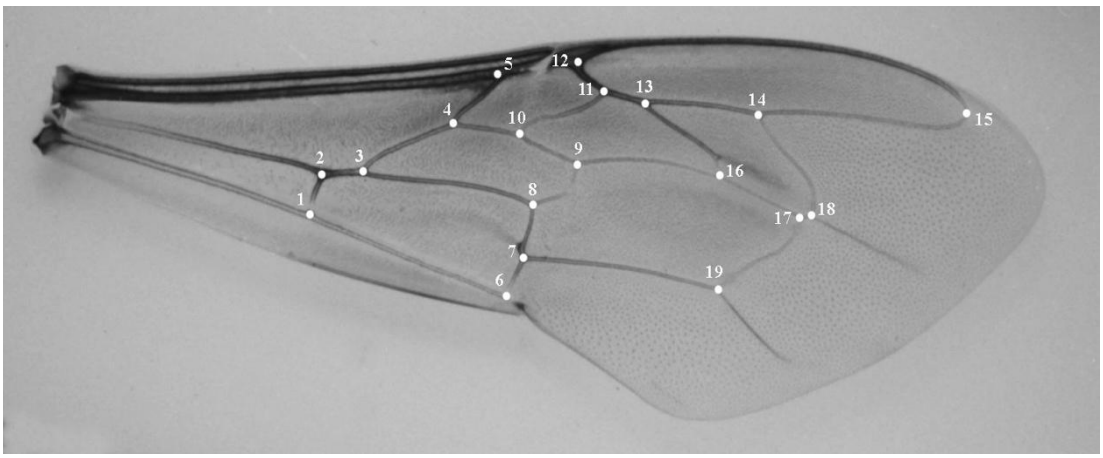

Figure 4.2 Right forewing of an *A. dorsata* worker bee showing (white circles) the respective position of each of the 19 plotted landmarks at the vein junctions.

RESULTS

The nineteen anatomic landmarks (Fig. 4.2) generated 34 relative warp measurements for each wing specimen. The three highest relative warps explained a total of 45.3% of the group‟s variability, with 23.7%, 12.8% and 8.8% being explained by the first, second and third relative warp, respectively. Based on the univariate *F* values, 12 relative warps were found to generate a significant contribution ($\alpha = 0.05$) to the discrimination of the group.

Six principal factors with Eigen values of greater than one were extracted in the PCA. Together, these six components explained 68.81% of the data set variability. The two main principal components explained 29.6% of the data set variability. The coordinate variable 3x mostly influenced the first factor and explained 16.2% of the

variability among the groups, whilst the coordinate variable 13x mostly influenced factor 2 and explained 13.4% of the variation.

Bee samples were first grouped into five major collecting geographical localities (Northern, Western, Central-East, Northeastern and Southern Thailand), as shown in Fig. 4.1 and Table 4.1, and subsequentially into the two groups of north of and south of the Kra of Isthmus, and then analyzed. With respect to the populations grouped into the five different geographic regions, based on the positions of the groups in the PCA plots of factor 1 (16.2%) and factor 2 (13.4%), no clear distinction among the groups was found (Fig. 4.3). The same result was obtained with PCA plots of all the other 15 combinations of the pair wise plots of the six principal factors (data not shown). Therefore, no subgroups of *A. dorsata* across these five principal geographic regions of Thailand were found by this analysis, but rather they all seem to belong to one group. Although it could theoretically be argued that the merging of the Central and Eastern Thailand populations may have compromised their differences, and perhaps those of the Western Thailand group, this would not be expected to affect the Northern, Northeastern and Southern Thailand populations. Moreover the analysis of the bee samples when divided into two groups (north and south of the Kra of Isthmus) showed the same trend of no significant differences (data not shown).

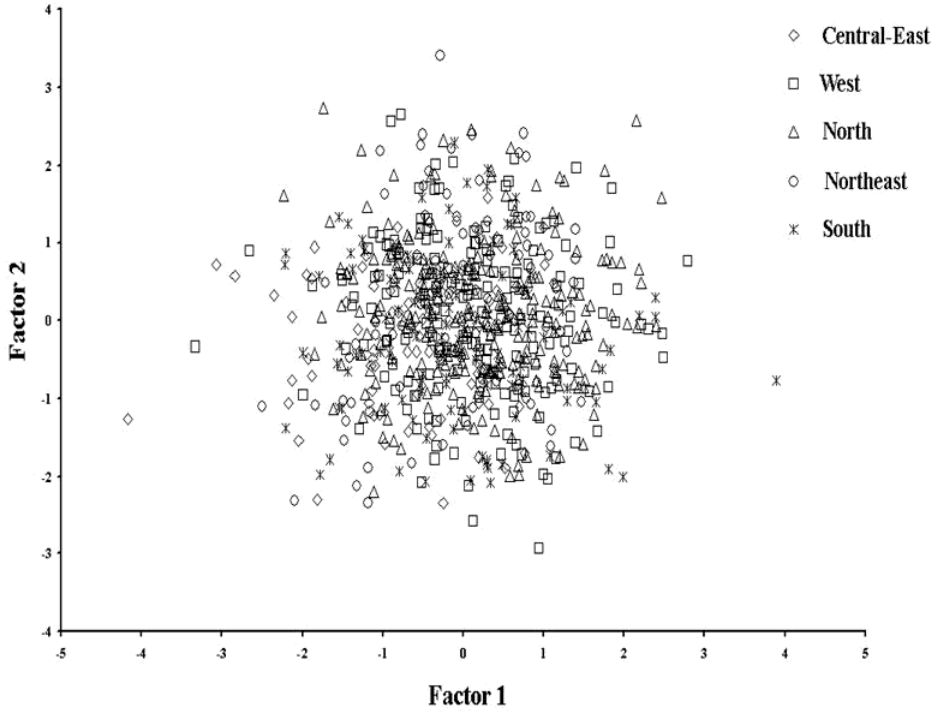

Figure 4.3 Scatter plot of the two most influential factors (variables 3x and 13x, respectively) from the PCA of 730 worker bees measured for 19 anatomical landmarks, and grouped into five geographical regions.

A dendrogram was constructed by a hierarchical cluster analysis of the squared Euclidian distances between the individual factor score values of the worker bees (Fig. 4.4) obtained from the indicated colonies and localities. The dendrogram revealed six distinct groups and subdivisions of the major groups (groups 1, 2, 3 and 5). Most bee colonies fell into either group 1 or group 2 (37.0% and 38.4%, respectively), and these were composed of the bees from all the major collecting localities. Indeed, the obtained cluster analysis results indicated no clear separation of *A. dorsata* into distinct geographic groups, be that the five geographic regions (Central-Eastern, Northern, Northeastern, West and Southern Thailand) or the two domains of north and the south of the Isthmus of Kra (12° N latitude). Therefore, the cluster analysis results are in close agreement with the factor analysis, which show no clear geographical based demarcation of populations but rather show a single group of *A. dorsata* in Thailand.

Multivariate analysis of variance (MANOVA) revealed no significant difference (Wilk‟s $\lambda = 0.995$; $P = 0.190$) between the *A. dorsata* populations from within the five different geographic regions in Thailand. Analysis of the Mahalanobis square distances between the centroids of the groups also revealed no significant difference between each of the other four groups (Table 4.2). In addition, the cross-validation test revealed a low level of correct classification (31.2%) of individual bees into their correct geographical group or population using the equations generated in the discriminant analysis (Fig. 4.5).

When the bee samples were reclassified into two groups, north and south of the Isthmus of Kra, the resulting MANOVA revealed no significant difference between the two groups (Wilk‟s $\lambda = 0.936$; $P = 0.252$). From the complete set of 730

wings, in the cross-validation test, 270 bees (37%) were misclassified to the wrong respective group (Fig. 4.6), and this did not significantly vary between the two regions, being 223 (37.2%) misclassifications from the north and 47 (36.2%) from the south. Thus, the discriminant analysis results also failed to identify different distinctive populations of *A. dorsata* in Thailand.

Table 4.2 Mahalanobis square distances between the centroid of the distribution of the five geographic groups of *A. dorsata* in Thailand.

Geographic group	**Central-East group**	**West group**	**North group**	**Northeast group**
Central-East group	-			
West group	3.184	-		
North group	3.141	2.929	-	
Northeast group	3.045	3.260	3.308	-
South group	3.576	3.409	3.549	3.461

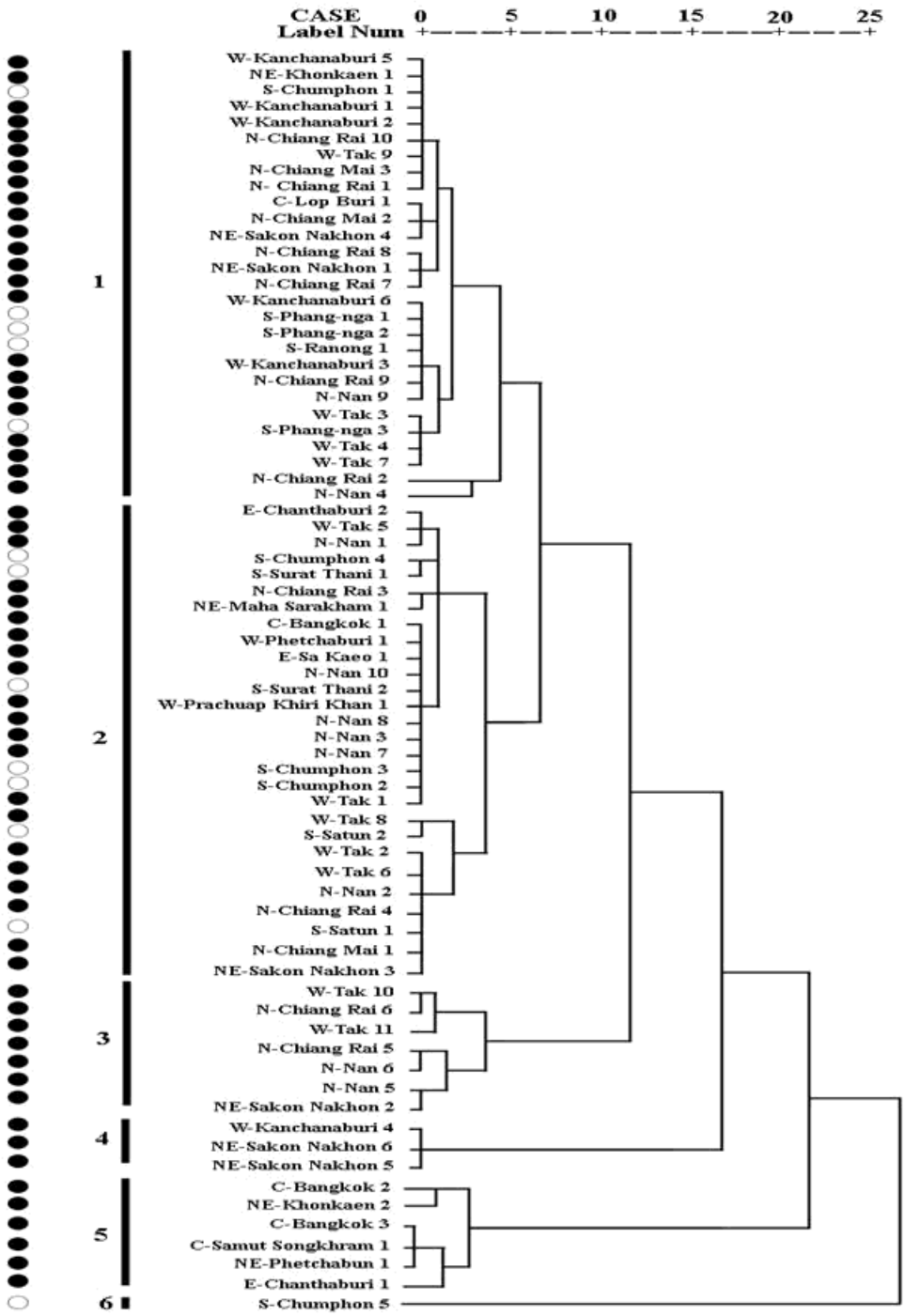

Figure 4.4 A hierarchical cluster analysis dendrogram constructed from the squared Euclidian distances. *A. dorsata* is classified by collection localities (see also table 1). Black and white circles indicate the colonies in terms of from the north and the south of the Kra of Isthmus (12° N latitude), respectively.

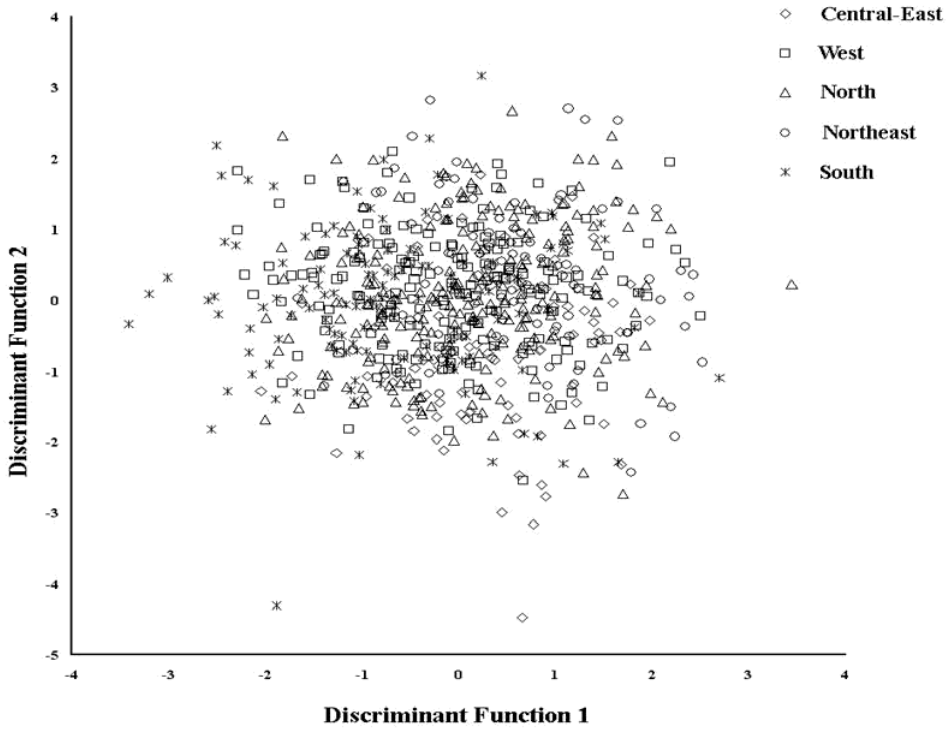

Figure 4.5 Linear discriminant analysis of the partial warps extracted from the right forewing of *A. dorsata* worker bees in Thailand, and analyzed as populations within five geographic regions.

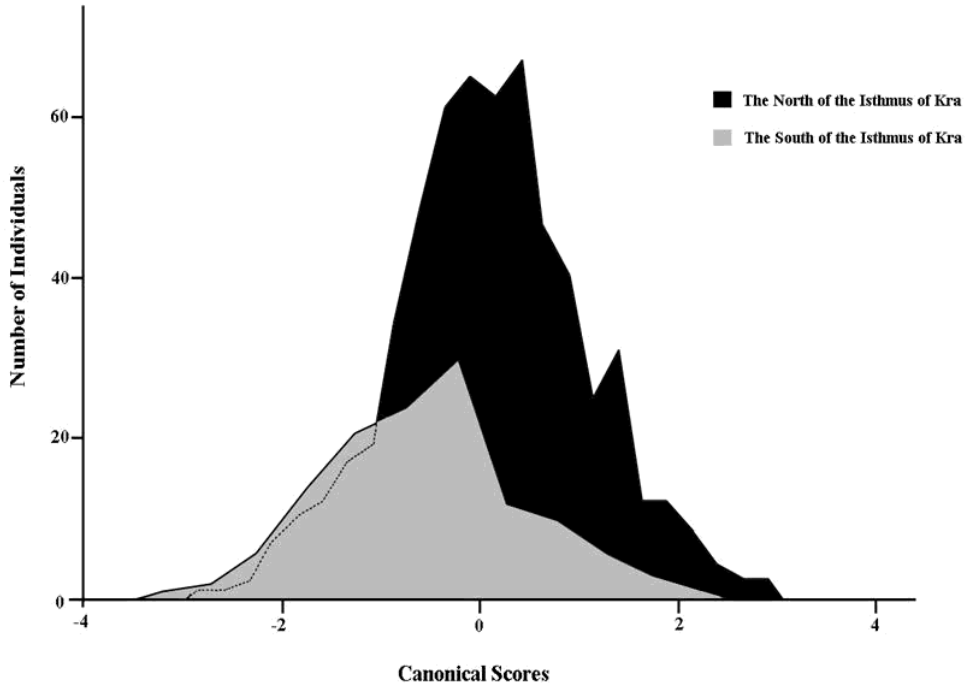

Figure 4.6 Canonical distributions of the individuals from the *A. dorsata* populations grouped as north or south of the Isthmus of Kra (12° N latitude), based upon measurements extracted from the wing venation.

DISCUSSION

Wing morphometry is a potentially powerful and sufficient methodology to discriminate bee species, subspecies and populations, including for honeybees (Francoy *et al.* 2006, 2008; Francisco *et al.* 2008; Tofilski 2008) and stingless bees (Mendes *et al.* 2007; Francoy *et al.* 2008, 2009). Tofilski (2008) reported that geometric morphometrics is marginally more reliable than standard morphometry for the ability to discriminate *A. mellifera* subspecies based upon forewing venation patterns. Furthermore, the geometric morphometric analysis of wing venation has been shown to be a very informative tool for the discrimination of bee populations (Mendes *et al.* 2007), and sensitive enough to discriminate between two populations of *Plebeia remota* (Francisco *et al.* 2008), a result which was also supported by analysis of the mtDNA by RFLP patterns and the cuticular hydrocarbon composition through gas chromatography-mass spectrometry.

In this study, PCA of the fore wing venation patterns of *A. dorsata* populations from five different geographic regions in Thailand suggested that they were closely related to each other with no significant differences between the regions, and hence no significant unique subpopulation structure (Fig. 4.3). Note that in this analysis *A. dorsata* from the Central and the Eastern regions of Thailand were grouped together into the one group (Central-Eastern), since both regions are similar in terms of having no geographic barriers between them, in contrast to the border between the Western and the Central regions, and between the Central and the Northwestern regions. Furthermore, discriminant analysis using the factor scores revealed no significant differences in Mahalanobis square distances between the centroids between samples

from the Central and the Eastern regions. Then, among all the five regional groups of bee samples, or between the north and south of the Kra of Isthmus, the cluster analysis indicated no clear separation of *A. dorsata* into distinct groups. For example, in terms of geographic distance colonies N-Chiang Rai 4 and S-Satun 1 are the furthest apart (Fig. 4.1), yet they are grouped together into the 2nd major group and are closely related to each other (Fig. 4.4). Therefore, our results suggest that *A. dorsata* in Thailand is panmictic and appears as a single but polymorphic population.

Similarly, Hepburn *et al.* (2005) and Rattanawannee *et al.* (2007) used more than 20 informative morphological characters to investigate the diversity of *A. florea* and *A. andreniformis* in Thailand, respectively. They found that the respective populations of the two dwarf honeybee species from across Thailand showed no geographical separation but rather appeared as one group (panmictic population).

The MANOVA results here revealed no significant difference between *A. dorsata* populations grouped into either the two regions of the north and south of the Kra of Isthmus, or into the five smaller geographic regions of Thailand. Moreover, cross-validation tests revealed a low level of correct classification of individual bees into each respective group when the populations were grouped either into the five geographical regions (31.2% correct) or into the two regions (north and south) of either side of the Kra of Isthmus (65.1% correct). Therefore, the results of the discriminant analysis supported those of the PCA and cluster analysis, in suggesting the *A. dorsata* populations in Thailand are panmictic. Indeed, this notion is in close agreement to a molecular analysis based upon the PCR-RFLP analysis of mitochondrial DNA combined with nuclear microsatellite DNA markers, which found

no significant population differentiation among *A. dorsata* populations throughout Thailand (Insuan *et al.*, 2007).

In contrast, in a different honeybee species, *A. cerana*, Limbipichai (1990) reported that by using standard morphometry the Thai-Malay peninsula *A. cerana* populations were comprised of distinct populations that come into contact at a sharp boundary at the Isthmus of Kra, a biogeographic transition area known as the Kra Ecotone. Moreover, this result was supported by that of Songram *et al.* (2006) who found, using PCR-RFLP of the mitochondrial *ATPase6-ATPase8* DNA fragment, large genetic distances between *A. cerana* populations from the north-to-central and southern peninsular mainland Thailand as well as the Koh Samui Island. Thus, they concluded that Thai *A. cerana* could be genetically differentiated into three distinct populations; Northern Thailand, peninsular Thailand and Koh Samui Island populations. However, Sittipraneed *et al.* (2001) used three microsatellite loci (A28, A107, and A113) to examine the genetic diversity of Thai *A. cerana* and although they reported a high level genetic diversity (H_o = 0.40 - 0.46) in mainland samples (Northern, Central, Northeastern and peninsular Thailand), they were allocated into four (not three) conspecific populations of the Northern-Central, Northeastern and Southern peninsular mainland, plus the low genetic diversity Koh Samui population.

My data here, for *A. dorsata* populations in mainland Thailand, demonstrate the potential for a high degree of gene flow and thus panmictic between *A. dorsata* populations across (mainland) Thailand. The overlapping of two peaks in Fig. 4.6, and the low rates of correct classifications indicate hybridization among the north and the south of the Isthmus of Kra groups. Consistent with this is that *A. dorsata* colonies are reported to potentially migrate up to some 200 km in distance (Crane *et al.*, 1993;

Koeniger and Koeniger, 1980). In addition, queens of *A. dorsata* are reported to be highly polyandrous (Moritz *et al.*, 1995; Oldroyd *et al.*, 1996) with average mating frequencies of up to 88.5 (Wattanachaiyingcharoen *et al.*, 2003). Thus, there is potentially no significant barrier to gene flow and geographical obstacles seem to play no significant role in directing the swarming migration of *A. dorsata* at the level of, and in, mainland Thailand. In summary, this geometric morphometric analysis of wing venation, together with previous molecular studies, conclude that the *A. dorsata* populations across Thailand are panmictic.

The giant honeybee, *A. dorsata*, is an economic source of honey and wax production for many rural people of Asia. In addition, they play an important role as a pollinator for many economic crops and wild plants throughout the tropical regions. Therefore, evaluating the patterns of distribution and biological diversity, and local and regional selection pressures upon *A. dorsata* is important in their management and conservation strategies, and to allow better utilization in neighboring agricultural practices.

CHAPTER V

Genetic structure of a giant honeybee (*Apis dorsata*) population in northern Thailand: implications for conservation

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ABSTRACT

1. The giant honeybee, *Apis dorsata,* is a keystone pollinator. The species is heavily hunted throughout Thailand. Furthermore, forest clearing, widespread use of pesticides and proliferation of street lighting (which attracts bees, often resulting in their death) are likely to have significant impacts on population viability.

2. I examined the relatedness and genetic variation within and between aggregations of *A. dorsata* nests. Microsatellite analysis of 54 nests in 3 aggregations showed that no colonies were related as mother-daughter. Thus, if reproduction occurred at my study site, daughter colonies dispersed. This suggests that rapid increases in *A. dorsata* colony numbers during general flowering events most likely occur by swarms arriving from other areas rather than by *in situ* reproduction.

3. The population has high levels of heterozygosity. F_{st} values between aggregations were not significantly different from zero ($P > 0.05$). This suggests that despite the formidable anthropogenic pressures that the *A. dorsata* population endures in northern Thailand, the species continues to enjoy a large effective population size and has high connectedness.

4. I conclude that *A. dorsata* is currently able to tolerate habitat fragmentation and annual harvesting. I speculate that the population is sustained by immigration from forested regions to the northwest of our study sites in Burma.

INTRODUCTION

The giant honeybee, *Apis dorsata* Fabricius, 1793, has a range that extends from the Indian subcontinent to Southeast Asia (Ruttner, 1988). Colonies build a massive single-comb nest, usually hanging beneath a tall tree branch, cliff overhang or eaves of a building (Oldroyd and Wongsiri, 2006). Throughout its range the nests are hunted for the honey, wax and brood they contain, providing an important source of household income (Lahjie and Seibert, 1990; Nath *et al.*, 1994; Soman and Kshirsagar, 1991 ; Strickland, 1982). Unfortunately harvesting is usually done at night in Thailand, which often results in the death of the harvested colony because the queen is killed or otherwise unable to re-join her colony (Oldroyd and Wongsiri, 2006).

The giant honeybee differs in behaviour and ecology from other species in the genus *Apis* (Oldroyd and Wongsiri, 2006). First, colonies are often found in dense aggregations. Over 200 individual colonies may occur on a single tree or rock face (Oldroyd *et al.*, 2000), and these are often separated by only a few centimetres (Paar *et al.*, 2004a). Second, nesting sites are often reoccupied annually for decades (Oldroyd *et al.*, 2000; Oldroyd and Wongsiri, 2006). Interestingly, queens often return to the same nest site even after an absence of up to 18 months (Neumann *et al.*, 2000; Paar *et al.*, 2000). Third, colonies frequently undergo seasonal migration between alternative nesting sites. Colonies usually occupy a nest site for 3-4 months (Paar *et al.*, 2004a). At the end of this period colonies abscond to an alternative nest site, leaving an empty comb. Absconding swarms probably migrate between locations with different blooming seasons (Crane *et al.*, 1993; Dyer and Seeley, 1994; Itioka *et al.*, 2001;

Koeniger and Koeniger, 1980; Liu *et al.*, 2007; Sheikh and Chetry, 2000; Underwood, 1990). In some areas the absconding swarms do not build comb at their alternate nesting site but enter a period of quiescence (Underwood, 1990). A broodless migration period may help to reduce infestation by *Tropilaelaps* parasitic mites, which need brood in order to reproduce (Kavinseksan *et al.*, 2003; Rinderer *et al.*, 1994).

The lowland forests of Asia are physically, though, not numerically dominated by the trees of the family Dipterocarpaceae. The pollination ecology of this forest type is characterized by infrequent (2-10 year) mass general flowering events, in which most trees of all species flower simultaneously at a random time of year (Ashton, 1988; Sakai *et al.*, 1999 and 2002). These forests, particularly the canopy trees, appear to be adapted for pollination by migratory honeybees that can rapidly increase in population size by both reproductive and migratory swarming (Corlett, 2011; Momose *et al.*, 1998b; Oldroyd and Nanork, 2009). No other potential pollinators (birds, bats, solitary bees or stingless bees) share the twin characteristics of migration and high rates of reproduction that are necessary for rapid population build up (Oldroyd and Wongsiri, 2006). In addition, because individual trees of each species tend to be widely spaced in dipterocarp forests, pollen must be transferred over long distances (Itioka *et al.*, 2001). This requires an animal vector that has species fidelity while foraging, a large foraging range, and the tendency to visit multiple trees. The giant honeybee has all these characteristics (Oldroyd and Nanork, 2009), and is therefore likely to be a keystone species in dipterocarp forests. For example, *A. dorsata* was the only social bee that foraged on (and presumably pollinated) several dipterocarp tree species at Lambir, Sarawak in which the flowers opened before sunrise (05:00–06:00 h) and after sunset (18:00–20:00 h) (Momose *et al.*, 1998b). This apparent adaptation of nocturnal-

flowering canopy trees to a migratory bee species that is normally absent from the site suggests that the relationship between *A. dorsata* migration and mass-flowering episodes in the lowland dipterocarp forest of Sarawak is an ancient one (Corlett, 2004; 2011).

In total, *A. dorsata* pollinated at least 15 species of emergent and canopy trees in the lowland dipterocarp forest at Lambir Borneo (Momose *et al.*, 1998b; Roubik, 2005). *A. dorsata* was also among the dominant pollinators of the upper strata in rainforest in peninsular Malaysia (Appanah, 1993) and for canopy dipterocarps in Sri Lanka (Dayanandan *et al.*, 1990). I conclude that any decline in *A. dorsata* populations caused by over-hunting or deforestation may lead to significant changes in the pollination ecology of these forests (Corlett, 2011; Itioka *et al.*, 2001; Oldroyd and Wongsiri, 2006). Furthermore, *Apis* species likely play an important pollinating role in disturbed habitats, as the dwarf and cavity-nesting honeybee species may be more resilient than solitary bee species to such disturbance (Corlett, 2001).

Population structure can arise via processes such as genetic drift and restricted gene flow that cause heterogeneous distribution of genetic variation within and among populations (Frankham, 1995; Frankham *et al.*, 2010). When gene flow is restricted, isolated populations can diverge genetically and suffer loss of heterozygosity and inbreeding depression. In most endangered species, habitat loss and degradation are the main causes of population isolation, decline, or extinction (Foin *et al.*, 1998).

A. dorsata colonies are hunted relentlessly across most if not all of Thailand. Thailand has also suffered significant deforestation (Sodhi *et al.*, 2004) and the remaining forest is often degraded by frequent forest fires that are lit by humans. Widespread use of pesticides and proliferation of street lighting (which attracts bees,

often resulting in their death, particularly when colonies nest on buildings) are likely to have significant impacts on population viability (Oldroyd and Wongsiri, 2006). An earlier broad-scale survey of genetic variation in the *A. dorsata* population showed limited genetic diversity across Thailand, potentially suggesting recent population bottlenecks arising from anthropogenic activity (Insuan *et al.*, 2007). Similarly, Tanaka *et al.* (2001) found remarkably low diversity among *A. dorsata* mitochondrial haplotypes in Borneo.

Here I assess population structure of *A. dorsata* colonies at a smaller scale in the province of Tak in northwest Thailand. Physiographically, this region is classified as continental highland. The dominant forest type is dry deciduous, which is characterized by the emergent dipterocarp tree species. I use DNA microsatellite analysis to assess the extent of gene flow among three relatively small colony aggregations, the degree of genetic divergence between the aggregations, and the relatedness of colonies within aggregations. By this means I determine if the population is beginning to show signs of inbreeding or fragmentation, that might be a prelude to local extinction, as has already occurred for the *A. dorsata* population on Bali Indonesia (personal observations of BPO).

MATERIALS AND METHODS

Collections

The study comprised samples from all colonies present in three bee trees situated in dipterocarp forest at Mae Tuen National Park, Tak province, northwestern Thailand (Fig. 5.1). These particular bee trees are extremely large, estimated to be more than 40 m high. *A. dorsata* colonies have occupied these three trees annually for at least 10 years, and colonies are (illegally) harvested annually by the local bee hunters using traditional methods. We collected adult workers from 54 colonies [bee tree 1 (N 17º 01.02, E 98º 57.06): 10 colonies; bee tree 2 (N 17º 01.06, E 98º 56.57): 23 colonies; and bee tree 3 (N 17º 03.56, E 98º 56.23): 21 colonies] in April 2010. At least 30 workers were sampled from each colony, from the curtain of bees on the lower edge of the comb. Samples were collected by local honey hunters who scaled the trees using traditional methods. All samples were taken carefully in the late evening without causing workers to take flight from the nest, thereby minimizing drift between colonies (Paar *et al.*, 2002). Sampled workers were preserved in 95% (v/v) ethanol until DNA was extracted.

DNA extraction

Total DNA was extracted from one hind leg of each worker bee using a 5% (w/v) Chelex solution (Walsh *et al.*, 1991). Leg was added to 400 µL of 5% (w/v) Chelex (Chelex®100; catalog no. 143-2832, BIO-RAD) solution in a 1 mL centrifugable 96 well plate (Greiner Bio-One, Applied Biosystems). Legs were homogenized by adding a stainless steel bead to each well and then inserted the plate into a TissueLyser (Qiagen) for at least 10 minutes at 25 Hz. Samples were boiled for

15 minutes, and precipitated by centrifugation for 50 minutes at 4300 rpm and 4 °C. Supernatants (200 µL) were transferred into a 600 µL centrifugable 96 well plate (Axygen, Applied Biosystems) and stored at 4 °C.

PCR amplification

The microsatellite loci used were A14, A24, A76, A88, BI225 and SV197 cloned from *A. mellifera* (Estoup *et al.*, 1993; Solignac *et al.*, 2007), B124 from *Bombus terrestris* (Estoup *et al.*, 1993), and Ad3 from *A. dorsata* (Paar *et al.*, 2004a). Genomic DNA (1 µL) was used in 5 µL PCR reactions [(1X TAQ-Ti polymerase reaction buffer (Fisher Biotect), 2 mM $MgCl_2$, 0.25 µL 50% (v/v) glycerol, 0. 5 mM of each dNTP, 0.2 mM of each forward and reward primer, and 0.25 unit of TAQ-Ti DNA polymerase (Fisher Biotech)]. All microsatellite loci were amplified using a standard polymerase chain reaction (PCR) program of 94ºC for 10 min, followed by 35 cycles of 94, 55, and 72ºC for 30 s each, and finally 72ºC for 9 min. PCR products were analyzed on a 3130xl Genetic Analyzer (Applied Biosystems, California). Resultant data files were analyzed using GENEMAPPER software (Applied Biosystems).

Data analysis

The genotype of the queen heading each colony was inferred from the worker genotypes (2000; Oldroyd *et al.*, 1996); plus the assumptions that (a) each colony was derived from a single queen *i.e.* it was not polymatrilineal (e.g. the original queen was superseded), (b) workers collected from that colony were daughters of the queen and not „foreign" or drifted workers and (c) for each loci examined, the queen did not carry a null allele. With a sufficient worker sample size per colony (at least 30 workers), the combination of these four rules will almost always unambiguously define the queen

genotype (Oldroyd *et al.*, 1996; Paar *et al.*, 2004a), and highlight any potential violations of the above assumptions.

The number of alleles, allele frequencies, effective number of alleles, and observed (H_0) and expected (H_e) heterozygosity (according to Hardy-Weinberg equilibrium assumptions) of each microsatellite locus were calculated using the GENEPOP 4.0.10 (Rousset, 2008), as were exact tests for Hardy-Weinberg equilibrium at each locus and genotypic linkage disequilibrium. We also used GENEPOP to test for genic differentiation and genetic distance (F_{ST}) between aggregations. We then performed a sibship reconstruction using COLONY version 2.0 (Wang, 2004) to examine the relatedness of queens within and between aggregations. All analyses were carried out with the inferred queen genotypes only (Paar *et al.*, 2004a).

RESULTS

The inferred genotypes of the queen heading each colony are shown in Table 5.1. Both within and between the aggregations, no queens were related as mother-daughter because no pair of inferred queens shared at least one allele at all of the eight microsatellite loci assayed. This result indicates that no adjacent colonies had been formed via a recent reproductive swarming event. The sibship reconstructions obtained from COLONY confirmed that none of the 54 queens were full-siblings. However, 35 queen pairs, involving 26 queens, were potentially related as half sisters (Table 5.2). These comprised 12 queen pairs within aggregations (three queen pairs in aggregation 1; four queen pairs in aggregation 2, and five queen pairs in aggregation 3). There were also 23 potential half sisters between aggregations (Table 5.2).

Tests for Hardy-Weinberg equilibrium were performed for each locus. There was no significant deviation from equilibrium for any one locus ($P > 0.05$) or over all loci ($P = 0.48$) (Table 5.3). There was no evidence of linkage disequilibrium between any pair of loci at a population ($P > 0.05$) level. Within populations three locus pairs showed significant linkage disequilibrium ($P < 0.05$) which I attribute solely to type I error. All F_{st} values between aggregations were not significantly different from zero ($P > 0.05$) (Fig. 5.1). Tests for allele frequency differences (genic differentiation) between aggregations also revealed no significant differences (Fig. 5.1).

Table 5.1 Inferred genotypes of 54 queen heading colonies within 3 aggregations in Tak province, north-western Thailand, at 8 microsatellite loci

Aggregation	Colony	Locus							
		A14	**A24**	**A88**	**B124**	**A76**	**Ad3**	**BI225**	**SV197**
A1	1	206/208	99/99	135/137	215/217	204/204	165/165	249/257	208/212
	2	204/210	105/105	135/139	215/217	206/216	163/165	253/253	210/214
	3	208/208	103/103	133/139	217/217	204/210	163/163	255/259	208/210
	4	204/210	99/99	129/141	215/215	204/210	171/199	235/247	210/210
	5	210/210	103/105	129/129	215/217	208/212	163/163	249/257	208/218
	6	204/210	103/?	143/146	217/217	202/214	165/171	243/253	212/220
	7	208/210	103/103	133/163	215/215	212/214	163/?	249/253	208/214
	8	206/208	97/105	129/146	215/217	208/210	163/171	253/257	208/212
	9	204/210	99/105	137/139	215/215	208/214	165/165	255/267	208/223
	10	210/210	103/105	129/129	215/215	204/204	161/163	255/257	210/212
A2	1	206/210	99/107	137/141	217/219	212/214	163/169	249/255	210/214
	2	206/211	99/109	135/137	215/215	210/214	163/163	257/257	208/214
	3	211/211	99/105	129/141	217/217	210/214	163/167	255/259	210/212
	4	204/204	101/103	139/146	215/217	204/210	163/165	257/259	212/220
	5	210/210	99/103	139/141	217/217	204/204	163/?	243/247	210/210
	6	204/210	99/103	135/150	215/217	204/208	165/?	257/269	208/208
	7	204/210	103/?	135/141	215/223	208/216	163/167	243/255	210/212
	8	210/210	99/101	129/133	215/217	208/210	165/167	243/247	210/212
	9	204/?	99/105	129/137	215/215	208/214	163/165	255/257	206/212
	10	210/211	103/107	139/141	215/?	204/206	165/169	249/255	212/214
	11	206/206	103/103	133/137	215/217	204/214	165/167	239/243	210/216
	12	210/211	99/99	135/135	215/217	204/212	163/167	249/253	212/243
	13	211/211	103/105	135/135	215/217	204/214	165/165	255/263	210/212
	14	204/208	99/?	139/141	215/217	204/210	163/163	257/275	208/212
	15	204/208	99/103	141/157	215/215	204/214	165/165	247/249	208/218
	16	204/206	105/107	133/133	215/217	204/212	161/165	249/255	212/224
	17	210/210	99/99	133/139	215/233	204/216	163/?	255/255	210/210
	18	208/210	103/111	141/148	215/217	204/204	167/198	255/263	210/210
	19	208/210	99/99	137/141	215/217	214/214	163/163	253/255	210/212
	20	204/208	99/103	135/139	215/215	204/216	163/163	249/255	216/?
	21	206/208	99/107	131/141	215/217	204/214	165/165	251/259	208/210

Table 5.1 (Continued)

Aggregation	Colony	Locus							
		A14	**A24**	**A88**	**B124**	**A76**	**Ad3**	**BI225**	**SV197**
A2	22	208/215	99/103	135/139	215/217	210/216	167/193	255/265	206/210
	23	204/211	103/105	129/148	215/217	206/214	165/169	249/255	212/214
A3	1	208/210	99/109	139/141	215/215	206/214	163/163	255/257	213/213
	2	208/210	99/105	150/153	215/215	204/216	165/165	249/253	205/215
	3	208/210	105/107	137/157	215/217	204/210	165/?	247/255	209/209
	4	210/210	101/103	129/135	215/215	210/216	161/163	249/253	207/211
	5	204/208	103/103	131/141	215/215	204/214	161/165	253/259	207/207
	6	210/210	99/107	129/139	215/217	204/206	165/169	243/261	211/217
	7	208/210	103/105	131/137	215/?	204/204	165/165	253/257	207/209
	8	204/206	99/101	137/141	215/217	204/214	163/165	235/251	207/207
	9	208/210	101/107	135/141	215/215	204/204	163/163	253/255	209/219
	10	204/210	101/103	133/137	217/217	204/204	163/165	249/255	207/207
	11	204/210	99/107	129/139	217/217	204/214	165/165	247/249	209/213
	12	204/204	99/103	131/141	215/215	204/204	163/165	245/255	209/209
	13	210/211	99/99	139/141	215/217	202/208	161/169	249/255	207/211
	14	204/210	99/103	139/141	215/217	204/212	163/163	255/267	207/211
	15	210/211	99/103	137/143	217/223	202/214	165/165	255/257	211/211
	16	206/206	99/103	133/159	215/215	214/?	163/165	247/261	207/211
	17	204/210	99/105	135/135	215/215	212/218	163/163	249/257	207/211
	18	210/210	99/107	129/141	215/215	204/204	167/167	247/255	211/211
	19	204/210	103/105	131/135	215/215	204/216	163/165	251/257	205/211
	20	204/211	99/109	129/131	215/217	204/208	163/169	255/259	207/217
	21	204/210	99/103	139/141	215/221	212/212	165/165	253/257	211/211

Table 5.2 Number of queens potentially related as half sisters of inferred queens within and between three aggregations of *A. dorsata*

	Aggregation 1	**Aggregation 2**	**Aggregation 3**
Aggregation 1	3	-	-
Aggregation 2	7	4	-
Aggregation 3	5	11	5

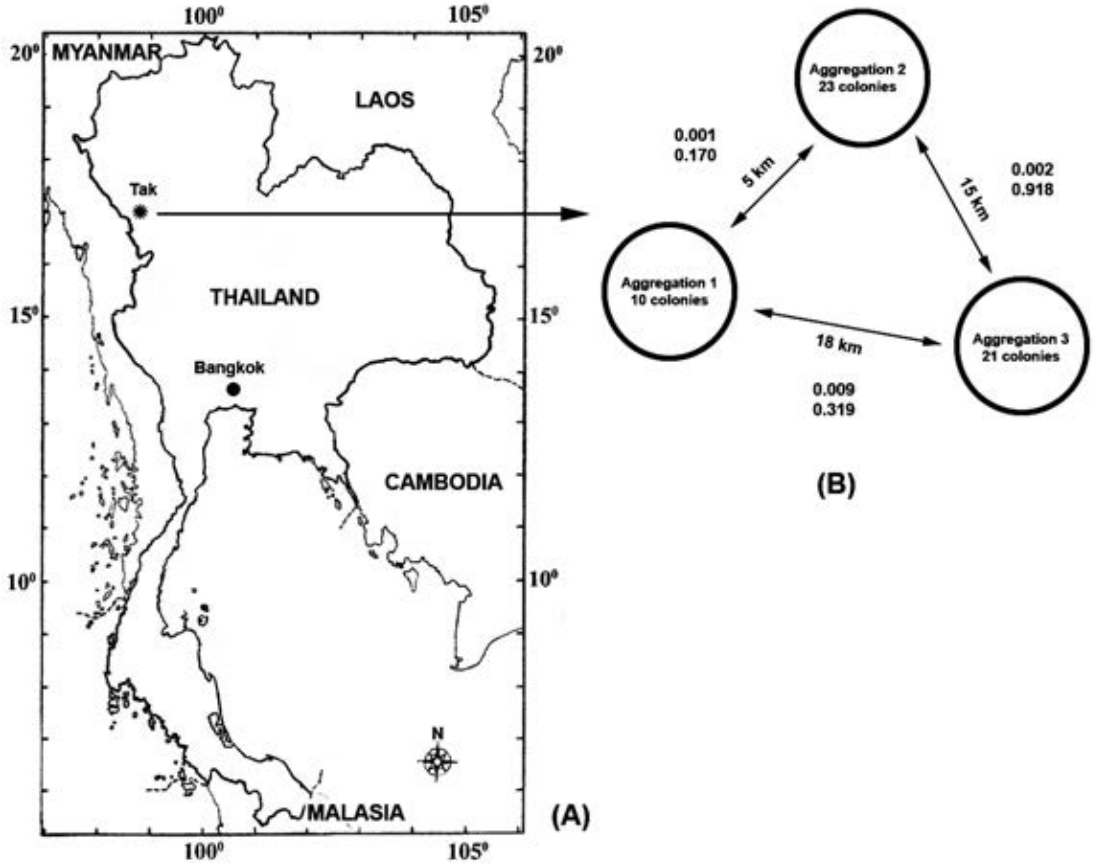

Figure 5.1 (A) Sampling sites for *A. dorsata* in Thailand. (B) F_{st} values (top) and degree of genic differentiation (bottom) for the three aggregations of *A. dorsata* in Tak province, northwest Thailand

Table 5.3 Observed (H_o) and expected heterozygosity (H_e) at 8 loci in *A. dorsata* populations within three aggregated colonies in Thailand

Locus		**Aggregation 1**	**Aggregation 2**	**Aggregation 3**	**All aggregations**
A14	Number of alleles	4	6	5	6
	Number of effective alleles	3.175	4.854	3.401	3.968
	H_o	*0.700*	*0.682*	*0.762*	*0.717*
	H_e	*0.685*	*0.794*	*0.706*	*0.748*
A24	Number of Alleles	4	7	6	8
	Number of effective alleles	3.311	3.676	4.444	4.016
	H_o	*0.444*	*0.809*	*0.905*	*0.784*
	H_e	*0.698*	*0.728*	*0.775*	*0.751*
A88	Number of Alleles	9	11	12	15
	Number of effective alleles	6.289	4.237	9.009	8.197
	H_o	*0.800*	*0.869*	*0.952*	*0.889*
	H_e	*0.841*	*0.764*	*0.889*	*0.878*
B124	Number of Alleles	2	5	4	6
	Number of effective alleles	1.923	2.267	1.901	2.070
	H_o	*0.400*	*0.727*	*0.400*	*0.538*
	H_e	*0.480*	*0.559*	*0.474*	*0.517*
A76	Number of Alleles	8	7	9	9
	Number of effective alleles	5.714	4.608	3.953	4.630
	H_o	*0.800*	*0.869*	*0.700*	*0.792*
	H_e	*0.825*	*0.783*	*0.747*	*0.784*

Table 5.3 (Continued)

Locus		**Aggregation 1**	**Aggregation 2**	**Aggregation 3**	**All aggregations**
Ad3	Number of Alleles	5	7	5	9
	Number of effective alleles	3.378	3.731	3.096	3.496
	H_o	*0.556*	*0.650*	*0.500*	*0.571*
	H_e	*0.704*	*0.732*	*0.677*	*0.714*
BI225	Number of Alleles	9	13	12	17
	Number of effective alleles	6.250	6.993	7.407	7.633
	H_o	*0.900*	*0.913*	*1.00*	*0.944*
	H_e	*0.840*	*0.857*	*0.865*	*0.869*
SV197	Number of Alleles	7	10	8	10
	Number of effective alleles	4.762	4.608	4.464	4.831
	H_o	*0.900*	*0.818*	*0.619*	*0.755*
	H_e	*0.790*	*0.783*	*0.776*	*0.793*

DISCUSSION

This study suggests that the *A. dorsata* population I sampled in north-west Thailand is panmictic, and characterized by limited genetic structure. Insuan *et al.* (2007) found surprisingly low matrilineal diversity in the *A. dorsata* population of Thailand at broad scales, with one mitochondrial type present at frequencies between 0.92 to 1.00 throughout the country. Similarly, nuclear microsatellites revealed no evidence of population structuring at broad scales (Insuan *et al.*, 2007). This homogeneity suggests a recent population bottleneck or selective sweep, affecting the whole country. Despite the large distances involved, frequent long distance migration apparently homogenizes allelic diversity, and has allowed a single mitochondrial lineage to colonize a broad area. As in Thailand, there is no detectable genetic structuring of the *A. dorsata* population in Borneo based on mitochondrial haplotypes (Tanaka *et al.*, 2001).

The present study reflects these trends at local scales. There was no genetic differentiation among aggregations, suggesting that matings may occur between aggregations (*i.e.* queens from one aggregation may mate with drones from another aggregation), and that migrating swarms that colonize a bee tree come from diverse sources (*i.e.* colonies from one aggregation do not all go to the same off-season locality after migration, Paar *et al.*, 2004). Lack of linkage disequilibrium also suggests that the population is panmictic.

This study shows that like *A. mellifera* (Oldroyd *et al.*, 1995) and *A. florea* (Wattanachaiyingcharoen *et al.*, 2008) aggregations of *A. dorsata* do not arise from the clustering of daughter colonies. No colonies in this study were related as mother-

daughter, as has been previously reported for aggregated *A. dorsata* colonies in Malaysia and India (Oldroyd *et al.*, 2000; Paar *et al.*, 2004a). The absence of offspring colonies suggests that reproductive swarms leave the vicinity of their mother colony, possibly moving to another aggregation (Wattanachaiyingcharoen *et al.*, 2008). Thus, the rapid increase in *A. dorsata* population size during general flowering events (Itioka *et al.*, 2001) is caused by migratory swarming rather than reproductive swarming events at the site.

Remarkably, I found no evidence for individuals that had drifted between nests – that is all worker genotypes within any one colony were compatible with being the daughters of a single polyandrous queen. Paar *et al.* (2002), found that the proportion of drifted workers in *A. dorsata* colonies ranged from 0 to 6.25% with an average of 1.27%. They also showed that there was no correlation between the direction of drift and the position of the nests. This study supports the conclusion that drifting is remarkably rare in *A. dorsata* aggregations, given the close proximity of the nests.

The results also suggest that the long distance migratory behavior of *A. dorsata* allows it to tolerate habitat fragmentation. Local extinction can be relieved by migration, perhaps from still-heavily forested areas in Burma. *A. dorsata* migration may also afford protection to remnant patches of dipterocarp forests which, if they are of sufficient size, may be able to attract *A. dorsata* colonies during general flowering events. This suggests that individual nesting trees are of extreme importance to the rapid increase in the number of colonies during a general flowering event in the dipterocarp forests of Southeast Asia, and should be fully protected.

As with *A. dorsata*, the dwarf honeybees *A. florea* and *A. andreniformis* are hunted heavily wherever they occur in Thailand (Oldroyd and Wongsiri, 2006). Both the *A. florea* (Hepburn *et al.*, 2005; Nanork, 2001) and *A. andreniformis* (Rattanawannee *et al.*, 2007) populations show remarkable uniformity throughout their range, with no evidence of population structuring. This uniformity may also reflect the ability of the dwarf bees to recolonize areas that are depleted by hunting. Thus, while we strongly caution that honeybee hunting may be at unsustainable levels, there is evidence that high rates of reproduction and migration currently allow Asian honeybees to endure it.

CHAPTER VI

No Evidence that Habitat Disturbance Affects Mating Frequency in the Giant HoneyBee, *Apis dorsata*

A version of this chapter was submitted to Apidologie (Recommended to publish: minor revision)

ABSTRACT

The giant honeybee (*Apis dorsata*) is a keystone pollinator within Asian lowland forests. Across its range, *A. dorsata* populations are impacted by heavy hunting pressure and habitat disturbance. These pressures have the potential to significantly impact the genetic structure of populations, particularly the ability of queens to find a large number of genetically diverse drones for mating. Here I compare queen mating frequency and allelic diversity between colonies sampled in disturbed and undisturbed areas in Thailand. Microsatellite analysis of 18 colonies in 6 aggregations showed no significant difference in paternity frequency at disturbed and undisturbed habitats. Measures of F_{ST} and between aggregations were not significantly different from zero ($P > 0.05$), and measures of allelic diversity showed no differences between disturbed and undisturbed sites. Our findings suggest that habitat disturbance has no effect on mating frequency or genetic diversity. This suggests that the mating behaviour of *A. dorsata* is robust to anthropogenic changes to the landscape.

INTRODUCTION

The giant honeybee, *Apis dorsata* Fabricius, 1793, is distributed from the Indian subcontinent to Southeast Asia (Ruttner, 1988). Colonies construct a massive single-comb nest, usually hanging in high, inaccessible places (e.g. beneath a tall tree branches, cliff overhangs and the eaves of a building) (Oldroyd and Wongsiri, 2006). The species is heavily hunted throughout its range for honey, wax and brood, providing an important source of household income to many local people (Lahjie and Seibert, 1990; Nath *et al.*, 1994; Soman and Kshirsagar, 1991 ; Strickland, 1982).

The ecology and behaviour of *A. dorsata* differ substantially from the other *Apis* species (Moritz *et al.*, 1995; Oldroyd and Wongsiri, 2006; Paar *et al.*, 2004a). Colonies are often found in dense aggregations, sometimes with over 200 colonies present on a single tree or rock face (Oldroyd *et al.*, 2000), often separated by only a few centimetres (Paar *et al.*, 2004a). *A. dorsata* is a peripatetic species (Moritz *et al.*, 1995), with colonies frequently undergoing a seasonal migration between nesting sites. Colonies usually occupy a nest site for 3-4 months (Paar *et al.*, 2004a). At the end of this period, colonies abscond to an alternative nesting or bivouac site, leaving an empty comb. Absconding swarms probably migrate between locations with different blooming seasons (Crane *et al.*, 1993; Dyer, 2002; Itioka *et al.*, 2001; Koeniger and Koeniger, 1980; Liu *et al.*, 2007; Sheikh and Chetry, 2000). Nesting sites are often reoccupied annually for decades (Oldroyd *et al.*, 2000; Oldroyd and Wongsiri, 2006).

Like other *Apis* species, the mating of virgin queens and drones in *A. dorsata* takes place during flight at some distance from the colony. Virgin queens and drones

leave their colonies at dusk, fly to well-defined drone congregation areas (DCAs), and return 15 - 30 min later (Koeniger *et al.*, 1994; Rinderer *et al.*, 1993; Tan *et al.*, 1999). *A. dorsata* virgin queens undertake 2 to 4 mating flights (Tan *et al.*, 1999). It is unknown if all the drones from all colonies within an aggregation area fly to a single DCA or if several aggregations service the same DCA. However Kraus et al. (2005) showed that the temporal genetic structure of an *A. dorsata* DCA showed significant genetic differentiation across three sampling days, supporting the hypothesis that the DCA was occupied by at least two subpopulations every day, but in varying proportions. In addition, the overall effective population size (N_e) was estimated to be as high as 140 colonies. This suggests that drones from the majority of colonies within the recruitment range of this DCA were represented (Kraus *et al.*, 2005a).

All *Apis* species have an extremely high but very variable level of polyandry (Oldroyd *et al.*, 1998; Palmer and Oldroyd, 2000; Strassmann, 2001; Tarpy *et al.*, 2004). Extreme paternity frequency in *A. dorsata* was first reported by Moritz *et al.* (1995). They found that queens mated on average with 30.17 ± 5.98 drones with a range from 19 to 53, while the average effective number of matings (i.e. corrected for variance in paternity skew between males) was 25.56 ± 11.63. Similarly, Oldroyd *et al.* (1996) reported that *A. dorsata* queens mated on average with 26 ± 5.42 drones and the mean effective mating frequency was 19.96 ± 6.63. Wattanachaiyingcharoen *et al.* (2003), who genotyped a larger sample size per colony, found 47 to 102 subfamilies per colony and an effective mating frequency ranged between 26.9 and 88.5. This indicates that there is a wide range of extreme mating frequencies in this species (Wattanachaiyingcharoen *et al.*, 2003).

The mating biology of *A. dorsata*, particularly the extreme mating frequency and the brief but multiple mating flights at dusk, suggests that *A. dorsata* queens and their offspring colonies may be placed at a fitness disadvantage if young queens are unable to locate nearby DCAs with large numbers of genetically diverse drones (Oldroyd *et al.*, 1995; Oldroyd *et al.*, 2000). Genetic diversity within colonies has multiple fitness benefits for honeybees, including increased disease resistance (Palmer and Oldroyd, 2000; Seeley and Tarpy, 2007; Tarpy and Seeley, 2006), improved task allocation (Jones *et al.*, 2004; Mattila and Seeley, 2007; Mattila and Seeley, 2011; Oldroyd and Fewell, 2007) and increased brood viability (Page, 1980). Indeed, colony aggregations in *Apis* may have evolved in order to facilitate mating with genetically diverse drones (McNally and Schneider, 1996; Oldroyd *et al.*, 1995; Oldroyd *et al.*, 2000).

I hypothesized that anthropogenic disturbance to the landscape might influence the mating biology of *A. dorsata* populations, reducing the number and genetic diversity of males available to queens for mating. To test this hypothesis I estimated the degree of genetic variation and the level of polyandry within *A. dorsata* colonies in disturbed and undisturbed areas of Thailand to assess whether habitat disturbance has an adverse effect on the mating frequency in this iconic species.

MATERIALS AND METHODS

Study sites and sample collection

Aggregations of *A. dorsata* were located in disturbed and undisturbed in northern Thailand (Table 6.1). I defined a disturbed habitat as an area substantially cleared for agricultural purposes. Undisturbed habitats were defined as a significant area of primary forest that had not been harvested. The aggregations studied were located at least 2 km within the borders of the defined habitat type.

A colony aggregation was defined as a group of colonies assembled on a single tree or building, including any solitary nests nearby (< 40m) the main group (Paar *et al.*, 2004a). I collected adult workers from 6 aggregations (3 aggregations in each habitat type) (Table 6.1). I sampled at least 300 workers (3 colonies per aggregation) from the curtain of bees on the lower edge of the comb. Samples were collected by local honey hunters who I paid to climb the trees or building. All samples were taken carefully in the late evening without causing workers to take flight from the nest, thereby minimizing drift between colonies (Paar *et al.*, 2002; Paar *et al.*, 2004a). Sampled workers were preserved in 95% (v/v) ethanol until used for DNA extraction. The number of *A. dorsata* colonies in each particular aggregation was counted and the location recorded using a GPS.

Table 6.1 Sample locations and number of *A. dorsata* colonies present in each of six aggregations in Thailand

Sampling location	Coordinate	Habitat type	Observed (*n* colonies)
Nan (north)	19 º 10.24‟ N 100º 55.41‟ E	Disturbed**:** Urban area and surrounded by rice fields	41
Sakon Nakhon (northeast)	16º 52.23‟ N 103º 56.28‟ E	Disturbed: Agricultural area (rice field)	20
Chaing Rai (north)	20º 17.162‟ N 99º 48.88‟ E	Disturbed: Urban area and surrounded by rice fields	18
Tak (northwest)	17º 01.06‟ N 98º 56.57‟ E	Undisturbed: Mae Tuen National Park	23
Tak (northwest)	17º 03.56 N 98º 56.23‟ E	Undisturbed: Mae Tuen National Park	21
Nan (north)	19º 11.21‟ N 100º 57.48‟ E	Undisturbed: Phu Ka National Park	28

DNA extraction and genotyping

DNA was extracted from the hind leg of each individual worker bee using a 5% (w/v) Chelex solution (Chelex®100; catalog no. 143-2832, BIO-RAD) (Walsh *et al.*, 1991). The microsatellite target sequences were amplified by multiplex polymerase chain reactions (PCR) with fluorescently labeled primers. The microsatellite loci used were A14, A76, A88, and BI225 (Estoup *et al.*, 1994; Estoup *et al.*, 1993; Solignac *et al.*, 2007). PCR condition is as in Chapter 5. Samples containing no DNA were included in all plates as negative controls. I then resolved PCR products in a 3130xl Genetic Analyzer (Applied Biosystems, California) according to methods described in Chapter 5. Resultant data files were analyzed to determine allele size using GENEMAPPER software (Applied Biosystems).

Reconstruction of queen genotypes and identifying patrilines

The genotype of the queen heading each colony was inferred from the worker genotypes (Oldroyd *et al.*, 2000; Oldroyd *et al.*, 1996). I excluded any worker that did not carry a queen allele as a drifted individual. After the genotype of the queen was determined, the genotype of the fathering drone was determined for each worker by subtraction (Oldroyd and Wongsiri, 2006). Where the paternal allele could not be distinguished from the maternal allele, paternity was allocated according to the proportion of homozygotes in the sample (Oldroyd *et al.*, 1997).

Genetic diversity measures

The number of alleles, number of effective alleles, allele frequencies, and observed (H_0) and expected (H_e) heterozygosity (according to Hardy-Weinberg

equilibrium assumptions) of each microsatellite locus were calculated using the GENEPOP 4.0.10 (Rousset, 2008), as were exact tests for Hardy-Weinberg equilibrium at each locus and genotypic linkage disequilibrium. I also used GENEPOP to test for genic differentiation and genetic distance (F_{ST}) between aggregations. All analyses were carried out with the inferred queen genotypes only (Paar *et al.*, 2004a).

Mating frequency determination

Effective mating frequency (m_e) within each colony with a correction for finite sample size was calculated according to Tarpy and Nielsen (2002) as:

$$m_e = \frac{(n-1)^2}{(\sum_{i=1}^{k} p_i^2)(n+1)(n-2)+3-n}$$

where k is the number of patrilines observed, p_i is the proportion of workers sired by the i^{th} male, and n is the number of workers scored.

Average relatedness, r, weighted according to the relative proportions of each subfamily and corrected for finite sample size, was calculated for each colony according to Oldroyd and Moran (1983) as = $\frac{1}{4}+\frac{1}{2m_e}$.

In order to make valid comparison of mating frequency between disturbed and undisturbed sites, and for comparison with previous studies of mating frequency in *A. dorsata*, observed paternity frequency, *k*, was adjusted to a common sample size of *n* according to Franck *et al.* (2000) as:

$$k_n = \sum_{i=1}^{k} (1 - C^n_{h-n_i} / C^n_h)$$

where k_n is the number of patrilines estimated for sample size *n*, *k* is the total number of patrilines observed, *n* is the number of workers in the i^{th} subfamily and *h* is the sample size. For each comparison I set *n* to the sample size of the colony with the lowest sample size.

Non-detection and non-sampling errors

The number of patrilines in a colony may be underestimated if a patriline goes undetected if two or more drones involved in the mating have identical genotypes (non-detection error), or if a patriline is not represented in the sample (non-sampling error). The probability that two drones in a population in Hardy-Weinberg will have identical genotypes at all loci studied, and thus be genetically indistinguishable (non-detection error, *nd*) was calculated according to Foster *et al.* (1999) as:

$$nd = \prod^{j} \left(\sum q_i^2 \right)$$

where q_i are the frequencies of the *i* alleles at each of the *j* loci. Allele frequencies were determined from deduced queen and drone genotypes from each of the 18 colonies of this study.

The probability of not sampling a particular patriline, *ns*, was calculated according to Foster *et al.* (1999) as:

$$ns = (1\text{-}p)^n$$

where p is the proportion of offspring in the patriline and n is the number of workers sampled.

Statistical tests

I tested for paternity skew for each colony using *G* tests (Palmer *et al.*, 2001). I compared paternity frequencies, effective paternity frequency, estimates of paternity frequency with common sample size n = 87, and intra-colonial relatedness between disturbed and undisturbed sites using ANOVA, with aggregation nested within habitat type. I also compared estimates of effective paternity frequency found in this study with those observed by Wattanachaiyingchareon et al. (2003) and Oldroyd et al. (1996) using the independent samples *t*-test using a common sample size n = 87 and 42 (k_{87} and k_{42},) respectively.

RESULTS

Population genetic structure

The number of alleles, number of effective alleles, allele frequencies, and observed (H_0) and expected (H_e) heterozygosity are shown in Table 6.2. There was no significant deviation from equilibrium for any one locus ($P > 0.05$) or over all loci ($P = 0.96$). There was no evidence of linkage disequilibrium between any pair of loci at

the population level ($P > 0.05$). Furthermore, no individual aggregation showed significant linkage disequilibrium ($P > 0.05$). All F_{ST} values between aggregations were not significantly different from zero ($P > 0.05$). Tests for allele frequency differences (genic differentiation) between aggregations also revealed no significant differences.

Paternity frequency in disturbed and undisturbed sites

The four microsatellite loci (A14, A76, A88, and BI225) had sufficient variability to differentiate the parental genotypes within the colonies (Table 6.2).

Observed mating frequency, k, ($F_{1,12} = 0.0004$, $P = 0.984$), effective mating frequency, m_e ($F_{1,12} = 0.232$, $P = 0.639$), mating frequency corrected for sample size, k_{87} ($F_{1,12} = 0.097$, $P = 0.760$) and intra-colonial relatedness, r, ($F_{1,12} = 0.480$, $P = 0.502$) were not significantly different between disturbed and undisturbed sites (Table 6.3). I found significant paternity skew in all colonies studied ($P < 0.01$).

The probability of failing to detect a patriline due to genetically identical inseminating drones (*nd*) (Boomsma and Ratnieks, 1996) was 0.0018 based on allele frequencies from the entire study. This means that fewer than two fathering males were expected to be undetected in all 18 colonies (Table 6.3).

A patriline that is well represented in a colony has a low probability of not being sampled. For our sample size, the probability of not sampling a patriline represented by 1/20 of the worker population ranged from 0.0001 to 0.0037. However, a patriline that represented by only a small proportion of a colony‟s worker population had a higher probability of non-sampling. For example, if a patriline was

represented by 1/50 of worker population, the probability of not sampling a worker from this patriline ranged from 0.027 to 0.172 with the sample sizes used in the present study. In some patrilines only one worker was identified, suggesting that some patrilines went undiscovered.

I found no significant difference in deduced paternity frequency (k_{87}) and effective paternity frequency (m_c) between disturbed and undisturbed sites ($F_{1,12}$ = 0.097, P = 0.760 and $F_{1,12}$ = 0.232, P = 0.639, respectively). Additionally, there was no significant difference in k_{87} and m_c values between aggregations within habitat types ($F_{5,12}$ = 0.128, P = 0.983 and $F_{5,12}$ = 0.399, P = 0.841, respectively).

The frequency of drifted workers was very low, ranging from 0 to 2.42% with an average of 0.83% (SE = 0.21) (Table 6.3).

I found significantly lower k_{87} and m_c values than those found by Wattanachaiyingcharoen *et al.* (2003) (P<0.001 2-tail t-test) (Table 6.3). I also found no significant difference in effective paternity frequency from that found by Oldroyd *et al.* (1996) (P=0.057 2-tail t-test).

Table 6.2 Number of alleles, allele frequencies, number of effective alleles and observed (H_o) and expected heterozygosity (H_e) at 4 loci in *A. dorsata* populations within six aggregated colonies in Thailand.

Locus	Parameter	Disturbed			All Disturbed	Undisturbed			All Undisturbed	All aggregations
		D1	D2	D3		UD1	UD2	UD3		
A14	Allele length (bp) 204	0.167	0.333	0.167	0.222	0.167	0.167	0.167	0.167	0.194
	206	-	-	0.333	0.111	0.333	-	-	0.111	0.111
	208	0.167	0.167	-	0.111	0.167	0.333	0.333	0.278	0.194
	210	0.667	0.500	0.500	0.556	0.167	0.500	0.500	0.389	0.472
	211	-	-	-	-	0.167	-	-	0.056	0.028
	Number of Alleles	3	3	3	4	4	3	3	5	5
	Number of effective alleles	1.997	2.572	2.572	2.610	4.495	2.572	2.572	3.677	3.214
	H_o	*0.667*	*0.667*	*1.00*	*0.778*	*0.667*	*1.00*	*0.667*	*0.778*	*0.778*
	H_e	*0.499*	*0.611*	*0.611*	0.617	*0.777*	*0.611*	*0.611*	*0.728*	*0.689*

Table 6.2 (Continued)

A76	Allele length (bp) 205	0.167	0.333	-	0.167	0.500	0.333	-	0.278	0.222
	207	0.167	-	-	0.056	-	0.167	0.333	0.167	0.111
	209	0.333	0.167	-	0.167	-	-	0.333	0.111	0.139
	211	-	0.167	0.333	0.167	-	0.167	-	0.056	0.111
	213	-	-	0.333	0.111	0.167	-	-	0.056	0.083
	215	0.333	0.167	0.167	0.222	0.333	0.167	0.167	0.222	0.222
	217	-	0.167	0.167	0.111	-	0.167	0.167	0.111	0.111
	Number of Alleles	4	5	4	7	3	5	4	7	7
	Number of effective alleles	3.603	4.495	3.603	6.222	2.572	4.495	3.603	5.394	6.183
	H_o	*1.00*	*0.667*	*1.00*	*0.889*	*1.00*	*1.00*	*1.00*	*1.00*	*0.944*
	H_e	*0.722*	*0.777*	*0.722*	0.839	*0.611*	*0.777*	*0.722*	*0.815*	*0.838*

Table 6.2 (Continued)

A88	Allele length (bp) 129	-	0.500	0.500	0.333	-	0.333	0.167	0.167	0.250
	131	0.167	-	-	0.056	-	0.167	0.167	0.111	0.083
	133	0.167	-	-	0.056	0.167	-	-	0.056	0.056
	135	0.167	-	-	0.056	0.333	0.167	0.167	0.222	0.139
	137	-	0.167	-	0.056	0.167	-	-	0.056	0.056
	139	0.333	0.167	0.167	0.222	-	-	0.167	0.056	0.139
	141	-	-	0.167	0.056	0.167	-	-	0.056	0.056
	145	0.167	0.167	0.167	0.167	-	-	0.167	0.056	0.111
	151	-	-	-	-	-	0.167	-	0.056	0.028
	153	-	-	-	-	-	0.167	-	0.056	0.028
	157	-	-	-	-	0.167	-	0.167	0.111	0.056
	Number of Alleles	5	4	4	8	5	5	6	11	11
	Number of effective alleles	4.495	2.997	2.997	4.908	4.495	4.495	5.976	8.080	7.437
	H_o	*1.00*	*0.667*	*1.00*	*0.889*	*0.667*	*1.00*	*1.00*	*0.889*	*0.889*
	H_e	*0.777*	*0.666*	*0.666*	*0.796*	*0.777*	*0.777*	*0.833*	*0.876*	*0.865*

Table 6.2 (Continued)

BI225	Allele length (bp) 235	-	0.167	-	0.056	-	-	-	-	0.028
	239	-	-	-	-	0.167	-	-	0.056	0.028
	243	-	-	0.167	0.056	-	-	-	-	0.028
	247	-	-	-	-	0.167	-	-	0.056	0.028
	249	0.333	0.167	0.167	0.222	0.167	0.333	0.167	0.222	0.222
	251	0.167	0.333	-	0.167	-	-	-	-	0.083
	253	0.167	-	-	0.056	0.167	0.333	0.500	0.333	0.194
	255	0.167	0.167	0.500	0.278	0.167	0.167	-	0.111	0.194
	257	-	-	-	-	-	0.167	0.167	0.111	0.056
	261	-	0.167	-	0.056	-	-	-	-	0.028
	263	0.167	-	-	0.056	0.167	-	-	0.056	0.056
	279	-	-	0.167	0.056	-	-	0.167	0.056	0.056
	Number of Alleles	5	5	4	9	6	4	4	8	12
	Number of effective alleles	4.495	4.495	2.997	5.771	5.976	3.603	2.997	5.067	6.907
	H_o	*1.00*	*1.00*	*1.00*	*1.00*	*1.00*	*1.00*	*0.677*	*0.889*	*0.944*
	H_e	*0.777*	*0.777*	*0.666*	*0.828*	*0.833*	*0.722*	*0.666*	*0.803*	*0.855*

Table 6.3 Paternity frequency in *A. dorsata* colonies from disturbed and undisturbed habitats in Thailand. Effective paternity frequency (m_c), observed paternity frequency (k), paternity frequency corrected to a standard sample size of 87 and 42 (k_{87} *and* k_{42}, respectively), average worker relatedness (r), patriline non-detection error (nd), the proportion of drifted workers, and number of workers analysed per colony (n) are listed for each colony within each aggregation. Bracketed numbers are the standard errors.

Habitat type	Aggregation	Colony	n	k	k_{87}	k_{42}	m_c	r	nd	Drift (%)
Disturbed	D1	1	130	39	35.59	25.90	37.61	0.319	0.002	0.59
		2	141	54	46.02	30.18	40.49	0.281	0.002	1.75
		3	178	61	45.69	29.63	40.62	0.284	0.002	0
	D2	1	160	67	50.88	31.57	46.04	0.271	0.001	0.61
		2	157	45	37.24	25.98	29.52	0.316	0.001	0.57
		3	153	30	27.35	20.49	12.35	0.464	0.003	0
	D3	1	160	55	43.88	29.07	38.32	0.288	0.001	1.67
		2	161	43	36.37	25.79	29.81	0.330	0.002	0
		3	133	49	43.06	29.17	37.96	0.287	0.002	2.42
Mean (SE)			152.56 (5.10)	49.22 (0.63)	40.68 (0.40)	27.53 (0.19)	34.75 (3.29)	0.316 (0.019)	0.0018 (0.00022)	0.84 (0.30)

Table 6.3 (Continued)

Habitat type	Aggregation	Colony	*n*	*k*	k_{87}	k_{42}	m_c	*r*	*nd*	Drift (%)
Undisturbed	UD1	1	166	50	40.20	27.12	30.62	0.304	0.001	0.55
		2	164	64	48.19	30.55	42.83	0.275	0.001	0
		3	163	40	31.89	22.82	22.41	0.352	0.002	0
	UD2	1	157	59	46.54	30.25	42.57	0.279	0.001	0
		2	109	54	48.28	30.54	37.13	0.268	0.001	2.24
		3	176	44	35.17	24.57	24.16	0.339	0.002	1.63
	UD3	1	116	34	31.38	23.34	22.50	0.333	0.002	0.56
		2	101	44	40.89	26.81	26.36	0.279	0.003	0
		3	87	55	55.00	33.29	43.25	0.261	0.003	2.30
Mean (SE)			137.67 (11.29)	49.33 (0.53)	41.95 (0.45)	27.70 (0.20)	32.43 (3.02)	0.299 (0.011)	0.0018 (0.00028)	0.81 (0.33)
Mean (total study) (SE)			145.11 (6.28)	49.28 (0.28)	41.31 (0.21)	27.61 (0.09)	33.59 (2.19)	0.307 (0.011)	0.0018 (0.00017)	0.83 (0.21)
Wattanachaiyingcharoen et al. (2003) Mean (SE)			207.4 (15.2)	80.1 (3.6)	51.78 (0.19)	-	63.0 (5.7)	0.259 (0.001)	-	-
Oldroyd et al. (1996) Mean (SE)			86 (33.4)	26.8 (5.4)	-	21.21 (0.87)	22.0 (6.5)	0.280 (0.009)	-	-

DISCUSSION

Mating frequency and environmental disturbance

My study shows that there is no significant difference in the mating frequency of *A. dorsata* queens sampled in disturbed and undisturbed habitats. This indicates that habitat disturbance has no measurable effect on mating frequency in *A. dorsata*, and so my hypothesis that mating frequency and intra-colonial genetic diversity might be adversely affected in disturbed areas can be rejected.

Franck *et al.* (2000) suggested that the documented differences in mating frequency between populations of *A. mellifera* probably arise from variance in the risks incurred on mating flights. The cost of multiple mating principally depends on the number and duration of mating flights (Franck *et al.*, 2000), as flights during bad weather are more likely to result in queen death and will reduce the likely levels of polyandry (El-Niweiri and Moritz, 2011; Kraus *et al.*, 2004; Neumann *et al.*, 1999; Schluns *et al.*, 2005). Franck *et al.* (2000) postulated that *A. mellifera* queens modulate the number of mating flights they undertake according to prevailing environmental conditions. For example, if a queen is obliged to mate during rainy weather, it most likely reduces the frequency and duration of mating flights, thereby reducing the genetic diversity of sperm in her spermatheca. Oldroyd *et al.* (1995) proposed that the genus-wide tendency for honeybee colonies to be aggregated may be driven by the need for proximity to drone congregation areas, thereby facilitating mating with a diversity of males. I suggested that this is especially the case in *A. dorsata* (Oldroyd *et al.*, 2000) where mating flights are brief (Rinderer *et al.*, 1993). However the results of the present study revealed no difference in the mating

frequency of *A. dorsata* queens in disturbed and undisturbed environments, suggesting that there is no significant difference in mating opportunities at disturbed and undisturbed sites.

My results suggest that current levels forest clearing in Thailand to make way for agricultural and urban areas has not had a significant effect on the population structure of *A. dorsata*, or on the mating frequency. A potential reason for this is that there is frequent exchange of colonies between forested and cleared areas. Thus a queen that mated in a disturbed habitat may found a colony in an undisturbed habitat and vice versa.

Genetic structure of *A. dorsata* in Thailand

There was no significant difference in the degree of genic differentiation between aggregations in disturbed and undisturbed habitats showed, implying that there are no significant barriers to gene flow between disturbed and undisturbed habitats. Rattanawannee *et al.* (2012) found no genetic differentiation in *A. dorsata* populations at local scales in north-western Thailand. These results support the theory that the long distance migratory behavior of *A. dorsata* allows it to tolerate habitat fragmentation (Rattanawannee *et al.*, 2012). Furthermore, there was no genetic differentiation among aggregations, suggesting that matings may occur between aggregations (Rattanawannee *et al.*, 2012), and that migrating swarms that join an aggregation come from diverse sources (Paar *et al.*, 2004a).

Levels of polyandry in *A. dorsata*

My results confirm that *A. dorsata* queens have an extremely high mating frequency (Moritz *et al.*, 1995; Oldroyd *et al.*, 1996; Wattanachaiyingcharoen *et al.*, 2003). The observed mating frequencies for queens in this study ranged from 30 to 67. The average effective mating frequency in this study (33.59 ± 2.19) was not significantly different from that found by Oldroyd *et al.* (1996) (25.60 ± 11.60), but is considerably lower than that observed by Wattanachaiyingcharoen *et al.* (2003) (63.0 ± 5.70). Despite our correction for sample size, our estimate of mating frequency may be underestimated due to undetected fathers. Wattanachaiyingcharoen *et al.* (2003) genotyped up to 288 workers per colony, providing better sampling coverage.

In conclusion, I have shown that the environment disturbance has no effect on mating frequency in *A. dorsata*. I confirm that the giant honeybee populations are panmictic despite current levels of bee hunting and habitat fragmentation. This is likely due to the highly mobile nature of the species, as well as their high reproductive rates.

CHAPTER VII

CONCLUSIONS

The giant honeybee, *Apis dorsata,* is one of the most important pollinators of Asian tropical forests (Itioka *et al*., 2001; Oldroyd and WongSiri, 2006; Paar *et al*., 2004). The species is heavily hunted throughout its range for the honey, wax and brood. In addition, predicted increases of urban and agricultural areas, widespread use of pesticides and proliferation of street lighting are likely to have significant impacts on population viability (Oldroyd and Wongsiri, 2006; Oldroyd and Nanork, 2009).

In this thesis I have made a detailed population genetic analysis of the *A. dorsata* population of Thailand. I conclude that despite the significant anthropomorphic pressures on this species caused by hunting and deforestation, the species shows remarkable genetic diversity and connectedness. I find no evidence that the population is fragmented. I also estimated the level of polyandry within *A. dorsata* colonies in disturbed and undisturbed areas using microsatellite analysis. I suggest that the reproductive biology of *A. dorsata* makes it remarkably robust to habitat disturbance. I conclude that the long distance migratory behavior and high reproductive rate of *A. dorsata* allows it to tolerate heavy hunting and habitat fragmentation pressure.

7.1 Geometric Morphometric Analysis in Honeybees

Due to the decline of insect pollinators in natural habitats, the development of accurate tools for the identification of species in a field study is of increasing importance. Morphometric analysis has been widely used for identifying honeybee subspecies and populations because of its practicality and low cost. Standard

morphometric methods involve multiple measurements of various body parts across many individuals and are therefore time consuming. Recently, a geometric morphometric method, which is based on the description of shape in Cartesian coordinates, has been developed and wildly used to identify honeybee species and subspecies level (Gumiel *et al.*, 2003; Slice, 2007).

In Chapter III, I showed that it is possible to distinguish honeybee species using geometric morphometric analysis using fifteen easily-identified landmarks of the right forewing of drones and workers. I found that within each sex the patterns of forewing venation of *Apis andreniformis*, *A. florea, A. cerana*, and *A. dorsata* were easily clustered by species. In addition, the sex of the individual did not obstruct identification. The results revealed that the wing venation pattern carried sufficient information to identify 99% of individuals to species level. I therefore suggest that geometric morphometric analysis of a single wing can be a useful tool for species and sub-species level studies of bees and other insects or even some fossils.

The application of geometry was then used to characterize *A. dorsata* population collected from 31 different localities in Thailand. I measured 19 landmarks from the digitized images of the right forewing of 10 workers from each colony (730 bees from 73 colonies in total). The Cartesian coordinates obtained from 19 landmarks were aligned and followed by partial warps analysis. A principal component analysis plots are in close agreement with a hierarchical analysis of the squared Euclidian distances that reveal no clear distinction geographically-based demarcation of populations. Multivariate analysis of variance results also showed no significant difference between *A. dorsata* populations grouped into either the two regions of the north and south of the Isthmus of Kra, or into the five smaller

geographic regions of Thailand. I conclude that the *A. dorsata* of mainland Thailand constitute a single panmictic population.

7.2 Genetic Structure of the Giant Honeybee in Thailand

To assess population structure of *A. dorsata* colonies at a regional scale, I collected adult worker bees from all colonies present in 3 aggregations at Mae Tuen National Park, Tak province, northwestern Thailand. I determined genetic variation and the relatedness within and between aggregations of *A. dorsata* nests. Analysis of 8 microsatellite loci in 54 nests in 3 aggregations showed no linkage disequilibrium, suggesting that the population sampled in northwest Thailand is panmictic. Furthermore, the high levels of heterozygosity and the fact that no pairs of aggregations showed significant F_{st} values suggests that gene flow within this *A. dorsata* population is high. These results indicate that *A. dorsata* is presently tolerating seasonal harvesting and habitat destruction. I surmise that the population is maintained by immigration from forested areas to the northwest of my study sites in Myanmar.

I also determined the relatedness within and between aggregations of *A. dorsata*. Among the 54 nests in 3 aggregations, no colonies were related as mother-daughter. Hence, if reproduction occurred at these study sites, the daughter colonies dispersed. The rapid increases in *A. dorsata* colony numbers during general flowering events are most likely occurred by swarms arriving from other areas rather than by *in situ* reproduction.

7.3 The Effect of Habitat Disturbance on Mating Frequency of the Giant Honeybee

Habitat fragmentation is likely to have a significant impact on genetic structure of wild honeybee populations, particularly on the ability of queens to find a

large number of genetically diverse drones for mating. As with all honeybees, mating of *A. dorsata* takes place during flight at drone congregation areas (DCA) (Koeniger *et al.*, 1994; Rinderer *et al.*, 1993; Tan *et al.*, 1999). Extremely high mating frequency in *A. dorsata* virgin queens has been repeatedly reported (Moritz *et al.*, 1995; Oldroyd *et al.*, 1996; Wattanachaiyingcharoen *et al.*, 2003). In Chapter VI, I tested the hypothesis that habitat disturbance might reduce the number and genetic diversity of males available to *A. dorsata* queens for mating. To test this hypothesis, I estimated the degree of genetic variation and the level of polyandry within *A. dorsata* colonies in disturbed and undisturbed areas using microsatellite analysis. I found that the observed mating frequencies for 18 *A. dorsata* queens in 6 aggregations ranged from 30 to 67, with an average effective mating frequency of 33.59 ± 2.19. These results confirmed that *A. dorsata* queens have an extremely high mating frequency (Moritz *et al.*, 1995; Oldroyd *et al.*, 1996; Wattanachaiyingcharoen *et al.*, 2003). Furthermore, microsatellite analysis showed no significant difference in paternity frequency at disturbed and undisturbed habitats. Measures of F_{ST} and between aggregations were not significantly different from zero ($P > 0.05$), and measures of allelic diversity showed no differences between disturbed and undisturbed sites. These findings suggest that habitat disturbance has no effect on mating frequency or genetic diversity. Thus I conclude that the mating behaviour of *A. dorsata* is robust to anthropogenic changes to the landscape

7.4 Suggestions for Future Work

7.4.1 **Maximum sustainable yield.** Oldroyd and Wongsiri (2006) developed models to estimate the maximum sustainable yield (MSY) for a hunted honeybee population based on the harvest rate, and the intrinsic growth rate of a population. In

the absence of data on the typical rate of reproduction of an *A. dorsata* colony and the harvest rate, Oldroyd and Wongsiri made assumptions about these parameters. Based on these assumptions they concluded that the harvest rate of the *A. dorsata* in Thailand is probably too high for the population to be sustained in the long term.

My study suggests that in fact, the *A. dorsata* population of Thailand is largely unaffected by hunting, suggesting that the population parameters assumed by Oldroyd and Wongsiri are in error. It is therefore more important than ever to obtain robust estimates of the harvest rate and reproductive rate so that we can be assured that the sustainable yield is not being exceeded. These critical parameters are (Oldroyd and Nanork, 2009):

a. Population size and population density.

b. Natural rate of increase of population (growth rate).

c. Harvest rate or the proportion of honeybee colonies that are harvested.

d. Survival rate or the proportion of colonies that survive harvest to reproduce.

7.4.2 Intrinsic population growth. To determine the natural rate of increase of wild honeybee population, we need to know its size, the number of swarms produced by a colony each year, the rate of immigration and emigration, and the length of life of colonies (Oldroyd and Wongsiri, 2006). These parameters cannot be readily determined directly, but they can be inferred. The growth rate can be estimated by determining the number of surviving daughter colonies a typical established colony produces (Oldroyd and Nanork, 2009). For the giant bees, which form dense aggregations, we need to study a colony aggregation for a complete reproductive season to determine the number of colonies present in aggregation at the beginning of the season and the number of immigrants and emigrants that join the

aggregation site. Furthermore, the number of offspring colonies within an aggregation must also be determined. Note that in Chapter V I showed that reproductive swarms do not remain within the aggregation in which they arose (see also Oldroyd *et al*. 2002).

7.4.3 **Harvest rate.** The harvest rate, the proportion of colonies that are harvested, is one of the most important parameters needed to determine if the rate of harvesting is sustainable. Oldroyd and Wongsiri (2006) suggested the goal of sustainable harvesting of wild honeybees is to maintain harvest rate less than growth rate of population – maximizing yields while reducing the effort required to locate colonies. Harvest rates can be obtained by surveys and questionnaires of local honey hunters. It will also be important to determine the method by which hunters harvest colonies, and if colonies are likely to survive.

7.4.4 **Population size.** Robust estimates of the density of colonies in the environment are essential if we are to determine if current harvesting rates are sustainable. Estimating the density of wild honeybee colonies by survey and direct count of colonies is likely to severely underestimate the total number of colonies present (Hepburn and Radloff, 1998). Therefore, a new method for estimating the relative size of wild honeybee populations has been recently developed (Jaffe *et al.*, 2010; Moritz *et al.*, 2008; Moritz *et al.*, 2007). The innovation in this new method is to genotype males (drones) with are collected from drone congregation area (DCA) at a series of tightly linked microsatellite loci. Strong linkage disequilibrium between loci means that a queen produces just two haplotypes, and her sons will therefore be of two kinds only (Fig. 7.1). Because of the number of alleles at each locus is very large, each haplotype is likely to unique or rare in the population, except among

relatives. Thus if a sample of drones is genotyped, and the number of haplotypes present is divided by two, an estimate of the number of colonies (queens) represented by the drone sample is obtained (Fig. 7.1). Kraus *et al*. (2005) and Moritz *et al*. (2007) presented a method where microsatellite data of haploid males, which were caught at DCA can be used to estimate the number of male producing queens (colony number) at a given locations an estimate of colony density.

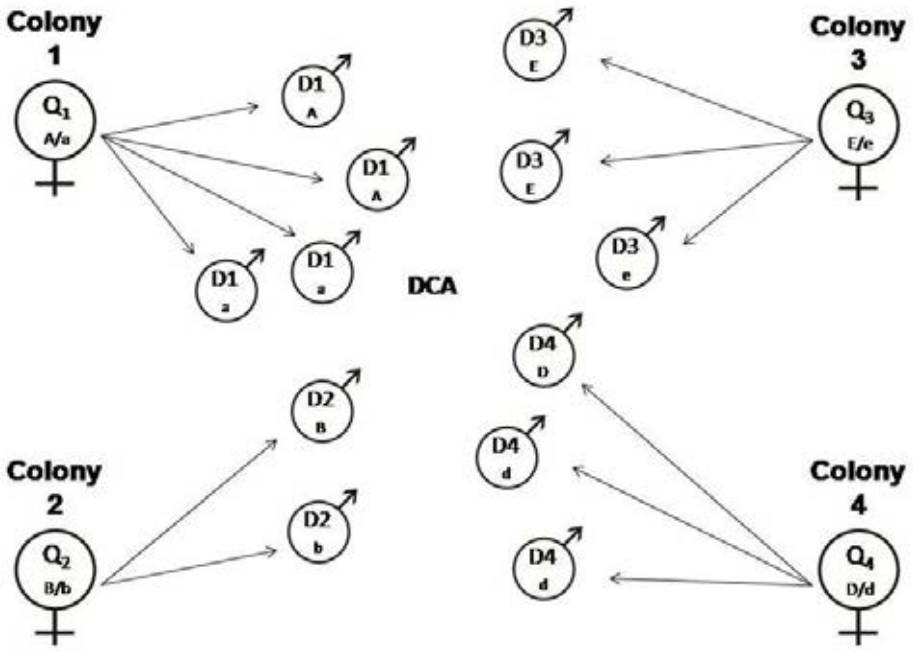

Figure 7.1 Honeybee drones are produced by several colonies for serving drone congregation area (DCA) to the queens for mating. Because queen produces just two haplotypes, two kinds of haploid drones are produced.

In the future study, the drone samples of *A. dorsata* will be collected directly from DCA (Koeniger *et al.*, 1994) at disturbed and undisturbed areas using a Williums‟s trap method (Moritz *et al.*, 2008; Moritz *et al.*, 2007; Williams, 1987). To calculate the density from the number of queens detected, it is necessary to know the distance over which drones fly to the DCA. In the European honeybee, *A. mellifera*, drones fly between 600 and 1200 m on average (Jaffe *et al.*, 2010). In *A. dorsata*, Koeniger *et al.* (1994) reported that the DCAs of *A. dorsata* are vary in distance from colony aggregation (700 m is a minimum distance). Therefore, 700 m might be used as the minimum flight distance of drones and 1.6 km^2 as the minimum mating area of drones (assuming the area is circular).

7.4.5 **Sampling drone congregation areas.** Honeybees have a complex mating system in which the drones and virgin queens meet at called drone congregation areas (DCAs) to mate in mid-air (Kraus *et al.*, 2005a; Oldroyd and Wongsiri, 2006; Ruttner, 1988). Koeniger *et al.* (1994) reported that *A. dorsata* uses isolated tall trees emerging from the canopy of the forest as the landmark for establishing its DCAs during a limited time window shortly before dusk. Samples of *A. dorsata* drones that are directly caught at a DCA can be used to obtain estimates of the density of colonies at a given locality as described above.

As part of my study I attempted to sample *A. dorsata* DCAs using a pheromone trap, (Koeniger *et al.*, 1994; Moritz *et al.*, 2007; Williams, 1987) as described by Koeniger *et al.* (1994). I was unable to catch drones at any location, suggesting that further research is required to develop new methods for directly harvesting drones at DCAs.

7.5 Conclusions

Although my study strongly suggests that the *A. dorsata* population of Thailand is not being greatly affected by hunting and deforestation, I wish to emphasise that a „business as usual" approach to conservation may still be inappropriate. In the absence of quantitative data on population size, harvest rate and intrinsic population growth rate, it is still possible that the population could collapse if hunting pressure increases. *A. dorsata* is one of the most charismatic insects on earth, and critical to the pollination of dipterocarp forests. If we are to continue harvesting, it is our responsibility to learn enough about them to do it in a responsible way.

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BIOGRAPHY

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1. **Rattanawannee, A.**, Chanchao, C., and Wongsiri, S. 2010. Gender and species identification of four native honey bees (Apidae: *Apis*) in Thailand based on wing morphometric analysis. *Annals of the Entomological Society of America*, 103(6): 965–970. (IF = 1.148)
2. **Rattanawannee, A.**, Chanchao, C., Lim, J., Wongsiri, S., and Oldroyd, B. P., 2012. Population structure of *Apis dorsata* in Thailand: implication for conservation. *Insect Conservation and Diversity*, DOI: 10.1111/j.1752-4598.2012.00193.x. (IF = 2.717)
3. **Rattanawannee, A.**, Chanchao, C., and Wongsiri, S. 2012. Geometric morphometric analysis of giant honeybee (*Apis dorsata* Fabricius, 1793) populations in Thailand. *Journal of Asia-Pacific Entomology*, *in press*.

Book

1. **Rattanawannee, A.** and Chanchao, C. (2011). *Bee Diversity in Thailand and the Applications of bee products*, In: *Changing Diversity in Changing Environment*, Grillo, O. and Venora, G., (Ed.), 133-162, InTech, ISBN 978-953-307-796-3, Rijeka, Croatia