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DNA METHYLATION OF GENES RELATED TO HONEY BEE WORKER REPRODUCTIVE PHYSIOLOGY

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ต่อมพิษผึ้งและรังไข่จัดเป็นอวัยวะที่สำคัญที่เกี่ยวข้องกับระบบสืบพันธุ์ในผึ้งงาน วัตถุประสงค์ของการศึกษาในครั้งนี้เพื่อตรวจสอบว่าดีเอ็นเอเมทิลเลชันมีความเกี่ยวข้องกับการควบคุมการทำงานของอวัยวะทั้งสองชนิดในผึ้งงานหรือไม่ ในการศึกษาส่วนแรก เกี่ยวข้องกับการค้นหาความถี่ของดีเอ็นเอเมทิลเลชันในผึ้งพื้นเมืองไทย 4 ชนิด คือ ผึ้งม้าน *A. andreniformis* ผึ้งมิ้ม *A. florea* ผึ้งโพรง *A. cerana indica* และผึ้งหลวง *A. dorsata* โดยเน้นที่ยีนที่เกี่ยวข้องกับพิษผึ้งคือยีนฟอสโฟไลเปสเอสอง (*PLA2*) จากการใช้เทคนิค reverse transcriptase polymerase chain reaction (RT-PCR) พบการแสดงออกสูงสุดของยีน *PLA2* ในผึ้งประจำรังของผึ้งพื้นเมืองไทยทุกชนิดในขณะที่มีการแสดงออกในระดับสูงของยีนดังกล่าวในผึ้งระยะหาอาหารของผึ้งมิ้มและผึ้งหลวง ในทางตรงกันข้ามไม่พบการแสดงออกของยีน *PLA2* เลยในผึ้งระยะดักแด้ในผึ้งพื้นเมืองไทยทุกชนิด แอกทิวิตีของ PLA2 และแอกทิวิตีจำเพาะในสารสกัดพิษผึ้งอย่างหยาบมีระดับที่สูงกว่าในผึ้งประจำรัง เมื่อเทียบกับผึ้งระยะดักแด้ตาดำในผึ้งทุกชนิดที่ทำการศึกษา ระดับของดีเอ็นเอเมทิลเลชันในผึ้งระยะดักแด้สูงกว่าในผึ้งประจำรังและผึ้งระยะหาอาหารของผึ้งมิ้มและผึ้งหลวง แต่พบว่าแตกต่างกันในผึ้งม้านและไม่แตกต่างกันในผึ้งโพรง ในการศึกษาส่วนที่สอง ภายใต้สภาวะที่ปราศจากผึ้งนางพญา ผึ้งงานจะถูกแบ่งเป็น 2 กลุ่ม คือ กลุ่มควบคุมและกลุ่มทดลอง (รมด้วยแก๊สคาร์บอนไดออกไซด์) เมื่อวิเคราะห์โดยใช้เทคนิค methylation-sensitive amplified fragment length polymorphism (MS-AFLP) พบว่าไม่มีความแตกต่างที่สามารถตรวจพบได้ในรูปแบบของดีเอ็นเอเมทิลเลชันในระดับจีโนมระหว่างผึ้งงานที่รังไข่พร้อมทำงานกับผึ้งงานที่รังไข่ไม่พร้อมทำงานในทุกการเปรียบเทียบ ในทางเดียวกัน ผึ้งงานในกลุ่มควบคุมและกลุ่มที่ถูกรมด้วยแก๊สคาร์บอนไดออกไซด์ไม่มีความแตกต่างอย่างมีนัยสำคัญของรูปแบบของดีเอ็นเอเมทิลเลชัน ในการศึกษาส่วนสุดท้าย ทำการตรวจหาดีเอ็นเอเมทิลเลชันที่บริเวณเป้าหมายของยีน *Krüppel homolog-1* (*Kr-h1*) จากผึ้งงานที่อายุ 7 วัน ในสภาวะรังที่ปราศจากผึ้งพญา จากเทคนิค bisulfite sequencing แสดงให้เห็นว่ามี 12 ตำแหน่งของ methylated CpG ที่สามารถตรวจพบได้ในบริเวณที่มีการเพิ่มจำนวนของยีน *Kr-h1* ในผึ้งงานที่ทำการศึกษาระดับดีเอ็นเอเมทิลเลชันของยีน *Kr-h1* โดยรวมมีระดับที่สูงขึ้นอย่างมีนัยสำคัญในรังไข่ผึ้งงานที่ไม่พร้อมทำงานเมื่อเทียบกับรังไข่ผึ้งงานที่พร้อมทำงาน การรมด้วยแก๊สคาร์บอนไดออกไซด์ไม่ได้ส่งผลอย่างมีนัยสำคัญต่อการเปลี่ยนแปลงของดีเอ็นเอเมทิลเลชันของยีน *Kr-h1* ในรังไข่ของผึ้งงาน นอกจากนี้ ยังพบว่า 1 บริเวณที่มีการขาดหายไปของนิวคลีโอไทด์มีความจำเพาะต่อ 1 ตำแหน่งของเมทิลเลชันที่เกิดที่ CpG

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

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MANLIKA KILASO: DNA METHYLATION OF GENES RELATED TO HONEY BEE WORKER REPRODUCTIVE PHYSIOLOGY. ADVISOR: ASSOC. PROF. CHANPEN CHANCHAO, Ph.D., CO-ADVISOR: PROF. BENJAMIN P. OLDROYD, Ph.D., 144 pp.

Venom gland and ovary are the important organs related to a reproductive system in honey bee workers. The aim of this study is to investigate whether DNA methylation is involved in the regulation of function role of both organs in workers. In the first part, the frequency of DNA methylation is determined in four Thai native honey bee species which are *A. andreniformis*, *A. florea*, *A. cerana indica* and *A. dorsata* by focusing on venom phospholipase A_2 (*PLA2*). Reverse transcriptase polymerase chain reaction (RT-PCR) revealed that the highest expression of *PLA2* is found in house bees of all four *Apis* spp. while the high expression of *PLA2* is observed in foragers of *A. florea* and *A. dorsata*. In contrast, expression of *PLA2* is undetectable in pupae from all *Apis* spp. The PLA2 activity and specific activity from crude venom extract shows the higher level in house bees than in black-eyed pupae in all studied bees. DNA methylation level of pupae is higher than that of house bees and foragers of *A. florea* and *A. dorsata* but it is not the case in *A. andreniformis* and *A. cerana indica*. In the second part, under the queenless condition, workers are divided into control and experimental (CO_2 narcosis) cages. Using methylation-sensitive amplified fragment length polymorphism (MS-AFLP) assay, there is no difference detectable in a pattern of genome-wide DNA methylation between workers with non-active and active ovaries in any comparisons. Similarly, non-treated and CO_2-treated workers are not significantly different in DNA methylation pattern. For the last part, DNA methylation at target region of *Krüppel homolog-1* (*Kr-h1*) is determined in 7-day old queenless workers. Bisulfite sequencing shows 12 methylated CpG sites found across amplified region of *Kr-h1* in examined workers. The overall DNA methylation level of *Kr-h1* is significantly higher in non-active ovaries than active ovaries. CO_2 narcosis does not have a significant effect to the DNA methylation change in *Kr-h1* in worker ovaries. In addition, it is found that there is one deletion site specific to one site of CpG methylation.

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Student's Signature .....
Advisor's Signature .....
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CHAPTER I
INTRODUCTION

Honey bees have agricultural and economic value as they produce honey and play an important role in pollination. Moreover, their other products such as propolis and venom contain various pharmaceutical properties those are useful for human health (Huh *et al.* 2010; Wu *et al.* 2011).

Honey bees are recognized as highly eusocial insect which their hive consists of three castes of drones, queen and workers (Wilson 1971). Queen and worker belong to female castes those have the same genome but they are different in morphology, behavior and reproductive physiology (Oldroyd and Wongsiri 2006).

A venom organ is related to a reproductive system in female honey bee. A sting evolves from an ovipositor using for laying eggs (Snodgrass 1956). This organ is not significant in a queen. In contrast, a worker uses a sting for a nest defense (Oldroyd and Thompson 2006). When a bee attacks an intruder, venom produced from a venom gland and stored in venom sac will then be released through a connecting sting (Dade 1994). Phospholipase A_2 is the main component in bee venom and important to a nest defense in worker (Schmidt 1995; Oršolić 2012). The regulation of *phospholipase A_2* expression and molecular characterizations of this enzyme in venom gland of workers are very interesting.

Besides a venom organ, an ovary is crucial to a reproductive system in female honey bee. A queen is highly fecund, whereas the workers are normally sterile (Ratnieks *et al.* 2006). However, around one third of young workers can activate their

ovaries and lay unfertilized haploid eggs after the absence of the queen (Winston 1987). This indicates that ovary activation in worker is plasticity and influenced by environmental factors.

Queen mandibular pheromone is the main factor involved in inhibiting ovary activation in workers (Conte and Hefetz 2008). Brood pheromone and effective mechanisms of policing are also involved in the regulation of worker sterility (Chapman *et al.* 2010; Traynor *et al.* 2014)). In addition to environmental cues in the colony, the inhibitory effects of CO_2 narcosis mimic those of queen signals on ovary activation in queenless workers (Berger *et al.* 2015). CO_2 narcosis is supposed to influence the expression of genes corresponding to sterility and fertility in worker.

Various studies have been tried to identify the gene networks those are involved in regulating worker sterility (Cardoen *et al.* 2011; Niu *et al.* 2014). Genes those are differentially expressed between different reproductive phenotypes of workers are candidates for the regulation of worker sterility. However, the underlying regulatory mechanism of those genes at molecular level of worker sterility remains elusive.

Epigenetic modification is a molecular mechanism that has an ability to alter gene expression and phenotype without changing in DNA sequence (Feil and Fraga 2012). Furthermore, DNA methylation is recognised as a crucial epigenetic mark to play an important role in modulation of gene expression (Duncan *et al.* 2014). This mechanism is implicating in various honey bee regulation such as caste determination, developmental process and phenotypic plasticity (Rasmussen and Amdam 2015). This suggests that DNA methylation can be deeply involved in the regulation of worker sterility and other important processes in the honey bee.

As described above, venom gland and ovary are important organs related to physiology and role in worker honey bees. In this project, whether epigenetic modification mediated by DNA methylation is associated with the regulation of the expression of *phospholipase A_2* in venom gland and sterility in worker honey bee is determined.

The objectives of this study are as below.

1. To reveal the expression and DNA methylation pattern of phospholipase A_2 (*PLA2*) in native honey bees of Thailand

2. To determine DNA methylation pattern at the genome-wide level between *A. mellifera* workers with non-active and active ovaries and the effect of CO_2 narcosis to the genome-wide DNA methylation using methylation-sensitive amplified fragment length polymorphism (MS-AFLP) technique

3. To examine whether DNA methylation of a candidate gene, *Krüppel homolog-1* (*Kr-h1*), is involved in the regulation of *A. mellifera* worker sterility including the influence of CO_2 narcosis to DNA methylation changes in *Kr-h1* using bisulfite sequencing

CHAPTER II
LITERATURE REVIEW

2.1 Honey bee

Honey bees are classified in the genus of *Apis*. The specific name of the western honey bee, *mellifera*, is the Latin word for honey produced from collected nectar (Winston 1987). Honey bees are important to human in various ways. In terms of agricultural importance, they serve as pollinators for crops and increase crop yield (Free 1970; vanEngelsdorp *et al.* 2008). They provide products such as beeswax, propolis, royal jelly, bee pollen and honey which is economic to human. Moreover, many bee products such as propolis, pollen and honey are found to have many bioactivities like anticancer, antioxidant and antimicrobial activities (Estevinho *et al.* 2008; Chantarudee *et al.* 2012; Teerasripreecha *et al.* 2012; Boonsai *et al.* 2014).

Honey bees are highly eusocial insects. Within a colony, there are three castes: queen, drones and workers. Also, there is cooperative brood care, reproductive division of labor and overlapping generation of workers (Wilson 1971). Based on morphology and anatomy, the defining characteristics of honey bees is pollen basket (corbicula) on the hind legs, hair covering head and thorax and eyes, strongly convex scutellum, sting evolved from an ovipositor in female and a venom gland (Ruttner 1988; Dade 1994; Engel 1999).

There are nine species of honey bees which are *Apis andreniformis, A. florea, A. cerana, A. koschevnikovi, A. nuluensis, A. nigrocincta, A. dorsata, A. laboriosa* and *A. mellifera* (Oldroyd and Wongsiri 2006). *Apis* spp. are thought to be originated in

Southeast Asia except *A. mellifera* which is thought to be originated in Africa or subtropical Europe (Culliney 1983; Winston 1987).

In Thailand, there are four native honey bee species which are *A. andreniformis*, *A. florea*, *A. cerana* and *A. dorsata* and one imported species which is *A. mellifera* (Wongsiri *et al.* 1990; Wongsiri *et al.* 1997; Rattanawannee *et al.* 2007). The classification of honey bee species in this study is shown as below.

Kingdom	Metazoa
Phylum	Arthropoda
Class	Insecta
Order	Hymenoptera
Super – family	Apoidea
Family	Apidae
Sub family	Apinae
Genus	*Apis*
Species	*Apis andreniformis, A. florea*, *A. cerana, A. dorsata* and *A. mellifera*

2.2 *Apis andreniformis* Smith, 1858

A. andreniformis (black dwarf honey bees) are the smallest honey bees. They are close to *A. florea* which are classified into the same subgenus *Micrapis* according to their nest characters with open and small single comb around small tree branches (Figure 2.1) (Wu and Kuang 1987; Oldroyd and Wongsiri 2006). In general, *A. andreniformis* looks black because it has black hair on the hind tibia and basitarsus, reddish-brown (rufous) scutellum, and black metasomal segment at the first segment (Rinderer *et al.* 1995; Wongsiri *et al.* 1997). However, *A. andreniformis* is separated from *A. florea* with some characters. For example, there is difference in morphology of male genitalia of both species. Hind barsitarsal extension (thumbs) in *A. andreniformis* drones is much shorter than in *A. florea* and cubital index in *A. andreniformis* is much larger (Wongsiri *et al.* 1990). A nest built by *A. andreniformis* has a midrib from a supporting branch while the crown of the nest of *A. florea* does not contain the midrib (Rinderer *et al.* 1996).

A. andreniformis is widely distributed in tropical and near subtropical regions of Asia including southern China, India, Burma, Laos, Vietnam, Thailand, Malaysia, Indonesia and the Philippines (Palawan) (Wongsiri *et al.* 1997).

Figure 2.1 *A. andreniformis* nest

2.3 *Apis florea* Fabricius, 1787

The body color of *A. florea* (red dwarf honey bees) differs from the color of *A. andreniformis. A. florea* has white hair at the hind tibia and basitarsus and black scutellum. They look generally rufous or toward yellow according to the first abdominal segment color (Wongsiri *et al.* 1997; Oldroyd and Wongsiri 2006)**.** Workers are very small with around 7 mm in length (Seeley *et al.* 1982) and normally build the nest around a small branch (Figure 2.2) on shrubs, bushes or small trees as do *A. andreniormis* (Wongsiri *et al.* 1997). The average size of nest of *A. florea* (~12 cm x 16.9 cm) is about 25 % larger than *A. andreniformis*'s (Rinderer *et al.* 1996; Oldroyd and Wongsiri 2006). An *A. florea* colony is relatively small with less than 5,000 individual bees (Michener 1974). Similar to nest of *A. andreniformis*, honey is stored on top surrounding a supporting branch and followed by the pollen storage area and brood cell area downward (Wongsiri *et al.* 1997).

A. florea performs dancing on the horizontal platform on the top of the comb (Oldroyd and Wongsiri 2006)**.** *A. florea* is commonly distributed from middle East to southeastern China and southeast Asia (Wongsiri *et al.* 1997; Hepburn *et al.* 2005)**.** The distribution of *A. florea* and *A. andreniformis* overlap in southeast Asia but *A. florea* has not been found in the southern peninsular Malaysia or Indonesia, Borneo and the Philippines or the surrounding islands (Wongsiri *et al.* 1997).

Figure 2.2 *A. florea* nest

2.4 *Apis dorsata* Fabricius, 1793

A. dorsata (giant honey bees) belong to the same subgenus, *Megapis*, as the Himalayan *A. laboriosa* (giant mountain honey bee) since they build an open and large single nest under a tree branch (Oldroyd and Wongsiri 2006). *A. dorsata* is the largest honey bee in Thailand. The size of worker body is approximately 17 mm in length and bigger than drone body which is opposite to other *Apis* spp. (Oldroyd and Wongsiri 2006). The overall appearance is rufous and their wings are fuscous and hairy. *A. dorsata* builds the largest nest (~1.5 m x 1 m) containing more than 50,000 individual bees (Oldroyd and Wongsiri 2006). *A. dorsata* usually builds a nest in the open area such as on high and large trees, under urban structure, water tower (Figure 2.3A) or cliff. Its colonies tend to be highly aggregated (Figure 2.3B), with up to 200 colonies on a same tree (Oldroyd *et al.* 2000; Paar *et al.* 2004). However, colony aggregations are not relatives (Rattanawannee *et al.* 2013). They can return to the same nest site of previous season (Paar *et al.* 2000).

A. dorsata is very aggressive and can defend the nest by performing shimmering behavior on the layer of curtain bees that cover the comb (Seeley *et al.* 1982; Woyke *et al.* 2008). *A. dorsata* is widespread from Indian subcontinent to southeast Asia (Ruttner 1988; Rattanawannee *et al.* 2013).

(A) (B)

Figure 2.3 *A. dorsata* nest on a water tower (A) and *A. dorsata* colony aggregation on a tree (B).

2.5 *Apis cerana* Fabricius, 1793

A. cerana (Eastern honey bees) are classified in the same subgenus *Apis* as *A. mellifera* since they generally build multiple combs inside the cavity. The color of *A. cerana* scutellum is variable from yellow, brown or black. They can have variation of yellow or black band at abdominal terga segments depending on seasonal factors (Peng *et al.* 1989). Their size is relatively medium with 10 mm in body length and a bit smaller than *A. mellifera* (Seeley *et al.* 1982; Oldroyd and Wongsiri 2006). Although *A. cerana* is relatively close to *A. mellifera*, they are morphologically distinct. *A. mellifera* lacks the distal abscissa of vein at hind wings and has more hamuli on the hind wings (Oldroyd and Wongsiri 2006). The average diameter of a *A. cerana* nest is 30 cm and consists of three or more parallel combs. The colony is relatively small, compared to *A. mellifera*, with population of 7,000 individual workers. A cavity such as a hollow tree and cave are the favorite nest site of *A. cerana* (Seeley *et al.* 1982).

As they build a nest in a cavity and are not seriously aggressive, they can be reared in a farm to harvest honey (Figure 2.4). *A. cerana* is distributed throughout Asia (Oldroyd and Wongsiri 2006). *A. cerana* can be classified into subspecies which are *A. cerana cerana* distributed in China, *A. cerana japonica* distributed throughout Japan and Korea and *A. cerana indica* distributed in India, Sri Lanka, Thailand, Malaysia and Indonesia (Wongsiri 1989; Crane 1992).

Figure 2.4 *A. cerana* nest in a bee farm

2.6 *Apis mellifera* Linnaeus, 1758

A. mellifera (Western honey bees) (Figure 2.5) are cavity-nesting honey bees which are native to Africa and Europe and have been introduced to other regions such as Asia, North and South America and Australia resulting in worldwide distribution (Winston 1987). Although the physical appearance looks similar to *A.cerana*, mating of *A. cerana* drone and *A. mellifera* queen cannot produce offspring. The colony population is more than 100,000 individual bees (Winston 1987). *A. mellifera* are likely to be docile and less prone to absconding than other species (Michener 1974). This enables *A. mellifera* to be managed in a hive box. This species is economically important and widespread for beekeeping (Wongsiri 1989; Crane 1992). According to

global distribution, there are various subspecies of *A. mellifera*. The main three groups of *A. mellfera* subspecies are African, European and near Eastern tribes (Winston 1987; Ruttner 1988).

Examples of European *A. mellifera* can be described as followed (Ruttner 1986; Winston 1987; Dietz 1992).

A. mellifera mellifera (German dark bees) are originated in northern Europe and west-central Russia. They have a short tongue, long hair and no yellow spot at the abdomen. They are usually crossbred with other subspecies resulting in reduced number of homozygous chromosomes.

A. mellifera ligustica (Italian bees) are originated in Italy. They have relatively smaller size of body than *A. mellifera mellifera*. They are the least aggressive and can build up a colony quickly. This makes *A. mellifera ligustica* the most popular for beekeeping throughout the world.

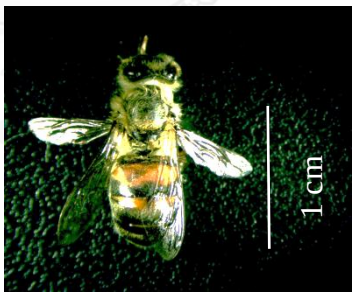


Figure 2.5 *A. mellifera*

2.7 Life cycle of honey bees

A honey bee undergoes a complete metamorphosis which consists of four stages of egg, larva, pupa and adult (Snodgrass 1956; Jay 1963; Winston 1987; Oldroyd and Wongsiri 2006; Seeley 2009). Although the overall of development

period is similar in all honey bee species, there is variation in time for each developmental stage for different castes of honey bees. Generally, the specification of each developmental stage would be summarized as the following.

The eggs are laid by a queen. Normally, there is one egg in a brood cell and it looks white, oval and long in shape. The incubation time for egg hatching is 3 days and during this period the development of embryo is occurred.

Then, the embryos develop to larvae which are milky white body and C-shape in appearance. In this stage, the external structure like legs, eyes, antennae, wings or sting do not appear yet. Larval stage takes 5-6 days for growth. For worker-destined larvae, they are fed with royal jelly by a nurse bee on only the first three days, while a queen-destined larva is fed with the same diet throughout the larval life. During larval development, molting occurs 5 times (5 larval instars) to make larvae grow continuously.

After the final molt, mature larvae stretch out their body and spin a cocoon of silk to change to pupal stage within the cell capped by a worker. The metamorphosis in the pupal stage lasts 7-14 days. In this stage, there is a big change in external structure to make it look like adult. Some external structures such as leg, antenna, eyes, head, thorax and abdomen will be obviously seen. Moreover, there is massive change in organ systems. After complete metamorphosis, the pupa becomes newly adult. It will bite the cell cap to emerge.

Considering a particular caste, the average time for development of a queen, a worker and a drone is 16, 21 and 24 days, respectively. A queen takes the shortest time for development followed by a worker and a drone.

2.8 Caste determination

As mentioned previously, the honey bee is highly eusocial. A honey bee colony consists of three castes which are a queen, workers and drones and each of them plays a specific role in the hive.

Drones are developed from haploid unfertilized egg (n) and are characterized by large compound eyes and thorax (Oldroyd and Wongsiri 2006). They only play a role in mating with a queen. Drones are produced during the mating season. Whenever they finish their mating role, they are usually removed from their colony (Wongsiri 1989). The average lifespan of drones is between 21-32 days (Winston 1987). There are approximately a hundred to a thousand of drones in a colony.

A queen is developed from diploid fertilized egg (2n) and has the largest size in a colony with short wings (Winston 1987). A queen has a well-developed ovary and its main role is to fertilize with drones. Later, it will lay fertilized eggs to produce female offsprings (Dade 1994; Oldroyd and Wongsiri 2006). In addition, a queen produces queen pheromone to control the worker performance (Free 1987; Keller and Nonacs 1993). Normally, there is only one queen in a colony. A queen lifespan is about 1-3 years (Winston 1987).

Workers are produced from diploid fertilized egg (2n) like a queen (Winston 1987). They are generally the smallest in a colony. An ovary of workers is not-well developed (Snodgrass 1956). Workers have some special morphological features such as pollen basket, honey crop, wax gland, sting and venom gland to fit their task (Dade 1994). A worker performs almost all tasks such as brood caring, guarding and foraging in the colony and their role is associated with their age (Robinson 1992). The colony population of workers is 10,000 – 60,000 bees (Moritz and Southwick

1992).The mean longevity of workers is 15-38 days for summer, 30-60 days for spring and 140 days for winter (Winston 1987).

2.8.1 Caste differentiation between a queen and a worker

The most notable characteristic of highly eusocial insects including honey bee is reproductive division of labour among female castes (Wilson 1971) which are a reproductive caste (a queen) and a non-reproductive caste (a worker). Despite arising from the same genome, a queen and workers differ in morphological appearances and behaviors. The major difference between both is reproductive physiology relating to ovary features positioning in the abdomen. A queen ovary is well developed with consisting of nearly 200 ovarioles per ovary while the worker ovary is rudimentary with containing less than 20 ovarioles per ovary (Snodgrass 1956; Velthuis 1970).

Larval diet is known as the external cue that generated the alternative morphologies between a queen and a worker (Page and Peng 2001). An underlying gene and molecular mechanism of this cue are thought to be an important determinant for a process of caste differentiation between a queen and a worker.

Evans and Wheeler (1999) found that six of seven clones (loci) differentially expressed between both castes corresponded to amino acids of the mammalian ETS-domain transcription factor ELK-3, fatty-acid binding proteins, hexamerins, λ crystallin and oxidoreductase found in GenBank database. The function of those clones is involved in the process of caste differentiation and a pathway of a queen or a worker development.

Later, by gene-expression array, it was found that queen larvae overexpressed genes encoding metabolic enzymes which were ATP synthase and cytochrome

oxidase, while worker larvae up-regulated genes encoding hexamerin 2, cytochrome P450 and dihydrodiol dehydrogenase (Evans and Wheeler 2000).

This is consistent with the report of Barchuk *et al.* (2007). They demonstrated queen larvae upregulated physiometabolic genes including genes coding for metabolic enzymes and genes involved in regulating the rate of mass-transforming. In contrast, worker larvae increased the expression of more genes participating in a developmental process than queen larvae.

The higher expression of genes involved in metabolic rates may associate with the increasing growth rate during larval development of queen. The up-regulation of developmental genes may involve in developing some specific organs such as pollen basket and wax gland leading to the task performance of workers.

2.9 Sterility and ovary activation in a worker

As described previously, in eusocial insects, a worker is normally assigned to be sterile and the activation of their ovaries is suppressed. However, in honey bees, workers retain functional ovaries with a reduced number of ovarioles, comparing to a queen (Figure 2.6A) (Linksvayer *et al.* 2009; Gotoh *et al.* 2013). This allows their ovaries to be activated under suitable conditions (Figure 2.6B).

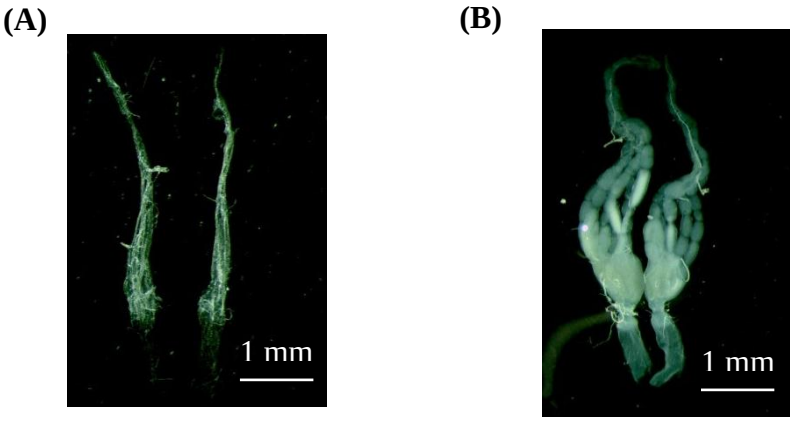


Figure 2.6 A functional worker ovary in a queenright colony (A) and an activated worker ovary in a queenless colony (B).

In a colony with the presence of a queen, it is very rare that workers activate their ovaries and lay haploid male-destined eggs (Oldroyd *et al.* 1994; Montague and Oldroyd 1998). Nevertheless, due to quantitative study of worker reproduction, approximately 0.12 % of drones produced in the *A. mellifera* queenright colony were developed from eggs laid by workers (Visscher 1989).

Ratnieks (1993) reported that the frequency of male-destined eggs produced by workers was very low. It was approximately estimated there was 1 egg per 16,000 drone cells. Meantime, there was only 1 worker out of 10,000 individuals that had fully-developed egg in its ovarioles in the queenright colony.

In addition, in the queenright colony of *A. cerana*, only 1-5 % workers presented eggs in their ovarioles (Oldroyd *et al.* 2001). However, worker reproduction was not observed in the queenright colony of *A. florea* (Halling *et al.* 2001) and *A. dorsata* (Wattanachaiyingcharoen *et al.* 2002).

Conversely, under a certain conditions such as queen loss from a colony, workers can activate their ovaries and lay male-destined eggs at much higher

frequency, comparing to a normal queenright colony (Crozier and Pamilo 1996). Miller and Ratnieks (2001) showed that, in the *A. mellifera* colony with absence of a queen, 5-24 % of collected workers from investigated colonies showed the fully-developed ovaries and they could lay eggs.

Barron and Oldroyd (2001) reported the effect of queenlessness to ovary development in different types of workers. They discovered that the wild type and anarchistic (mutant strain with high rate of ovary activation) worker had higher frequency of active ovary in a queenless colony, comparing to a queenright colony.

In *A. cerana*, Nanork *et al.* (2007) reported the higher proportion of natal workers with activated ovaries reached 36.3 % of total natal workers in the examined colonies after dequeening.

This indicates that the different circumstance whether a queen is present or absent in a colony affects the ovary activation in a worker. Besides a queen signal in a colony, there are other major causes that have an effect to ovary activation in a worker such as brood presence and policing behavior by workers.

2.10 Factors affecting ovary activation in a worker

Queen mandibular pheromone (QMP)

The presence of a queen conducts its pheromones appearing in a hive. Queen pheromone is composed of blended chemicals produced from mandibular glands of a queen (Plettner *et al.* 1996). Thus, it is called “queen mandibular pheromone (QMP)” which is consisted of five components including (E)-9-keto-2-decenoic acid (9ODA), (R,E)-(-)- and (S,E)-(+)-9-hydroxy-2-decenoic acid (9HDA), methyl *p*-hydroxybenzoate (HOB) and 4-hydroxy-3-methoxyphenylethanol (HVA) (Winston

and Slessor 1998). The primary role of QMP is to maintain a colony organization and control workers to perform their proper tasks such as attracting a retinue of workers surrounding the queen, inhibiting rearing a new queen and influencing comb-building (Keeling *et al.* 2003; Beggs *et al.* 2007).

A significant role of QMP is to prevent worker reproduction (Slessor *et al.* 2005; Oi *et al.* 2015). *A. mellifera* workers which were exposed to synthetic QMP and in the real queen treatment had a lower level of ovary development than unexposed workers (Hoover *et al.* 2003).

The inhibitory effect of synthetic and real QMP to the level of ovary activation in honey bee workers was confirmed by Backx *et al.* (2012) . Moreover, *A. cerana* QMP acts as an honest signal for worker sterility as in *A. mellifera* (Tan *et al.* 2015).

Interestingly, the presence of a queen also inhibits oogenesis in a worker ovary. Tanaka and Hartfelder (2004) found that queen and queenless workers had a similar structure of ovary in early oogenesis but queenright workers showed signs of programmed cell death in their ovary. Similarly, Ronai *et al.* (2015) revealed that the young workers exposed to queen pheromone had inactivated ovaries, however, in the early stage of oogenesis, they can produce some oocytes in their ovarioles prior to degrading. The physical death of germ cells in ovarioles of queenright workers is associated with the expression of programmed cell death genes such as *Ark* and *Buffy* which were differentially expressed between queen and worker castes (Dallacqua and Bitondi 2014). This indicates that the presence of queen is an importantly external cues which could lead to worker sterility.

Brood pheromone

Brood pheromone is a chemical signal emitted from larvae. It is a mixture of 10 ethyl and methyl esters of common fatty acids (Le Conte *et al.* 2001). Larvae use this signal to stimulate workers to do many activities corresponding their needs such as brood cell capping (Maisonnasse *et al.* 2010), pollen foraging (Pankiw 2004) and brood rearing (Sagili and Pankiw 2009). Significantly, brood pheromone also plays a role in controlling reproduction of workers by inhibiting their ovary development (Mohammedi *et al.* 1998). Maisonnasse *et al.* (2009) tested the effect of E-*β*-ocimene, a volatile young brood pheromone, from different larval instars to workers and found that E-*β*-ocimene-treated workers had the significant lower level of ovary development than control worker. In addition, a high dose of E-*β*-ocimene significantly decreases ovary activation in workers, comparing to worker in control or low dose pheromone treatment (Traynor *et al.* 2014).

This can confirm that larval pheromone is one of factors that can suppress ovary activation in workers. Nonetheless, if a worker's ovary fails to respond to larval and queen pheromones, it will get some responses from other workers in a colony instead.

Policing behavior

Because queens mate with multiple drones, a colony contains various patrilines of female offspring (Palmer and Oldroyd 2000). Considering genetics, a worker is more closely related to a drone offspring produced from a queen than to a drone offspring produced from other workers. This relatedness asymmetry evolves worker policing behavior of male reproduction by sibling workers (Ratnieks 1988).

Workers perform an aggressive behavior towards a reproductive worker (Visscher and Dukas 1995) and police (eat) eggs laid by unmated workers (Ratnieks and Visscher 1989; Visscher 1996; Wenseleers *et al.* 2004b). This leads to evolution of functional sterility of workers according to acquiescence of the colony (Wenseleers *et al.* 2004a).

2.11 Differential gene expression between non-activated and activated worker ovary

To understand the molecular basis of worker sterility, a set of genes which were involved in creating the different phenotype of non-activated and activated ovaries has to be identified. There have been many reports revealing the gene network underlying the regulation of worker sterility in honey bees. One approach to specify the involving genes is to compare gene expression profiles and patterns between workers with non-activated and activated ovaries.

Using a cDNA microarray, Thompson *et al.* (2006) found two *major royal jelly protein* (*MRJP2* and *MRJP7*) and *Niemann–Pick type C2 protein (NPC2-like)* were up-regulated in wild type workers but these genes were down-regulated in anarchist workers (strain with higher rate of ovary activation). The higher expression of *MRJP2* and *MRJP7* in sterile workers corresponds to their role in producing royal jelly protein to rear broods in a colony.

Later, Thompson *et al.* (2008) conducted similar study with additional population and database from honey bee genome. Twelve differentially expressed genes (DEGs) were identified to be involved in ovary activation in workers. For example, *Drosophila* CG6004 homologues and two unknown genes were up-regulated in the wild type workers. *Vitellogenin, secretory phospholipase A2, secapin*

and *AdoHycase* were up-regulated in anarchist workers. These genes are most likely to be involved in suppression and activation of ovaries in workers.

A whole genome microarray revealed total of 1,292 genes those were differentially expressed between honey bee workers with non-activated and activated ovaries (Cardoen *et al.* 2011). Workers with non-activated ovaries overexpressed genes participating in energy metabolism and respiration, flight and foraging behavior, detection of visible light, flight and heart muscle contraction, comparing to workers with activated ovaries. On the other hand, workers with activated ovaries overexpressed genes implicated in oogenesis relative to the workers with non-activated ovaries. An overexpression of particular genes in each worker with non-activated and activated ovaries was consistent with their performance in a hive.

Transcriptome comparison by RNA sequencing found 2,104 genes those show a differential expression pattern between inactivated and activated ovaries of honey bee workers in a queenless colony (Niu *et al.* 2014). Among these genes, 409 genes were up-regulated and 1,695 genes were down-regulated in activated worker ovaries. Many differentially expressed genes were majorly related to cell, binding, biological regulation and metabolic processes.

2.12 CO_2 narcosis

Ovary activation of honey bees can be manipulated by CO_2 narcosis. Thus, this technique has been widely used in honey bees. Interestingly, CO_2 narcosis has a contrary effect to queen and worker ovaries. CO_2 narcosis stimulates the initiation of oviposition and development of ovary in virgin queens (Mackensen 1947; Abdalla and Berger 2005). In contrast, CO_2 narcosis suppresses ovary activation in queenless

workers (Harris and Harbo 1990; Harris *et al.* 1996). This suggests the effect of CO_2 narcosis imitates the effect of queen pheromone to workers. In addition, it is feasible that CO_2 narcosis may increase the expression of genes associating to sterility or decrease the expression of genes for fertility in workers.

Previously, Koywiwattrakul *et al.* (2005) showed that CO_2-treated workers had a lower level of ovary activation than control workers in a queenless condition. Eight candidate genes with a presumptive role in ovary activation were examined for expression between CO_2-treated and control groups. Two genes encoding vitellogenin and transferrin were down-regulated in the CO_2-treated workers relative to control workers. Therefore, the expression of these genes may relate to the gene network in the ovary activation of workers.

Thompson *et al.* (2007) used CO_2 narcosis to manipulate ovary activation of honey bee queens and workers. Both castes were assigned into two groups of treatment and control. It was found that 10 of 25 selected candidate genes of ovary activation were differentially expressed between treatments and controls in at least one caste. Among these, two genes, *ribosomal protein* (*rpl26*) and *tyramine receptor* (*amtyr 1*), showed a difference in expression between treated and control group in both castes.

CO_2 narcosis also caused the differential changes in the expression of candidate genes thought to be related to either foraging or reproductive behavior in queens and workers. In workers, it seems that CO_2 narcosis caused precocious foraging and suppressed ovary activation, whereas CO_2 treatment enhances ovary activation in unmated queens. The result supports the hypothesis that foraging and reproductive traits are causally related in the honey bees (Brito *et al.* 2010).

Although a mechanism of CO_2 narcosis to ovary activation has been unclear, this suggests the relationship among CO_2 narcosis, ovary activation status of workers and gene expression.

2.13 *Krüppel homolog-1* (*Kr-h1*)

Krüppel homolog-1 (Kr-h1) encoding C_2H_2 zinc-finger type transcription factor plays an important role in insect metamorphosis (Minakuchi *et al.* 2011). This gene participates in juvenile hormone (JH) signaling pathway during insect metamorphosis (Hiruma and Kaneko 2013). Kr-h1 acts as a repressor of insect metamorphosis, and interacts with methoprene-tolerant (Met), the other repressor of metamorphosis, in an upstream way to repress the expression of *broad-complex* (*BR-C*) encoding an important transcription factor for initiating metamorphosis in insects (Minakuchi *et al.* 2009; Konopova *et al.* 2011; Kayukawa *et al.* 2012; Kayukawa *et al.* 2016). *Kr-h1* is up-regulated by JH during pupal-adult development in *Drosophila melanogaster* (Minakuchi *et al.* 2008). Suppression of the expression of *Kr-h1* leads to a precocious of larval-pupal metamorphosis in the beetle *Tribolium castaneum* (Minakuchi *et al.* 2009).

Grozinger *et al.* (2003) studied the effect of queen mandibular pheromone (QMP) to a brain of honey bee workers. They found that QMP enhanced the expression of genes involving in nursing behavior and suppressed that of genes involving in foraging behavior in workers. Also, workers those were exposed to QMP showed the significant lower level of *Kr-h1* like other genes involving in foraging behavior. This suggests that the expression of *Kr-h1* is modulated by QMP.

Furthermore, microarray study showed that *Kr-h1* is more significantly highly expressed in foragers than in nurse bees (Whitfield *et al.* 2003).

Kr-h1 is thought to have a significant role in ovary activation due to its modulation by QMP. Its expression has been used as a marker for detecting the effect of CO_2 narcosis (Thompson *et al.* 2007).

2.14 Phospolipase A_2 in honey bee venom

Honey bee venom is produced from a venom gland and is stored in a venom sac connecting directly to a sting (Figure 2.7) (Snodgrass 1956). A sting is an organ in an abdomen modified from an ovipositor in female to use as a defensive tool (Winston 1987). A sting is composed of two lancets which were joined with the hardened plate and strong muscle (Dade 1994). Although a sting is only found in a queen and a worker, it has an important defensive role in workers. When a worker stings a victim, its sting barb is embedded in the skin. Later on, the venom from venom reservoir is released through the lancets (Winston 1987; Dade 1994). However, the bees will die shortly. After stinging, the abdominal organ is torn apart because the bee tries to pull its sting out of the victim's skin. A venom gland in workers is small, relative to that of queens. (Snodgrass 1956; Bachmayer *et al.* 1972).

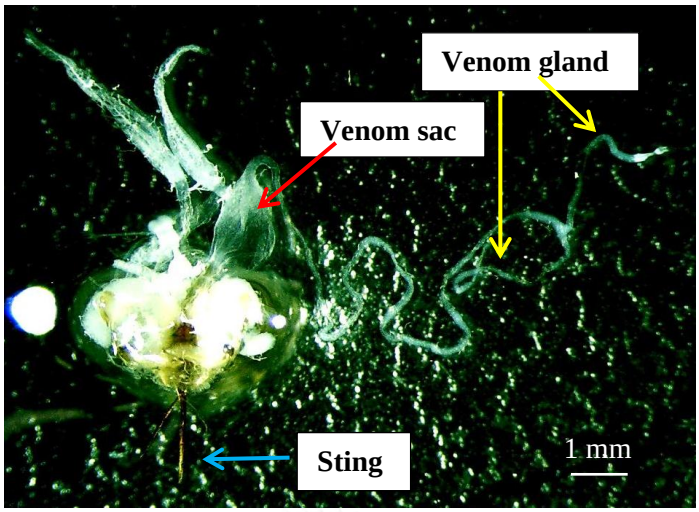


Figure 2.7 A sting chamber. A sting, venom sac and venom gland are indicated by light blue, red and yellow arrows, respectively.

Bee venom is a mixture of protein and peptides. The main components of bee venom are melittin, phospholipase A_2, hyaluronidase, acid phosphatase and histamine (Schmidt 1982). In combination, these components generate an allergic reaction to cells. As known, bee venom has long been used in traditional medicine to treat various sicknesses such as pain, arthritis and rheumatism. Bee venom has been reported to have many pharmaceutical properties such as anti-inflammatory, anti-arthritis activities (Lee *et al.* 2001; Park *et al.* 2004; Son *et al.* 2007). In addition, bee venom showed the cytotoxicity to cancer cells (Lee *et al.* 2007; Tu *et al.* 2008; Huh *et al.* 2010). Focusing on bee venom components, phospholipase A_2 (PLA2) was the major component (12 % of dry weight venom) (Habermann 2013) which had various biological effects.

Putz *et al.* (2006) studied the effect of PLA2 to tumor cell growth. The result revealed that such PLA2 reduced the proliferation rate of renal, breast and prostate

cancer cells. In addition, the inhibitory effect increased when those cells were treated with PLA2 and phosphatidylinositol-(3,4)-bisphosphate.

PLA2 also presented antibacterial activity by reducing survival of two-hour and twelve-hour cultures of *Enterobacter cloacae*, *Escherichia coli* and *Citrobacter freundii*. PLA2 displayed antimicrobial activity to the parasitic protozoan *Trypanosoma brucei brucei* (Boutrin *et al.* 2008).

PLA2 (EC 3.1.1.4.) is an enzyme that catalyzes the hydrolysis of sn-2 ester bond of phospholipid locating on the cell membrane to release free fatty acid and lysophospholipid. One of key free fatty acids released is arachinoic acid which acts as a precursor of eicosanoid (inflammatory mediator) such as prostaglandins, leukotrienes, thromboxanes and lipoxins (Altmann *et al.* 1991; Balsinde *et al.* 2002; Murakami and Kudo 2002). Releasing products of PLA2 result in nociceptive pain and cell membrane damages (Yue *et al.* 2005). It was reported that PLA_2 has a synergistic effect with melittin for erythrocyte lysis (Owen *et al.* 1990).

PLA2 can be found in other organisms such as snakes (Mukherjee 2014), mammals (Suzuki *et al.* 2000; Liu *et al.* 2013). PLA2 in bee venom is classified into group III of secretory PLA2 family with low molecular weight (14-17 kDa), high disulfide linkage and Ca^{2+} requirment (Kudo and Murakami 2002). PLA2 is implicated in several cellular processes such as phospholipid digestion and metabolism, host defense and signal transduction (Dennis 1994).

Schmidt (1995) studied toxicology of bee venom. The result showed that bee venom of all *Apis* spp. had similar lethal activity to mice. The activity of bee venom from a young queen is 50 % less than the activity from a worker. Interestingly, PLA2 was the main venom component that exhibited the most lethal activity.

This is related to Marz et al. (1981). They found that honey bee queen venom was nearly lacked of PLA2, comparing to mature worker venom.

PLA2 from *A. mellifera* venom gland had been already characterized with the 540 bp of nucleotide sequence determined from positive clones of cDNA library. The deduced amino acid sequence of mature enzyme comprised of 134 amino acids (Kuchler *et al.* 1989). This amino acid sequence was later deduced to be a synthetic gene (*PLA2*). The clone of this synthetic gene showed the high level of expression in *Escherichia coli* and the derived recombinant enzyme revealed the similar properties to the native enzyme (Dudler *et al.* 1992). Moreover, it was reported that *PLA2* from *A. cerana cerana* was manipulated to be expressed in *E. coli* expression system (Shen *et al.* 2004) and in insect cell expression system (Shen *et al.* 2010).

2.15 Epigenetic modification

Epigenetic modification is a regulatory mechanism at a genome level which is not involved in changing nucleotide sequence (Zhang and Meaney 2010; Feil and Fraga 2012). This mechanism is fundamentally mediated through the process of histone modification (acetylation, phosphorylation and so on) and DNA methylation to alter the gene expression in the cell (Hirst and Marra 2009; Biémont 2010). This alteration can result in phenotypic change (Duncan *et al.* 2014). Epigenetic modification is essential for various biological processes including regulation of tissue-specific expression, embryonic differentiation and development, genomic imprinting, X-chromosome inactivation and is implicated in several human diseases including cancer (Heard *et al.* 1997; Egger *et al.* 2004; Meissner 2010; Ballestar 2011; Ferguson-Smith 2011). In addition, environmental stimuli such as stress and

exposure to chemical may influence epigenetic regulation (Jaenisch and Bird 2003). Epigenetic modification can sometimes be transmitted to the offspring (Bird 2007).

2.16 DNA methylation

DNA methylation is a crucial epigenetic mark that is prevalent in eukaryotes including plants, insects and humans (e.g. Li *et al.* 2008; Laurent *et al.* 2010; Glastad *et al.* 2013). Methylation of DNA mostly involves the addition of a methyl group (CH_3) to the fifth carbon of cytosine (Auclair and Weber 2012) (Figure 2.8). Nonetheless, methylation of other nucleotides such as adenine has been found in higher plants and lower eukaryotes (Hattman 2005; Vanyushin and Ashapkin 2011). Cytosine methylation is typically found in CpG dinucleotides in animals (Lee *et al.* 2010; Pelizzola and Ecker 2011), however, methylation of cytosine in non-CpG context is distributed through embryonic stem cells of mammals as low frequency, comparing to the whole cytosine methylation (Lister *et al.* 2009; Stadler *et al.* 2011). In contrast, methylation of cytosine in plants is extensively located in CpG, CpHpG and CpHpH (H = A, C or T) contexts (Henderson and Jacobsen 2007).

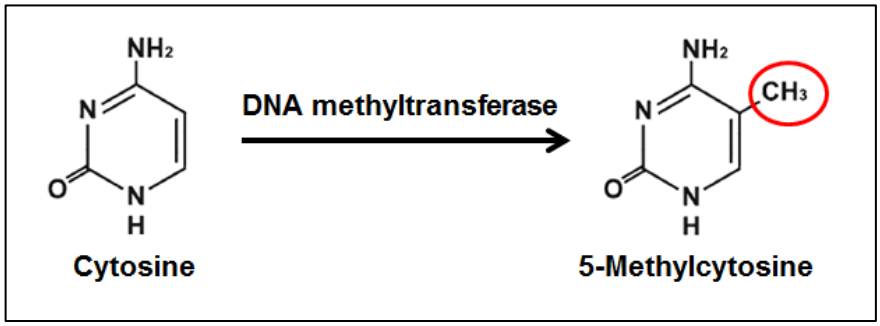


Figure 2.8 A mechanism of DNA methylation at cytosine.

DNA methylation is catalyzed by a conserved family of DNA methyltransferase (DNMTs) (Siedlecki and Zielenkiewicz 2006). DNMT1 is involved in the maintenance of the methyl tags to newly synthesized DNA in post-DNA replication (Goll and Bestor 2005). DNMT3 plays a key role in *de novo* DNA methylation. Thus, *DNMT3* is highly expressed at the early developmental stage (Okano *et al.* 1999). In contrast, DNMT2 is not responsible for DNA methylation but implicated in tRNA methylation (Goll *et al.* 2006). DNMT1 and DNMT3 were thus likely to be highly required for DNA methylation. Interestingly, there have been not found *DNMT3* in some organisms such as fruit fly *Drosophila melanogaster* and flour beetle *Tribolium castaneum* resulting in no extensive DNA methylation in genome of these organisms (Drewell *et al.* 2012).

2.17 A functional role of DNA methylation

A function of DNA methylation, regarding to modulation of gene expression, depends on the methylated CpG targeted regions. However, the distribution of CpG methylation through the genome varies in invertebrates and vertebrates. Generally, in vertebrates (most research is on mammals), CpG methylation is found throughout the genome (Suzuki and Bird 2008). DNA methylation in invertebrates is primarily targeted to a gene body which refers to exon and intron (Sarda *et al.* 2012). A gene body is also one of the main targets for methylation in plants (Zhang *et al.* 2006). In addition, in mammals, approximately 60-90 % of all CpGs in genome is methylated (Lister *et al.* 2009) while in invertebrates, the methylation is very low (Glastad *et al.* 2011).

The functional role of DNA methylation in gene regulation appears to be typically associated with transcriptional repression and alternative splicing of a target gene.

Transcriptional repression

Transcriptional repression or gene silencing is known as the major role of DNA methylation. This function is primarily focused on DNA methylation at a promoter region which is commonly CpG rich in mammalian and cancer cells (Herman and Baylin 2003; Weber *et al.* 2007). DNA methylation at promoter can impede the binding of transcription factors to their recognition DNA sequence or involve in recruiting co-repressors of methyl-CpG-binding proteins (MBP) to silence gene expression directly and to modify chromatin structure (Klose and Bird 2006; Elango and Soojin 2008). This could lead to suppression of transcription.

Furthermore, it has been found that the methylation of gene body can decrease gene expression by inhibiting transcriptional elongation (Lorincz *et al.* 2004). This mechanism was presumed to be similar to that of promoter methylation with the assistance of MBP to directly inhibit elongation or affect the chromatin structure (Klose and Bird 2006). Although methylation of gene body was thought to be reliable to suppress gene expression (Zilberman *et al.* 2007), the actual mechanism of gene body methylation is poorly understood.

Alternative splicing of target genes

DNA methylation of gene bodies can bring about alternative transcription. The association between DNA methylation and alternative splicing of target genes has

been recently proposed in invertebrates and mammals. Shukla *et al.* (2011) studied the effect of CCCTC - binding factor (CTCF) to exon 5 of *CD45* splicing. They found that binding of CTCF to exon 5 of *CD45* influenced the inclusion of exon 5 in transcript through promoting RNA polymerase II pausing. Enrichment of DNA methylation at exon 5 inhibited the binding of CTCF resulting in reduced exon inclusion. This suggest methylation contributes to alternative splicing.

Li-Byarlay *et al.* (2013) used the honey bee as a model to study the functional role of DNA methylation to alternative splicing. Knockdown of *DNMT3* expression decreased the overall methylation and caused various changes in alternative splicing form such as exon skipping and intron retention in fat tissue. This indicates that regulation of alternative splicing can be one of the functional roles of gene body methylation.

This demonstrates the ability of DNA methylation to alter gene expression. DNA methylation could therefore lead to the phenotypic plasticity that is observed in various organisms such as plants, humans and insects (e.g. Lim *et al.* 2012; Valena and Moczek 2012; Kooke *et al.* 2015).

2.18 DNA methylation in the honey bee

The importance of environmental cues to create different phenotypes of female castes in social insects leads to an interest in studying the role of epigenetic regulation by DNA methylation to this phenomenon including other important regulating processes.

Interestingly, methylation systems appear to be common among social insect species such as ants, wasps and honey bees (Kronforst *et al.* 2008).

The discovery of a functional CpG methylation system in the honey bee combined with its extreme phenotypic plasticity means that this species is a good model system in which to study DNA methylation. Wang *et al.* (2006) showed that the honey bee methylation system consists of three standard DNA methyltransferases: DNMT1, DNMT2 and DNMT3. *DNMT1* and *DNMT3* showed high similarity in sequence to the same genes in human. They also reported that non-CpG methylation in honey bee was extremely rare or nonexistent.

The significant study of DNA methylation in honey bee was conducted by Kucharski *et al.* (2008). They used RNA interference (RNAi) to silence the expression of *DNMT3,* which is known to be crucial for *de novo* methylation, in the newly-hatched larvae. The result demonstrated that RNAi-treated larvae majorly emerged as adults (72%) with queen-like phenotype of fully-developed ovaries while the remaining (28%) were typical workers with basic ovaries. This suggests suppression of DNA methylation imitates the effect of enriched larva diet and DNA methylation is a prospective mechanism to generate the phenotypic plasticity between queen and worker caste. This finding contributes to many studies in DNA methylation of honey bees.

Lyko *et al.* (2010) studied brain methylome of an adult queen and an adult worker based on whole-genome bisulfite sequencing. They reported that there was a significant difference in methylation patterns between queen and worker brains highlighting the relationship between DNA methylation and caste differentiation. In this study, they also provided the important basis of methylation in honey bee which nearly all methylated cytosines were found in a CpG dinucleotide context. Furthermore, methylation is mainly congregated in intragenic regions, particularly in

exon regions of the genome. In addition, the contribution of DNA methylation to alternative splicing was proposed in this study.

It is in accordance with the study of Foret *et al.* (2012). The result suggested that queen and worker larvae differed in the methylation patterns. Also, the number of differentially methylated genes (DEGs) in larval heads (2,399) of both castes was higher than that in adult brains (561) of both castes from the previous report (Lyko *et al.* 2010). Considering DEGs in larvae, a worker showed higher methylation than a queen. In addition, there was differential methylation pattern in a subset of selected DEGs between worker and queen castes. Critical metabolic and signaling pathways such as tricarboxylic acid (TCA) cycle, the ubiquitin–proteasome pathway, the spliceosome and the cell division in honey bee contained methylated genes with the high number.

Furthermore, methylation is involved in behavioral transition state of worker subcastes. DNA methylation in workers appeared to change from nursing to foraging and, after the reverse state, from foraging to nursing (Herb *et al.* 2012). This indicates that change in methylation is associated with changing behavioral phenotypes of worker subcastes.

Moreover, methylation is implicated in honey bee development. Whole-genome bisulfite sequencing revealed the genome-wide DNA methylation level was significantly higher in an unfertilized egg than in sperm (Drewell *et al.* 2014). There were 381 genes which were differentially methylated between egg and sperm methylomes. More were methylated in eggs. This suggests there are dynamic changes of DNA methylation in genome through adult drone development and the DNA methylation acts as a epigenetic marker to gamete.

Foret *et al.* (2009) characterized methylated genes in honey bee. They further reported genes which were ubiquitously expressed were the main targets for DNA methylation. Those functions were related to a housekeeping role in the main conserved biological processes. In addition, genes in *A. mellifera* could be divided into two classes of low-CpG and high-CpG contents, resulting from CpG depletion through evolutionary time (Elango *et al.* 2009). The low CpG content containing gene was predicted to be hypermethylated in germline and involved in basic biological processes. In contrast, the high-CpG content containing gene was predicted to be hypomethylated in germline and associated with developmental processes including caste differentiation (Elango *et al.* 2009).

As demonstrated, epigenetic regulation mediated by DNA methylation has been implicated in honey bee in various aspects such as caste differentiation, phenotypic plasticity, development and behavioral transition.

2.19 Bisulfite sequencing

DNA methylation can be assessed by various methods such as methyl-CpG affinity chromatography, methylation-sensitive restriction enzyme analysis and bisulfite sequencing (Suzuki and Bird 2008).

Among these, bisulfite sequencing is widely used to detect DNA methylation in several organisms such as plants (Cokus *et al.* 2008), animals (Gavery and Roberts 2013) and humans (Deng *et al.* 2009). According to a sequencing-based method, this technique is convenient and rapid. This technique can be used for detecting DNA methylation in various contexts such as promoters (Hegi *et al.* 2004), transcribed regions (Si *et al.* 2016) and genomes (Meissner *et al.* 2008). A bisulfite method has

been incorporated with advance technology systems such as high-throughput sequencing (Taylor *et al.* 2007; Gu *et al.* 2011) and microarray (Gitan *et al.* 2002; Reinders *et al.* 2008) to increase high efficiency of detection and reduce time consumption.

Basically, bisulfite sequencing consists of two main procedures which are sodium bisulfite treatment and sequencing determination. Target DNA is primarily treated by sodium bisulfite to provide a deamination reaction to cytosine (unmethylated cytosine) but not to 5-methylcytosine (5mC) (Figure 2.9) (Frommer *et al.* 1992). This results in converting cytosine to uracil while 5-methycytosine remains unchanged (Figure 2.9) (Harrison and Parle-McDermott 2011).

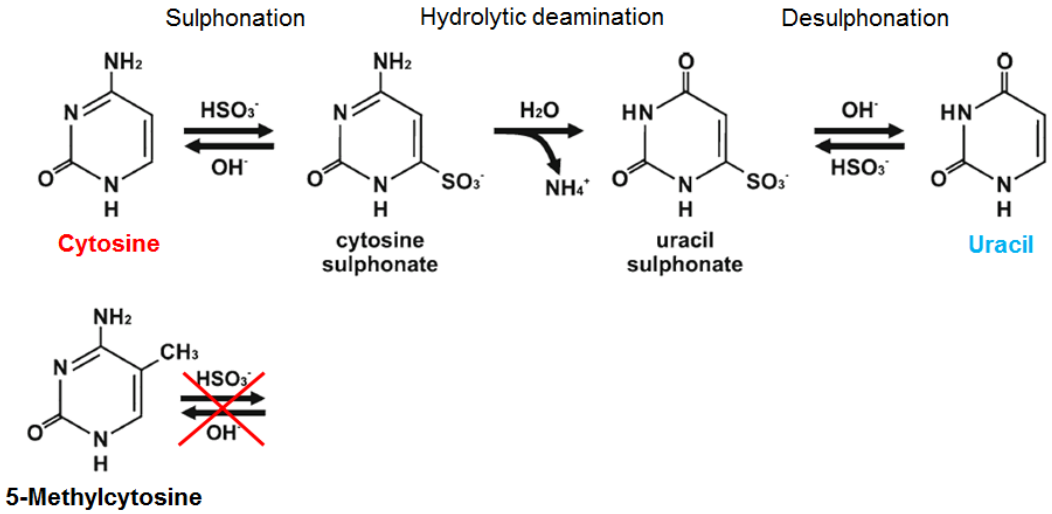


Figure 2.9 A chemical reaction for bisulfite conversion of unmethylated cytosine to uracil [(modified from Hatakeyama *et al.* (2013)].

Bisulfite-converted DNA is then used as a template for PCR to amplify a desired fragment. In a PCR process, uracil in the treated DNA sample is subsequently detected as thymine in the final product (Zilberman and Henikoff 2007) (Figure 2.10). The obtained PCR product can be analysed by Sanger sequencing (Shang *et al.* 2016) or pyrosequencing (de Boni *et al.* 2015). Methylation status is assessed by comparing bisulfite sequence profile with control (untreated) sequence profile to detect methylated cytosine sites.

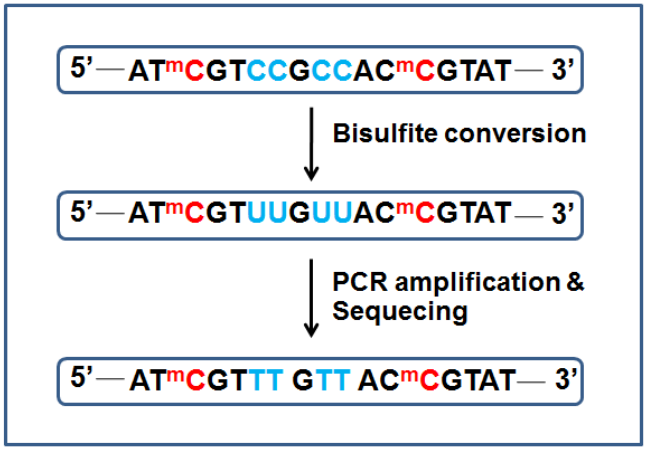


Figure 2.10 Schematic of bisulfite PCR.

Sanger sequencing

This technique is developed by Sanger *et al.* (1977) for determining the nucleotide sequence using the 2', 3' dideoxyribonucleoside triphosphate (ddNTP) which are analogues of deoxyribonucleoside triphosphate (dNTP) to act as chain-terminating inhibitors of DNA polymerase. Generally, there are four separated polymerase chain reactions (PCR) which their mixture contain dNTPs (dATP, dGTP, dCTP and dTTP) and each of ddNTPs, either ddATP, ddGTP, ddCTP or ddTTP).

When DNA polymerase incorporates ddNTP into the newly synthesized DNA strand, the reaction will be terminated. All fragment products from each reaction are then analysed together by acrylamide gel electrophoresis and visualized according to radioactively labeled dNTPs or primer to determine the sequence. At present, using automated sequencing, each ddNTP is labeled with different fluorescent dye (Pettersson *et al.* 2009). The resulting fragment product could be analysed by capillary electrophoresis providing electropherogram that can be translated into nucleotide sequences (Morozova and Marra 2008).

Pyrosequencing

Pyrosequencing is based on detecting the incorporation of one dNTP in the synthesizing DNA strand. The success of nucleotide incorporation by DNA polymerase results in releasing pyrophosphate which is then converted into adenosine triphosphate (ATP) by ATP sulfurylase (Ronaghi *et al.* 1998). Next, the ATP drives the reaction that is catalysed by luciferase to change luciferin into oxyluciferin resulting in emitting the light of which the signal is converted as peak (Shendure and Ji 2008; Metzker 2010). The height of peak is proportional to the amount of incorporated nucleotide (Harrington *et al.* 2013). Apyrase degrades the remaining ATP and unincorporated dNTP in the reaction. Also, the new dNTP is added to initiate the cycle of sequencing (Ahmadian *et al.* 2006). Pyrosequencing is accounted as high resolution for nucleotide detection and widely used to determine DNA methylation since it can detect the amount of thymine or cytosine as the proportion of both nucleotides at the interesting CpG regions (Tost and Gut 2007).

However, a sequencing technique needs to know the target sequence background. Also, the cost of sequencing is quite high especially if it is a high-throughput technique. Besides sequencing, there are alternative methods to detect DNA methylation at a genome-wide scale without having any information of genome sequence. For example, using different restriction enzymes those have the same recognition site but have differential cleavages on the methylated cytosine in that site can be fruitful.

2.20 Methylation-sensitive amplified fragment length polymorphism (MS-AFLP)

MS-AFLP is a useful method that is widely used to examine genome-wide methylation. This technique is based on using a rare cutter like *Eco*RI and methylation-sensitive restriction enzymes as frequent cutters like *Hpa*II and *Msp*I (Reyna-Lopez *et al.* 1997). *Hpa*II and *Msp*I are isoschizomers which recognize and cut the same tretranucleotide sequence of 5'-CCGG-3' but have differential sensitivity to methylation at external and internal cytosine (Herrera *et al.* 2013). *Hpa*II cleaves the site where external cytosine is hemimethylated (^{HMe}CCG) while *Msp*I cleaves the site where internal cytosine is hemi or fully methylated (^{HMe}CG or ^{Me}CG). Both enzymes can cleave the site where is not methylated (Table 2.1) (McClelland *et al.* 1994; Schulz *et al.* 2013). Therefore, comparison of two different profiles from both enzymes of individuals reflects the differentially methylated cytosine status in cleavage site.

Table 2.1 Sensitivities of isoschizomers, *Hpa*II and *Msp*I, to the CCGG 3 methylation status ("+" represents "cut by enzyme" while "-" represents "uncut by enzyme"). The methylated cytosine is indicated by red shadow[modified from Schulz *et al.* (2013)].

	Methylation status	***Hpa*II**	***Msp*I**
C C G G G G C C	No methylation	+	+
C C G G G G C C	Full-methylation of internal C	-	+
C C G G G G C C	Hemi-methylation of internal C	-	+
C C G G G G C C	Hemi-methylation of external C	+	-

Generally, the method consists of two separated digestion reactions of *Eco*RI-*Hpa*II and *Eco*RI-*Msp*I (Díaz-Freije *et al.* 2014). The digested product is ligated to a compatible adaptor to use as a template for PCR amplification (Hanai *et al.* 2010). Pre-selective amplification is performed using a primer specific to an adaptor with one additional selective nucleotide (A, C, T or G) to obtain the corresponding fragment (Xu *et al.* 2000). This is followed by a selective amplification using selective primers with two or three additional selective nucleotides to reduce the number of fragments to be easily readable (Yaish *et al.* 2014; Gupta *et al.* 2015). The obtained product is separated by polyacrylamide gel electrophoresis (Liu *et al.* 2012; Mastan *et al.* 2012) or genetic analyzer (Marconi *et al.* 2013; Busconi *et al.* 2015). The banding type is then scored for methylation status.

MS-AFLP is used to determine the whole DNA methylation variation across genome with no requiring whole genome sequence background in various organisms such as plants and animals (e.g. Sun *et al.* 2014; Avramidou *et al.* 2015; Covelo-Soto

et al. 2015; Roy *et al.* 2015; Zhang *et al.* 2015). This technique has been proven to be successful in examining the difference in methylation pattern in several social insects such as ants (Kronforst *et al.* 2008), wasps (Weiner *et al.* 2013) and bees (Amarasinghe *et al.* 2014).

CHAPTER III

MATERIALS AND METHODS

3.1 Expression and methylation of *Phospholipase* A_2 (*PLA2*) in Thai native honey bees

3.1.1 Honey bee collection

Apis florea, *A. cerana indica* and *A. dorsata* were collected from a garden in Samut Songkram province, Thailand. On the contrary, *A. andreniformis* was collected from Kanchanaburi province, Thailand. Two late stage pupae, red-eyed and black-eyed pupae, were directly pulled out of cells from a hive (Figure 3.1). House bees were caught while they were in their hive. Foragers were caught by a net while they were foraging (Figure 3.1). All bee samples were kept at -20 °C until use.

Figure 3.1 The red-eyed pupa, black-eyed pupa, house bee and forager from *A. florea* (A), *A. cerana* (B) and *A. dorsata* (C).

3.1.2 Determination of expression of *PLA2* transcript and PLA2 enzyme

3.1.2.1 RNA extraction

Total RNA was extracted from whole body of red-eyed pupae and black-eyed pupae and abdomen of house bees and foragers (30 mg each) using RNeasy® mini kit (Cat# 74104, Qiagen, Germany) or RNeasy® plus mini kit (Cat# 74134, Qiagen, Germany) following the manufacturer's instructions. The quality of extracted RNA was determined by 1.2 % (w/v) formaldehyde/ agarose gel electrophoresis. The purity of RNA was assessed by the ratio of the absorbance at 260 nm (A_{260}) and 280 nm (A_{280}). The ration should be in the range of 1.8-2.0 indicating the good quality of extracted RNA. The concentration of RNA was calculated from A_{260} as following formula.

$$\text{Concentration of RNA (ng/µl)} = A_{260} \times \text{dilution factor} \times 40^{*}$$

*40 is defined as one absorbance unit at A_{260} that corresponds to 40 µg/ml solution of single-stranded RNA.

3.1.2.2 Primer design for *PLA2* mRNA

Forward and reverse primers for *PLA2* mRNA amplification of ~393 bp were designed by eyes from the conserved regions of *PLA2* cDNA of *A. cerana cerana* (GenBank, accession# AF321087.1) and *A. mellifera* (GenBank, accession# NM_001011614.1, AF438408.1, JQ900376.1 and JQ900380.1). The control primers were designed from the regions of *28S rRNA* of *A. mellifera* (GenBank, accession# AB126808) and *elongation factor-1 alpha* (*EF-1α)* of *A. mellifera* (GenBank, accession# NM001014993). The sequences of primer were reported in Table 3.1.

Table 3.1 The sequences of primer for reverse transcriptase-PCR amplification of *PLA2*.

Name of primers	Sequences of forward primers (5'⟶3')	Sequences of reverse primers (5'⟶3')	Expected size of product (bp)
PLA2 mRNA	TATCC AGGAA CGTTG TGGTG	CTTGC GAAGA TCGAA CCATT	393
28S rDNA	AAAGA TCGAA TGGGG AGATT C	CACCA GGTCC GTGCC TCC	358
EF-1α	TCGCT TTTAC TCTTG GTGTG A	AAACT TCCAA CATAT TATCT CCA	198

3.1.2.3 Reverse transcriptase-PCR amplification

Reverse transcriptase-PCR was performed using the access RT-PCR system kit (Cat# A1250, Promega, USA). The negative control without RNA template was performed to detect DNA contamination. The reaction mixture for 25 µl of final volume was consisted of 1X reaction buffer, 0.4 µM of each forward and reverse primer, 1 mM of $MgSO_4$, 0.2 mM of dNTP, 2.5 U of *Tfl* DNA polymerase and AMV reverse transcriptase and 20 ng of RNA template from individual bees. The reaction of *PLA2* amplification was performed under condition as follow: 1 cycle of 48 °C for 45 min and 94 °C for 2 min; 30 cycles of 94 °C for 30 sec, 56 °C for 30 sec, and 72 °C for 45 sec; and 1 final cycle of 68 °C for 7 min. The condition for *EF-1α* and *28S rRNA* amplification was as follow: 1 cycle of 48 °C for 45 min and 94 °C for 2 min; 30 cycles of 94 °C for 30 sec, 48 °C for 30 sec and 68 °C for 1 min; and 1 final cycle of 68 °C for 7 min. The reverse transcriptase-PCR product was mixed with 0.5x of UltraPower™ DNA safedye (Cat# UP2001, Gellex, Japan) and resolved on 1.2 % (w/v) agarose gel electrophoresis. The gel was visualized under U.V. light. The

expected PCR product was purified using Qiaquick PCR purification kit (Cat# 28104, Qiagen, Germany) and sent for direct sequencing at AIT Biotech, Singapore. The derived cDNA sequences were searched for homology via BLASTn program (www.ncbi.nlm.nih.gov).

3.1.2.4 Crude extraction for PLA2 enzyme

Crude extract was prepared by modifying the method of Li *et al.* (2005). Ten honey bees of each black-eyed pupae and house bees were individually grinded in 2 ml of 100 mM phosphate buffer saline (PBS), pH 7.4. Then, the mixture was centrifuged at 12,000 rpm (13,040x g) for 5 min at RT. The supernatant was obtained and was kept at 4 °C as venom extract until use.

3.1.2.5 Enzyme activity assay of PLA2

The activity of PLA2 was detected using secretory phospholipase A_2 assay kit (Cat# ab133089, Abcam®, UK). A reaction was prepared by mixing 5 µl of assay buffer, 10 µl of DTNB (5,5'-dithio-bis-(2-nitrobenzoic acid), and 10 µl of enzyme sample in 96 well microtiter plate. After that, 200 µl of diheptanoyl thio-PC as substrate was added to the mixture and the plate was shaken carefully to mix. Non-enzymatic control (blank) and positive control (synthesized bee venom PLA2) were assayed at the same time. Then, the absorbance was measured at 414 nm every min for at least five times. The change in absorbance at 414 nm per min (ΔA_{414}/min) of protein sample (after subtraction from the rate of blank) was used for calculating PLA2 activity as a formula below. One unit of enzyme was defined as an activity of enzyme that could hydrolyze one µmol of diheptanoyl thio-PC per min at 25 °C.

$$\text{PLA2 activity (µmol/min/ml)} = \frac{\Delta A_{414}/\text{min} \times 0.225 \text{ ml}}{*10.66 \text{ mM}^{-1}} \times \frac{\text{Sample dilution}}{0.01 \text{ ml}}$$

$10.66\ mM^{-1}$ is the extinction coefficient for DTNB at 414 nm.

3.1.2.6 Protein determination

The concentration of protein was determined using Bradford assay (Bradford 1976) with protein standard, bovine serum albumin (BSA). The concentration of BSA was prepared as 0, 20, 40, 60, 80 and 100 µg/ml. Each 10 µl of BSA and protein sample was separately mixed with 200 µl of Bradford solution in microtiter plate and incubated at RT for 5 min. The mixtures were measured for the absorbance at 595 nm. The standard curve of BSA was constructed with Y-axis indicating the absorbance at 595 nm and X-axis indicating various concentrations of BSA. The concentration of protein sample was determined from the standard curve.

3.1.3 Determination of DNA methylation of *PLA2*

3.1.3.1 DNA extraction

Genomic DNA was isolated from black-eyed pupae, house bees and foragers (25 mg each) following the protocol of QIAamp® DNA mini kit (Cat# 51304, Qiagen, Germany). The quality of isolated DNA was checked by 1.2 % (w/v) agarose gel electrophoresis. The DNA purity was estimated by the ratio of A_{260}/A_{280}. DNA concentration was calculated by A_{260} using a formula below. The obtained DNA was used as a template for PCR amplification and bisulfite treatment in the following steps.

$$\text{Concentration of DNA (ng/µl)} = A_{260} \times \text{dilution factor} \times 50^{*}$$

*50 is defined as one absorbance unit at A_{260} that corresponds to 50 µg/ml solution of double-stranded DNA.

3.1.3.2 Primer design for full-length of *PLA2* DNA

The primers for PCR amplification of full-length of *PLA2* DNA were designed from the DNA sequence of *PLA2* from *A. mellifera* (GenBank, accession# EF373554.1) in conjunction with mRNA sequence of *PLA2* from *A. mellifera* (GenBank, accession# NM_001011614.1, AF438408.1, JQ900376.1 and JQ900380.1) to encompass the open reading frame (ORF) region. Three pairs of primers and their sequences were shown in Table 3.2.

Table 3.2 The sequences of primers for PCR amplification of full-length of *PLA2* DNA.

Name of primers	Sequences of forward primers (5'→3')	Sequences of reverse primers (5'→3')	Expected size of product (bp)
F1 and R1	CTTCT CCCAC GATGC AAGT CG	TCAAT ACTTG CGAAG ATCGA ACC	1,271
F1 and R2	CTTCT CCCAC GATGC AAGT CG	AGCAT GCATC CGTGT GCT	283
F2 and R1	CAACA GGTTG TCGTG CGACT G	TCAAT ACTTG CGAAG ATCGA ACC	356

3.1.3.3 PCR amplification

PCR reaction (20 µl of final volume) was performed by mixing 1x of EmeraldAmp GT PCR master mix (PCR buffer, PCR enzyme and dNTP mixture) (Cat# RR310Q, Takara, Japan), 0.5 µM of forward primer, 0.5 µM of reverse primer and 200 ng of DNA template. PCR amplification was performed under the suitable condition (1 cycle of 94 °C for 2 min, 30 cycles of 94 °C for 30 sec, 56 °C for 30 sec and 72 °C for 1.5 min and 1 cycle of 68 °C for 7 min). The PCR product was mixed with 0.5x UltraPower^{TM} DNA safedye and resolved on 1.2 % (w/v) agarose gel electrophoresis. The gel was then visualized under U.V. transmission. The PCR product was purified and sent to AIT Biotech, Singapore for DNA sequencing.

3.1.3.4 Sequence analysis

The derived DNA fragment sequences from each pair of primers were assembled by ClustalX program and manually by eyes to obtain the full-length of *PLA2* DNA. The obtained full-length of DNA was used for primer design for subsequent DNA methylation analysis using PCR amplification.

3.1.3.5 Bisulfite treatment

Target DNA was treated by EpiTech® bisulfite kit (Cat# 59104, Qiagen, Germany) according to the protocol. Briefly, the methylation status of DNA could be determined using sodium bisulfite. Bisulfite reaction was prepared by mixing target DNA (1 ng – 2 µg), RNase-free water, bisulfite mix and DNA protecting buffer. Bisulfite conversion was performed using thermal cycler under condition as follow: denaturation at 99 °C for 5 min, incubation at 60 °C for 25 min, denaturation at 99 °C for 5 min, incubation at 60 °C for 85 min, denaturation at 99 °C for 5 min, incubation

at 60 °C for 175 min and hold indefinitely at 20 °C. This allowed the unmethylated cytosine residue to be converted to uracil and kept the methylated cytosine residue unchanged. After wash and elution, converted DNA were kept at -20 °C and used as template for downstream PCR amplification.

3.1.3.6 Primer design for selected *PLA2* region of bisulfite-converted DNA

The CpG rich regions in the full-length *PLA2* DNA sequence were examined using MethPrimer program (www.urogene.org/methprimer). Primers were designed to encompass these regions and the product size of amplification was not over than 200 bp. Primers were attached with biotin in order to pyrosequence. The sequences of primer for detecting *PLA2* DNA methylation were described in Table 3.3. The location of these primers for each *Apis* spp. was demonstrated in Figure 3.2

Table 3.3 The primers for detecting DNA methylation of *PLA2* by PCR amplification from the four native Thai *Apis* species.

Species[a]	Sequences of forward primers (5'⟶ 3')	Sequences of reverse primers (5'⟶ 3')	Expected size of product (bp)
A.a.	GTTAT TTAGG AAYGT TGTGG	AACCR AATTA ATCAA ATTAT AC	158
A.f.	GTTAT TTAGG AAYGT TGTGG	AACCR AATTA ATCAA ATTAT AC	158
A.c.	TTTGT TGTGT TAGGA AYGTT GTG	CTTCR ATTCA CCAAC TAACA TC	141
A.d.	GTTTA TTTGT TGCGT TAGGA A	CTTCR ATTCA CCAAC TAACA TCA	146

[a]Species abbreviations: *A.a.* for *A. andreniformis*; *A.f.* for *A. florea*; *A.c.i.* for *A. cerana indica* and *A.d.* for *A. dorsata*. R includes all purines while Y included all pyrimidines.

(A)

(B)

(C)

Figure 3.2 Location of primers for detecting DNA methylation of *PLA2* by PCR amplification of *A. andreniformis* and *A. florea* (A), *A. cerana indica* (B) and *A. dorsata* (C). The upper sequence is the submitted *PLA2* DNA sequence while the lower sequence is the predicted CpG sites (++ and grey highlight), unchanged nucleotide (|) and converted C to T (:) in *PLA2* DNA sequence from MethPrimer. The sequences of primer were underlined.

3.1.3.7 PCR amplification of bisulfite-converted DNA

PCR reaction (20 µl of final volume) consisted of 1x EmeraldAmp GT PCR master mix (PCR buffer, PCR enzyme and dNTP mixture) (Cat# RR310Q, Takara, Japan), 0.5 µM of each forward and reverse primer that was attached with biotin and 5 µl of bisulfite–converted DNA. PCR amplification was processed under the suitable condition (1 cycle of 94 °C for 3 min, 45 cycles of 94 °C for 30 sec, 54 °C for 30 sec and 68 °C for 30 sec and 1 cycle of 68 °C for 7 min). The PCR product was mixed with 1 µl of 0.5x U.V. UltraPower DNA safedye and resolved on 1.2 % (w/v) agarose gel electrophoresis. The gel was visualized under U.V. light. The PCR product containing an expected band was purified and sent to Chula GenePRO Center, Faculty of Medicine, Chulalongkorn University, Thailand for pyrosequencing.

3.2 DNA methylation in worker sterility of honey bee *A. mellifera*

3.2.1 Honey bee management

Two replicates of experiment were performed in September 2014 and December 2014 consisting of eight and ten experimental cages, respectively. For each replicate, to obtain worker adult bees, emerging worker brood frames from a single colony were incubated at 35 °C for overnight. On the following day, 150 newly-emerged adult workers were transferred to an individual experimental cage (approximately 7 x 8.5. x 11.5 cm^3) (Rothenbuhler *et al.* 1979) prepared with a foundation comb of beeswax (7 x 4 cm^2) and important honey bee diet (water, royal jelly mixed with honey (1:1) and bee-collected pollen *ad libitum*) (Figure 3.3). All

cages were observed every day and the food was replenished every three days. Cages were kept in an incubator at 35 °C.

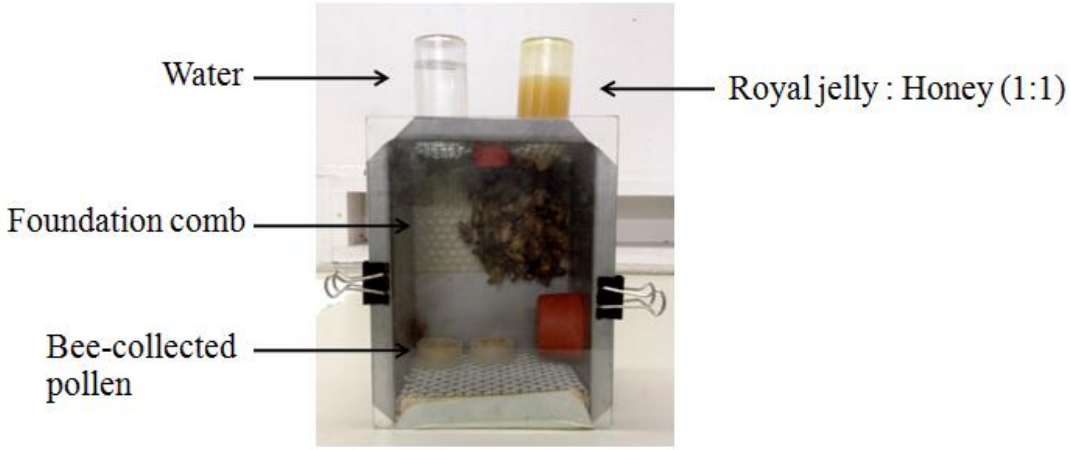


Figure 3.3 Experimental cage.

3.2.2 CO_2 narcosis

This method was modified from Koywiwattrakul *et al.* (2005) as follow. In each replicate, experimental cages were divided into control and treatment groups in half of all cages. Four days old workers would be narcotized with CO_2 to reduce ovary activation. In detail, at RT, each treatment cage was placed in a large zip-lock plastic bag and flushed with compressed CO_2 until the bees were immobilized (Figure 3.4). The bag was then sealed for 5 min. The control cages were left outside the incubator simultaneously but not obtained CO_2 treatment. The same treatment was performed again after 24 h. After the second treatment, bees from control and experimental cages were collected at 4 h as 5 days old samples (20 bees), 2 days as 7 days old samples (60 bees) and 7 days as 12 days old samples (the remaining bees). Bee samples were frozen at -80 °C until dissection.

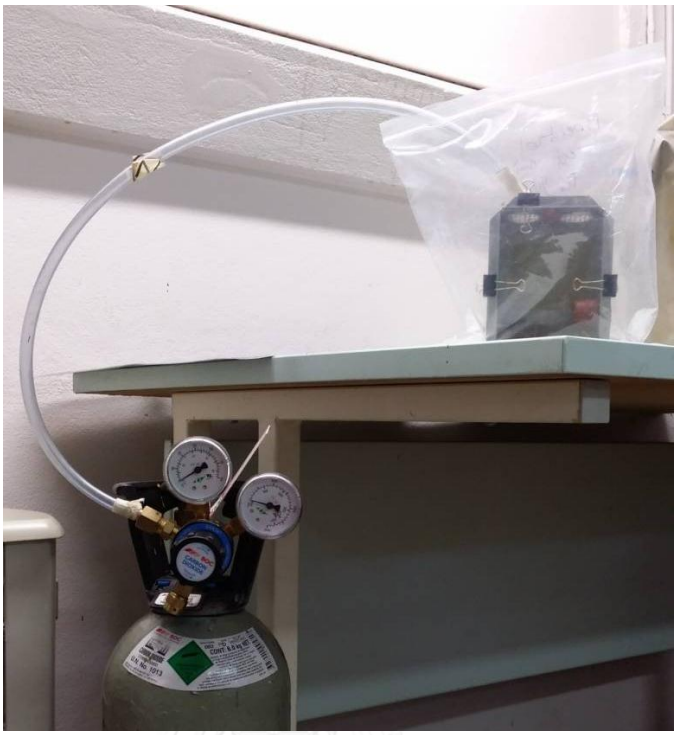


Figure 3.4 Necrotized bees by CO_2.

3.2.3 Ovary dissection and DNA extraction

Collected bees were dissected and the extent of ovary activation was assessed according to Koywiwattrakul *et al.* (2005). Thin ovarioles with lacking defined ova (egg cells) were denoted as "non-active" (Figure 3.5A) while thick ovarioles with clearly defined ova were defined as "active" (Figure 3.5B). Four active ovaries from four individual bees were collected from each cage at each collection time point and pooled in one tube. Four non-active ovaries from four individual bees were collected as the same manner. DNA was extracted from each pooled sample of ovaries using DNeasy® blood and tissue kit (Cat# 69506, Qiagen, Germany). The concentration of

DNA was measured by NanoDrop® ND-1000 spectrophotometer (ThermoFisher Scientific, USA).

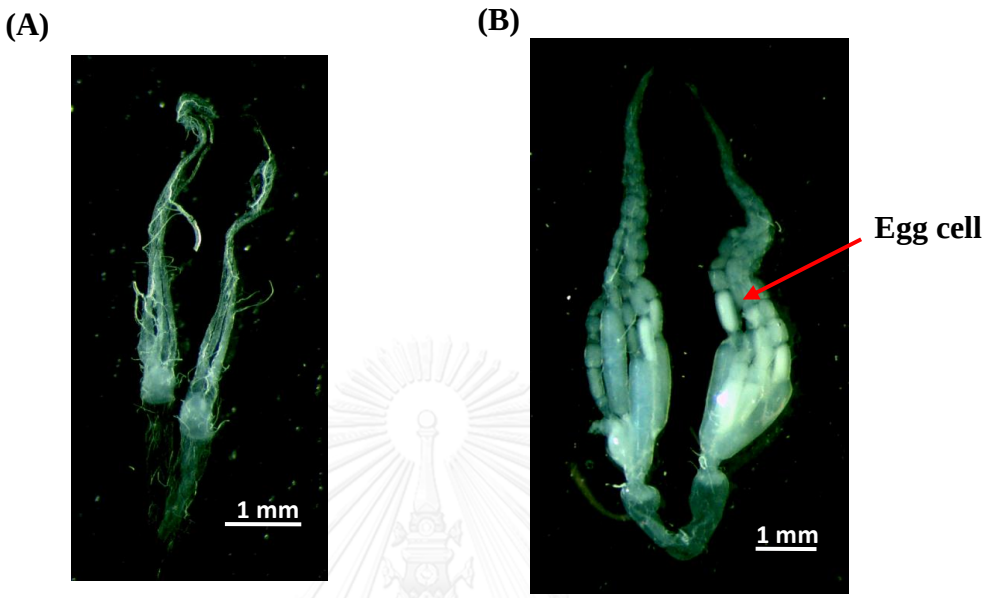


Figure 3.5 The non-active ovary (A) and active ovary (B) derived from individual bees. The red arrow indicates the egg cell.

3.2.4 Methylation-sensitive amplified fragment length polymorphism (MS-AFLP) assay

MS-AFLP assay was modified according to Xu *et al.* (2000) and Lo *et al.* (2012). The sequences of *Eco*RI adaptor, *Hpa*II–*Msp*I adaptor and primers for PCR amplification were described in Vos *et al.* (1995), Xu *et al.* (2000) and Lo *et al.* (2012), respectively (Table 3.4).

Table 3.4 Adaptors and primers used in MS-AFLP.

Step	Adaptor/ Primer name	Sequence (5'⟶ 3')
Ligation	*Eco*RI adaptor_F	CTCGTAGACTGCGTACC
	*Eco*RI adaptor_R	AATTGGTACGCAGTCTAC
	*Hpa*II – *Msp*I adaptor_F	GACGATGAGTCTAGAA
	*Hpa*II – *Msp*I adaptor_R	CGTTCTAGACTCATC
Pre-selective amplification	*Eco*RI pre-primer	GACTGCGTACCAATTCT
	*Hpa*II – *Msp*I pre-primer	GATGAGTCTAGAACGGT
Selective amplification	*Eco*RI selective primer (E primer)	
	E-TGA	GACTGCGTACCAATTCTGA
	E-TAG	GACTGCGTACCAATTCTAG
	E-TCT	GACTGCGTACCAATTCTCT
	*Hpa*II – *Msp*I selective primer (HM primer)	
	HM-TAG	GATGAGTCTAGAACGGTAG
	HM-TAC	GATGAGTCTAGAACGGTAC
	HM-TGC	GATGAGTCTAGAACGGTGC
	HM-TGG	GATGAGTCTAGAACGGTGG
	HM-TGT	GATGAGTCTAGAACGGTGT
	HM-TCG	GATGAGTCTAGAACGGTCG

3.2.4.1 Enzyme digestion

DNA (100 ng) from each pooled-ovary sample was digested separately in a reaction of *Eco*RI/*Hpa*II and a reaction of *Eco*RI/*Msp*I together with negative control. The *Eco*RI/*Hpa*II digestion reaction (25 µl of final volume) was comprised of 1X CutSmart^{TM} buffer (New England Biolabs, USA), 0.05 µg/µl of BSA (New England Biolabs, USA), 3 U of *Eco*RI and 3 U of *Hpa*II (Lo *et al.* 2012). The *Eco*RI/*Msp*I digestion reaction (25 µl of final volume) consisted of 1X NEB buffer 4 (New

England Biolabs, USA), 0.05 µg/µl of BSA, 3 U of *Eco*RI and 3 U of *Msp*I (Lo *et al.* 2012). The reaction mixture was incubated at 37 °C for 1 h.

3.2.4.2 Adaptor ligation

Digested DNA product from each digestion reaction was ligated to *Eco*RI adaptor (50 µM final concentration) and *Hpa*II –*Msp*I adaptor (50 µM final concentration). In detail, each adaptor was prepared by heating the two complementary strands at 95 °C for 5 min, then, 65 °C for 10 min. Five µl of ligation solution [1X ligation buffer, 0.5 µM *Eco*RI adaptor, 5 µM *Hpa*II–*Msp*I adaptor and 4 U of T4 DNA ligase (New England Biolabs, USA)] was added to the digested DNA. The ligation reaction was performed at 18 °C for overnight. The obtained adaptor-ligated DNA fragment was diluted at 1: 5 with dH_2O to use as a template for pre-selective amplification in the next step.

3.2.4.3 Pre-selective PCR

Pre-selective PCR was performed in a final volume of 20 µl as follow: 1X PCR reaction buffer (Fisher Biotec, Australia), 2.5 mM $MgCl_2$, 0.2 mM dNTP, 0.3 µM each of *Eco*RI pre-primer and *Hpa*II-*Msp*I pre-primer (Table 3.4), 0.4 U of *TAQ-Ti* DNA polymerase (Fisher Biotec, Australia) and 10 µl of 5X dilution of ligated-DNA template. The PCR condition was as follow: 1 cycle of 94 °C for 5 min and 72 °C for 2 min; 20 cycles of 94 °C for 30 sec, 56 °C for 30 sec and 72 °C for 1 min; and 1 final cycle of 72 °C for 10 min. The pre-selective product was diluted at 1: 5 with dH_2O for use as the template in selective PCR.

3.2.4.4 Selective PCR

The selective PCR was prepared using 2.5 µl of 1: 5 diluted pre-selective product in a final volume of 10 µl containing 1X PCR reaction buffer (Fisher Biotec, Australia), 2.5 mM $MgCl_2$, 0.2 mM dNTP, 0.25 µM single *Eco*RI selective primer (hereafter "E primer", Table 3.4) which its 5'-end was labeled with blue fluorescent dye (FAM, Sigma-Aldrich, USA), 0.25 µM single *Hpa*II-*Msp*I selective primer (hereafter "HM primer", Table 3.4) and 0.4 U of *TAQ-Ti* DNA polymerase (Fisher Biotec, Australia). In this step, each of 3 E primers was paired with each of 6 HM primers. Thus, 18 combinations of selective primers were obtained. The condition for selective amplification was as follow: 1 cycle of 95 °C for 10 min; 12 cycles of 94 °C for 30 sec, 65 °C for 30 sec for the first cycle (Later, the annealing temperature was decreased by 0.7 °C per cycle in the following 11 cycles) and 72 °C for 1 min. Then, it was followed by 23 cycles of 94 °C for 30 sec, 58 °C for 30 sec and 72 °C for 1 min. At last, it was followed by 1 final cycle of 72 °C for 10 min.

3.2.4.5 DNA fragments analysis

One µl of selective PCR product was mixed with 10 µl of the mixture of GeneScan™ – 500 LIZ® size standard (orange fluorescent dye labeling) (Cat# 4322682, Applied Biosystems, UK) and Hi-Di™ formamide (Cat# 4311320, Applied Biosystems, UK) (1: 100). The entire solution was electrophoresed on 3130*xl* Genetic Analyzer (Applied Biosystems, USA) to analyse DNA fragment product. The obtained DNA fragments between 100 and 600 bp were then analysed by GeneMapper v3.7 (Applied Biosystems, USA) with AFLP default setting. The

fragments were primarily screened as follow. The fragments were excluded from analysis when their peak height was less than 15 % of the relative highest peak in the same reaction. The fragments with no variation found in the profile were not considered as well. The presence (1) or absence (0) of each fragment from *Eco*RI/ *Hpa*II and *Eco*RI/ *Msp*I digestions in each sample were manually recorded.

3.2.4.6 Data analysis

"Presence or absence" data from MS-AFLP fragment analysis was analysed using *msap* package v. 1.1.8 in R environment (R Development Core Team 2011) according to Pérez-Figueroa (2013). Methylation state at each fragment in each sample was assessed based on fragment patterns as follow. The presence of fragment from both *Eco*RI / *Hpa*II and *Eco*R I/ *Msp*I digestions (pattern 1/ 1) was defined as unmethylated state. The presence of one fragment from either *Eco*RI/ *Hpa*II digestion (pattern 1/ 0; hemimethylation) or *Eco*RI/ *Msp*I digestion (pattern 0/ 1; internal cytosine methylation) was defined as methylated state. The absence of fragment from both *Eco*RI/ *Hpa*II and *Eco*RI/ *Msp*I (pattern 0/ 0) digestions was considered as an uninformative state because it could be resulted from (i) full methylation at both cytosine or external cytosine or (ii) absence of fragments.

Each fragment was assessed to be methylation-susceptible locus (MSL) or non-methylated locus (NML) (Herrera and Bazaga 2010) depending on whether the observed proportion of methylated state across all samples was exceeded 5 % (error rate-based threshold) (Pérez-Figueroa 2013). Polymorphic MSL where there was variation from common methylation state in at least two samples were subsequently

analysed. Epigenetic variation at polymorphic MSL was measured with Shannon's diversity index (*I*) for each locus (Pérez-Figueroa 2013). The overall epigenetic variation was reported as mean diversity calculated by averaging the diversity index across all loci. Epigenetic differentiation between groups of samples were evaluated by principal coordinates analysis (PCoA). The difference in methylation frequency between workers with active and non-active ovaries and between control and treatment cages was examined using an analysis of molecular variance (AMOVA) (Excoffier *et al.* 1992).

3.3 DNA methylation of *Kr-h1* in regulating worker sterility of honey bee *A. mellifera*

3.3.1 Sample collection

Adult workers were obtained by incubating worker brood frames at 35 °C for overnight. On the following day, ninety newly emerged workers were transferred to each experimental cage provided with a beeswax foundation comb (7 x 4 cm^2) and honey bee diet [water, royal jelly mixed with honey (1: 1) and bee-collected pollen *ad libitum*]. In this study, there were four experimental cages which half of them were control cages and other half was treatment cages. All experimental cages were maintained in an incubator at 35 °C and were checked every day. The diet was filled up every three days.

3.3.2 CO_2 treatment

When workers were four days old, they were subjected to CO_2 narcosis. At RT, the treatment cages were placed in a large plastic bag. The compressed CO_2 was applied to the cages until the bees did not move, then, the narcosis had been continued for 10 min. The control cages were placed outside the incubator but were not narcotized at the same time. After 24 h, the treatment cages had been secondly narcotized for 10 min and the control cages were done similarly to the previous time. After the second narcosis, 10 workers from control and treatment cages were collected at 4 h (5 days old samples), 1 day (6 days old samples), 2 days (7 days old samples) and 3 days (8 days old samples). All of them were kept at - 80°C until dissection.

3.3.3. Ovary assessment

Bees were dissected and the extent of ovary activation was examined. Non-active ovaries were defined from thin ovarioles without defined ova while active ovaries were defined from thick ovarioles with clearly defined ova. One ovary from each sample was collected in a separate tube using as one ovary sample and kept at -80 °C.

3.3.4. DNA extraction and bisulfite conversion

DNA was extracted from individual ovaries of 7 days old bees (2 days of collection after the second narcosis) and was performed bisulfite modification in one step using EZ DNA methylation-direct^{TM} kit (Cat# D5021, Zymo Research, USA) as

manufacturer's instruction. Briefly, DNA was primarily extracted from ovary tissue. It was then treated by bisulfite conversion reagent to convert unmethylated cytosines into uracils which allowed to detect thymines instead in the following PCR amplification. The obtained bisulfite-converted DNA was used as a template in bisulfite PCR amplification. This was done in three replicates for the same phenotype of individual ovaries (non-active or active) in each cage.

3.3.5 Methylation prediction

Based on the previous study, the genes involved in ovary regulation were examined for methylation status using the whole genome bisulfite sequence data from honey bee. The multiple candidate genes consisted of *vitellogenin* (*vit*; GB13999), *tyramine receptor* (*amtyr1*; GB17991), *major royal jelly protein1* (*mrjp-1*; GB14888), *Krüppel homolog-1 (Kr-h1*; GB14867), *cGMP-dependent protein kinase known as foraging* (*amfor*; GB18394), *secreted protein acidic and rich in cysteine* (*SPARC*; GB11432), *phosphoinositide-dependent kinase* gene (*pdk1*; GB15780) (Koywiwattrakul *et al.* 2005; Thompson *et al.* 2007; Brito *et al.* 2010), *Gemini* (GB11947) (Jarosch *et al.* 2011), dopamine receptor *Amdop3* (GB14561) (Vergoz *et al.* 2012) and the programmed cell death genes *Anarchy* (GB13621) and *Buffy* (GB18455) (Ronai *et al.* 2016). Their DNA sequences were searched for methylated CpG sites via the UCSC genome browser *A. mellifera* Baylor 2.0/apiMel2 assembly (http://genome.ucsc.edu/cgi-bin/hgGateway) in conjunction with the *A. mellifera* genome assembly 2.0 (Amel_2.0) available at BeeBase (http://hymenopteragenome.org/cgi-bin/gb2/gbrowse/bee_genome2/). These assemblies contain the information of methylation from five available honey bee

methylomes; egg, sperm and drone tissues (Drewell *et al.* 2014) and queen and worker brain (Lyko *et al.* 2010), respectively.

Only *Gemini* and *Kr-h1* were found to have some methylated CpG sites in these previous methylomes. *Gemini* had three CpG sites of methylation in egg and drone tissue and five CpG sites of that in sperm but not in queen and worker brains (Figure 3.6A). *Kr-h1* had three CpG sites of methylation in egg, sperm and worker brain and four CpG sites of that in drone tissue and queen brain (Figure 3.6B). *Kr-h1* was selected for subsequent study according to its prevalence of methylation in all five methylomes with total of six methylated CpG sites. In addition, it was known in responsiveness to queen mandibular pheromone (QMP) and CO_2 narcosis that presumably linked to ovary activation (Grozinger *et al.* 2003; Brito *et al.* 2010).

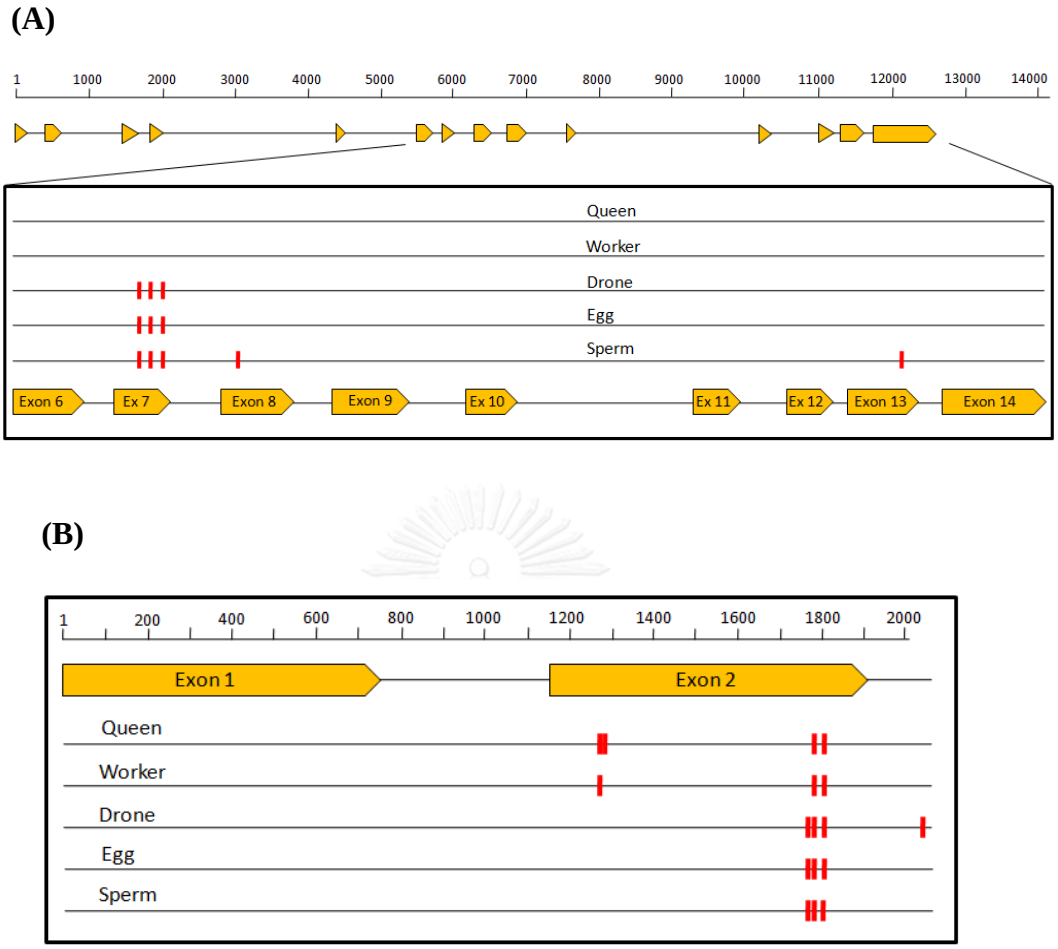


Figure 3.6 The CpG methylation sites of *Gemini* (GB11947) (A) and *Kr-h1* (GB14867) (B), in five sequenced methylomes: egg, sperm, drone, worker and queen. The exons are indicated by the thick yellow box. The methylated CpG sites are shown in vertical red line.

3.3.6 Primer design for *Kr-h1* DNA

To analyse CpG methylation in methylation region of *Kr-h1* by bisulfite PCR amplification, *Kr-h1* DNA sequence obtained from Amel 2.0 (GB14867) and Amel 4.5 (GB45427) assemblies in BeeBase were together analysed. The genome assembly

around the *Kr-h1* gene contained a gap of 328 bases which were manually filled with the *Kr-h1* mRNA sequence (GenBank, accession# NM_001011566.1) by Sequencher. All predicted methylated CpG sites were found around the gap in exon 2 and the 3' untranslated region (3' UTR) of genome assembly (Figure 3.7). The primers were designed to encompass the predicted methylated CpG sites in genome assembly (Figure 3.7). The forward and reverse primer sequences were 5' GTTTTAGGTTGTTTATTATGGTGAAAAAG 3' and 5' CAACCCATCACTATCCCAACATA 3', respectively providing a product of 1,088 bp.

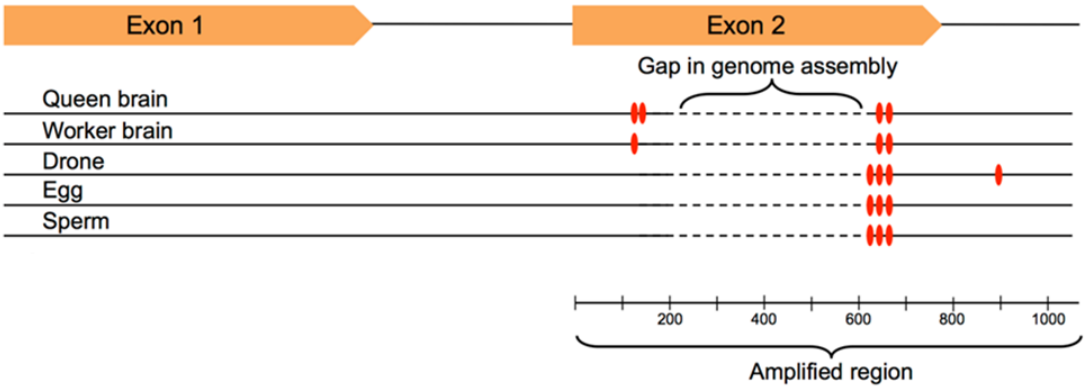


Figure 3.7 CpG sites subject to methylation of *Kr-h1* and region targeted for amplification. The exons are indicated by the thick orange box. The methylated CpG sites from five honey bee methylomes (queen and worker brain, drone tissues, egg and sperm) are indicated in red. The gap in genome assembly is indicated by a dashed line.

3.3.7 Bisulfite PCR amplification

PCR amplification of bisulfite-treated DNA was performed using the KAPA2G robust HotStart PCR kit (Cat# KK5514, KAPA Biosystems, South Africa). A reaction (25 µl of final volume) contained 1X KAPA2G buffer A, 1X KAPA enhancer 1, 0.2 mM dNTP mix, 0.4 µM of each forward and reverse primers, 1 U of KAPA2G robust HotStart DNA polymerase and 1 µl of bisulfite-treated DNA. The condition for amplification was as follow: 1 cycle of 94 °C for 10 min, 38 cycles of 94 °C for 30 sec, 55.6 °C for 30 sec and 72 °C for 1.5 min. Then, it was followed by 1 cycle of 72 °C for 7 min. The PCR product was resolved on 1.2 % (w/v) agarose gel mixed with 1X SYBR®Safe DNA gel stain (Cat# S33102, Invitrogen™ by Life Technologies™, USA) and visualized under U.V. light. The expected PCR product was purified using QIAquick® PCR purification kit (Cat# 28104, QIAGEN, Germany).

3.3.8 PCR cloning

The purified product was cloned for sequencing using TOPO TA cloning® kit (Cat# 45-0030, Invitrogen™ by Life Technologies™, USA). Cloning reaction was set up by mixing 0.5 µl of salt solution, 2 µl of fresh purified PCR product and 0.5 µl of pCR®4-TOPO® vector. The reaction mixture was incubated for 20 min at RT. Three µl of cloning reaction were added to the 25 µl of One Shot® TOP 10 competent *E. coli* and mixed gently. The reaction solution was incubated on ice for 10 min. The cell solution was then heat-shocked at 42 °C for 30 sec and was immediately placed on ice for 2 min. The transformed cells were added with 150 µl of S.O.C medium and

shaken at 200 rpm at 37 °C for 1 h. Later, 100 µl of the culture was spread on LB agar plate containing 100 mg/ml of ampicillin and incubated at 37 °C for overnight.

3.3.9 Colony PCR

Forty to fifty colonies from each PCR product were picked with a pipette tip to do colony PCR to check the inserted DNA fragment. A reference plate of selected colonies were prepared simultaneously and incubated at 37 °C for overnight. The residue of colony at the tip was immersed in the PCR reaction mixture (10 µl of final volume) containing 1X reaction buffer, 2.5 mM $MgCl_2$, 2.5 % glycerol, 0.2 mM dNTP mix, 0.4 µM each of universal M13 forward and reverse primers and 0.4 U of *TAQ-Ti* DNA polymerase (Fisher Biotec, Australia) and the reaction was mixed well. PCR amplification was performed due to the following condition: 1 cycle of 94 °C for 5 min, 35 cycles of 94 °C for 30 sec, 56 °C for 30 sec and 72 °C for 1 min. At last, it was followed by 1 cycle of 72 °C for 7 min. The PCR product was electrophoresed via 1.2 % (w/v) agrarose gel mixed with 1X SYBR®Safe DNA gel stain (Cat# S33102, Invitrogen™ by Life Technologies™, USA). At least ten positive colonies containing the target gene (range of 10 - 18 clones) were selected from the reference plate and cultured in liquid media at 200 rpm at 37 °C for overnight. The plasmid DNA were purified using Wizard® plus SV minipreps DNA purification system (Cat# A1460, Promega, USA) and sent for sequencing at Macrogen Inc., Korea.

3.3.10 Bisulfite DNA sequence and methylation status analysis

The obtained bisulfite-converted DNA sequences (which were already converted unmethylated C to T) were aligned with *Kr-h1* sequence to investigate the methylated CpG site using Sequencher. The frequency of methylated CpG (methylation level) at each site was determined by the ratio of the number of methylated CpG clone to the total number of clones. The difference in frequency of methylation for overall and each site of methyalated CpG were compared between worker with non-active and active ovaries and between worker from control and CO_2-treatment cages using Chi-square test.

CHAPTER IV

RESULTS

4.1 Expression and methylation of *Phospholipase A_2* (*PLA2*) in Thai native honey bees

4.1.1 The expression pattern of *PLA2* transcript

Red-eyed pupae, black-eyed pupae, house bees and foragers of native *Apis* spp. (*A. andreniformis*, *A. florea*, *A. cerana indica* and *A. dorsata*) in Thailand were selected to determine the expression profile of *PLA2* transcript. With an assumption, pupa, which is known as an intermediate organism before becoming an adult, is likely to express this gene. House bees and foragers, which are adults, are expected to express this transcript at the high level. Due to reverse transcriptase-PCR amplification, it revealed that the expression level of *PLA2* was the highest in house bees of all *Apis* spp. and highly detectable in foragers of *A. florea* and *A. dorsata* (Figure 4.1). There was no detectable gene expression in pupae of all *Apis* spp. In addition, the reverse transcriptase-PCR product derived from amplifications of *28S rDNA* and *EF-1α,* which were housekeeping genes, were obtained from all samples (Figure 4.1).

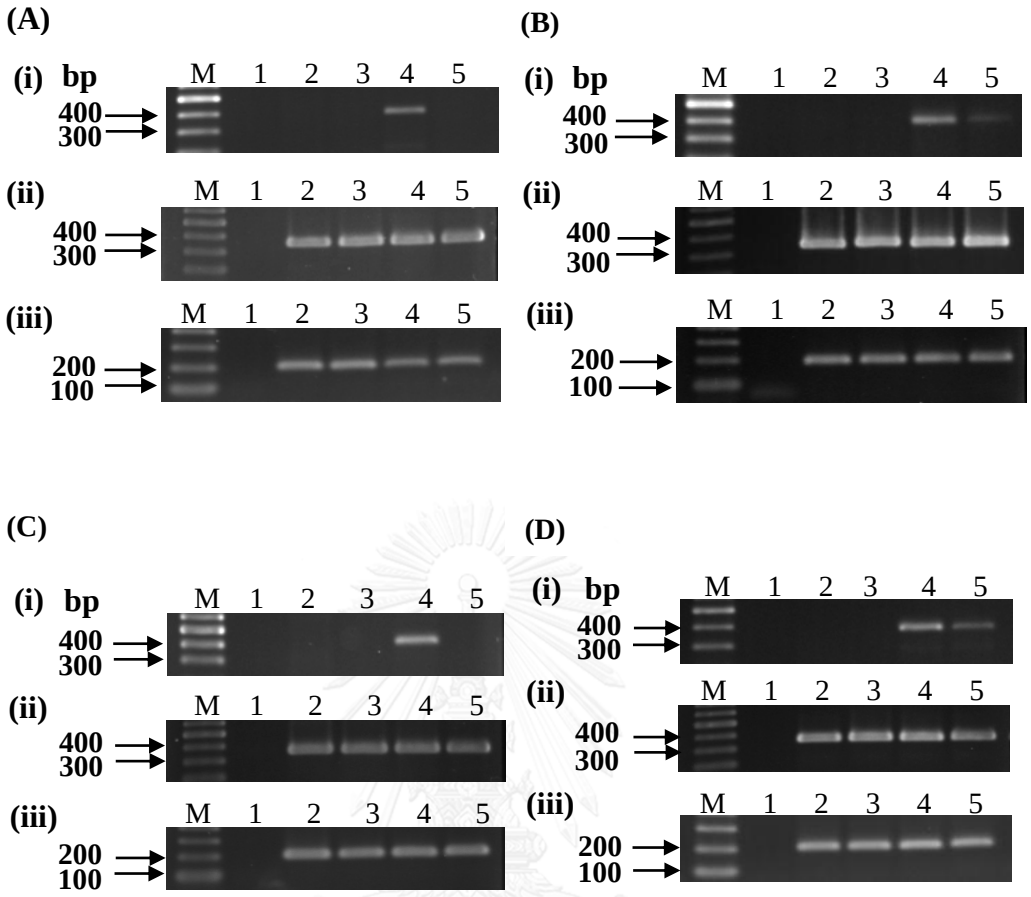


Figure 4.1 The expression profile of *PLA2* of *A. andreniformis* (A), *A. florea* (B), *A. cerana indica* (C) and *A. dorsata* (D). The reverse transcriptase-PCR products were derived from primers for amplification of *PLA2* (i), *28S rDNA* (ii) and *EF-1α* (iii) gene fragments. In each panel, lane M contained a 100 bp ladder marker. Lane 1 contained negative control with no RNA template. Lanes 2, 3, 4 and 5 contained the reverse transcriptase-PCR products from red-eyed pupae, black-eyed pupae, house bees and foragers, respectively. The expected sizes of products are 393 bp for *PLA2* (i), 358 bp for *28S rDNA* (ii) and 198 bp for *EF-1α* (iii).

The quantity of amplified *PLA2* transcript was estimated by comparing the intensity of a band to that of DNA ladder marker (Table 4.1). The concentration of *PLA2* amplicons of *A. andreniformis* (8 ng/µl) was only detected in house bees. The reverse transcriptase-PCR product of *PLA2* in *A. florea* showed the higher concentration in house bees (8 ng/µl) than in foragers (1.5 ng/µl). The highest concentration of amplified *PLA2* products from *A. cerana indica* (12.5 ng/µl) was found in house bees. In *A. dorsata*, the highest concentration of amplified *PLA2* transcripts was in house bees (22.5 ng/µl). However, the highly detectable product in foragers (8 ng/µl) was detected. The concentration of amplified target genes was standardized with the concentration of amplified products of control genes which were *28S rDNA* and *EF-1α* (Table 4.1).

Table 4.1 The estimated concentration of reverse transcriptase-PCR product of *PLA2* and its standardized concentration ratio to *28S rDNA* and *EF-1α.*

Species	Stage	Concentration of DNA product (ng/µl)	Concentration ratio of *PLA2/28S rDNA*	Concentration ratio of *PLA2/EF-1α*
A. andreniformis	House bee	8	0.46	0.53
A. florea	House bee	8	0.32	0.55
	Forager	1.5	0.06	0.1
A. cerana indica	House bee	12.5	0.83	0.63
A. dorsata	House bee	22.5	1.13	1.5
	Forager	8	0.4	0.53

4.1.2 Analysis of partial *PLA2* cDNA sequence

After cDNA sequencing, the partial *PLA2* cDNA of *A. andreniformis*, *A. florea*, *A. cerana indica* and *A. dorsata* were obtained at 393 bp, respectively. By BLASTn analysis, the similarity of derived *PLA2* sequence to the same gene of European honey bee or *A. mellifera* (GenBank, accession# NM_001011614.1) was 89%, 90%, 95% and 94% for *A. andreniformis* (GenBank, accession# KU203672), *A. florea* (GenBank, accession# XM_012489077.1), *A. cerana indica* (GenBank, accession# KU203674) and *A. dorsata* (GenBank, accession# KU203673), respectively. It can be seen that the *PLA2* sequence of *A. cerana indica* exhibited the highest similarity to that of *A. mellifera*. All obtained partial cDNA sequences of *PLA2* from *Apis* spp. including the partial cDNA sequence of *PLA2* from *A. mellifera* (GenBank, accession#NM_001011614.1) were aligned by ClustalX and BioEdit program (Figure 4.2).

```
                    10         20         30         40         50         60
A. c. indica        TATCCAGGAA CGTTGTGGTG CGGGCATGGT AACGTGTCGT CCGGCCCAAA CGAGCTAGGT
A. mellifera        TATCCAGGAA CGTTATGGTG CGGGCATGGT AACAAGTCGT CCGGCCCGAA CGAGCTAGGT
A. dorsata          TATCCAGGAA CGTTGTGGTG CGGGCACGGT AACGTGTCGT CCAGTCCGAA CGAGCTGGGT
A. andrenif         TATCCAGGAA CGTTGTGGTG CGGGCAAGGT AACAAGGCGT CCGATCCGAA CCAACTAGGC
A. florea           TATCCAGGAA CGTTGTGGTG CGGGCATGGT AACAAGGCGT CCGATCCGAG CCAACTAGGC
Clustal Co          ********** **** ***** ****** *** ***  * *** **   ** *  * * ** **

                    70         80         90        100        110        120
A. c. indica        TGGTTCAAGC ACACGGATGC ATGCTGTCGA ACCCACGACA TGTGCCCGGA CGTGATGTCA
A. mellifera        CGGTTCAAGC ACACGGATGC ATGCTGTCGA ACCCACGACA TGTGCCCGGA CGTGATGTCA
A. dorsata          CGGTTCAAGC ACACGGATGC ATGCTGTCGA AGCCACGACA TGTGCCCGGA CGTGATGTCA
A. andrenif         TGGTTAAAGC ACACGGATGC ATGCTGTCGA ACTCACGATA TGTGCCCCGA CGTGATGTCA
A. florea           TGGTTAAAGC ACACGGATGC ATGCTGTCGA ACTCACGATA TGTGCCCCGA CGTGATGTCA
Clustal Co           **** **** ********** ********** *  ***** * ******* ** **********

                    130        140        150        160        170        180
A. c. indica        GCTGGTGAAT CGAAGCACGG TCTGACCAAT ACGGCCTCCC ACACCAGGTT GTCGTGCGAC
A. mellifera        GCTGGTGAAT CGAAGCACGG CCTGACCAAC ACGGCCTCCC ACACCAGGTT GTCGTGCGAC
A. dorsata          GCTGGTGAAT CGAAGCACGG CCTGACCAAC ACCGCCTCCC ACACCAGGTT GTCGTGCGAC
A. andrenif         GCTGGTGAAT CGAAGCACAA TCTGACCAAC CCGGCCTCCC ACACCAGGTT GTCGTGCGAC
A. florea           GCTGGTGAAT CGAAGCACAA TCTGACCAAC CCGGCCTCCC ACACCAGGTT GTCGTGCGAC
Clustal Co          ********** ********    ********   * ******* ********** **********

                    190        200        210        220        230        240
A. c. indica        TGCGACGACA CGTTCTACGA TTGTCTTAAA AATTCGGGGG ACAAGATTAG CTCGTATTTC
A. mellifera        TGCGACGACA AGTTCTATGA TTGTCTTAAA AATTCGGCGG ACACGATTAG CTCGTATTTC
A. dorsata          TGTGACGATA AGTTCTACGA TTGTCTTAAA AATTCGGCGG ACACGATTAG CTCGTATTTC
A. andrenif         TGTGACGACG AGTTCTATAC TTGTCTGAAA AATTCGGGAG ACACGATTAG CGCGTATTTC
A. florea           TGTGACGACG AGTTCTATAC TTGTCTGAAA AATTCGGGAG ACACGATTAG CGCGTATTTC
Clustal Co          ** *****    ******    ****** *** *******  * *** ****** * ********

                    250        260        270        280        290        300
A. c. indica        GTAGGAAAGA TGTACTTCAA TCTGATAGAC ACCAAGTGTT ACAAACTGGA GCATCCTGTG
A. mellifera        GTAGGGAAGA TGTACTTCAA TCTGATAGAC ACGAAGTGTT ACAAACTGGA GCATCCTGTC
A. dorsata          GTAGGACAGA TGTACTTCAA TCTGATAGAC ACGAAATGTT ACAAATTGGA GCATCCTGTC
A. andrenif         GTAGGAAATA CGTATTTCAA CCTGATAGAC ACGAAGTGTT ACAAATTGGA GCATCCCGTC
A. florea           GTAGGAAATA TGTACTTCAA TCTGATAGAC ACGAAGTGTT ACAAATTGGA GCATCCCGTC
Clustal Co          *****  * *  *** *****  ********* ** ** **** ***** **** ****** **

                    310        320        330        340        350        360
A. c. indica        ACCGGGTGCG GTGAGAGGAC CGAGGGTCGT TGTCTTCACT ACACGGTGGA CAAAAGCAAG
A. mellifera        ACCGGGTGCG GTGAGAGAAC CGAGGGTCGT TGTCTTCACT ACACCGTGGA CAAAAGCAAA
A. dorsata          ACCGGATGCG GTAAGAGTGT CGGGGGTCGT TGTCTTAACT ACACGGTGGA CAAAAGCAAA
A. andrenif         ACCGGTTGCG GTGAGAAGGT CGAGGGTCGT TGCCTCCACT ACACGGTGGA CGAAAGCAAG
A. florea           ACCGGTTGCG GTGAGAAGGT CGAGGGTCGT TGCCTTCACT ACACGGTGGA CGAAAGCAAG
Clustal Co          ***** **** ** ***    ** ******* ** **  *** **** ***** * *******

                    370        380        390
A. c. indica        CCAAAAGTGT ACCAATGGTT CGATCTTCGC AAG
A. mellifera        CCGAAAGTGT ACCAATGGTT CGATCTTCGC AAG
A. dorsata          CCGAAAGTGT ACCAATGGTT CGATCTTCGC AAG
A. andrenif         CCGAAAGTCT ACCAATGGTT CGATCTTCGC AAG
A. florea           CCGAAAGTCT ACCAATGGTT CGATCTTCGC AAG
Clustal Co          ** ***** * ********** ********** ***
```

Figure 4.2 The multiple alignment of partial *PLA2* cDNA sequences of *Apis* species (*A. cerana indica*, *A. mellifera*, *A. dorsata*, *A. andreniformis* and *A. florea*). The asterisk indicated the same base residues among the aligned sequences.

4.1.3 Crude venom protein extract and activity assay of PLA2 enzyme

Black-eyed pupae which are the last pupal stage and house bees were used as subjects for crude protein purification. The result showed that there was no detectable expression of *PLA2* transcript in the black-eyed pupae. Black-eyed pupae were therefore chosen for a representative of pupal stages to determine the expression of PLA2 at the translational level. Since house bee was the stage that showed the highest expression of *PLA2* transcript, it was selected to study at the translational level. After colorimetric activity assay, the activity and specific activity were obtained (Table 4.2). The activity of PLA2 in pupae for all of four honey bee species was much lower than in house bees. The lower activity of PLA2 led to undetectable specific activity from pupae of all honey bee species. From the obtained data, it suggested that the translational profile of PLA2 from crude venom protein extract was coincided to the transcriptional profile of *PLA2*. For all species, the house bees showed much higher PLA2 activity (105 – 146 fold greater in *A. andreniformis, A. cerana indica* and *A. dorsata* but 9.7 fold in *A. florea*) comparing to the pupae.

Table 4.2 Activity and specific activity of the expressed PLA2 among *Apis* spp.

Sample	PLA2 activity (µmol/min/ml)		Specific activity (U/mg)	
	Blacked-eyed pupae	House bees	Blacked-eyed pupae	House bees
A. andreniformis	0.01±0.01	1.46±0.03	0	1.78±0.03
A. florea	0.03±0.00	0.29±0.01	0	0.72±0.03
A. cerana indica	0.03±0.03	3.16±0.13	0	2.26±0.09
A. dorsata	0.05±0.01	5.64±0.18	0	1.82±0.06

Data are shown as the mean ± 1 SD and are derived from 3 independent repeats.

4.1.4 Determination of DNA methylation of *PLA2*

4.1.4.1 Full-length of *PLA2* DNA sequence

In order to analyse methylation in *PLA2* gene by PCR amplification, the full-length of *PLA2* DNA sequence before bisulfite conversion in both pupae and house bees of *Apis* spp. was primarily determined. The amplified fragment of *PLA2* DNA from various pairs of primers (F1/R1, F1/R2 and F2/R1) of *Apis* spp. were 1271, 283 and 356 bp (Figure 4.3). After contig assembling, the derived full-length of *PLA2* DNA sequences of *A. andreniformis*, *A. florea*, *A. cerana indica* and *A. dorsata* were 1,197, 1,158, 1,220 and 1,261 bp, respectively.

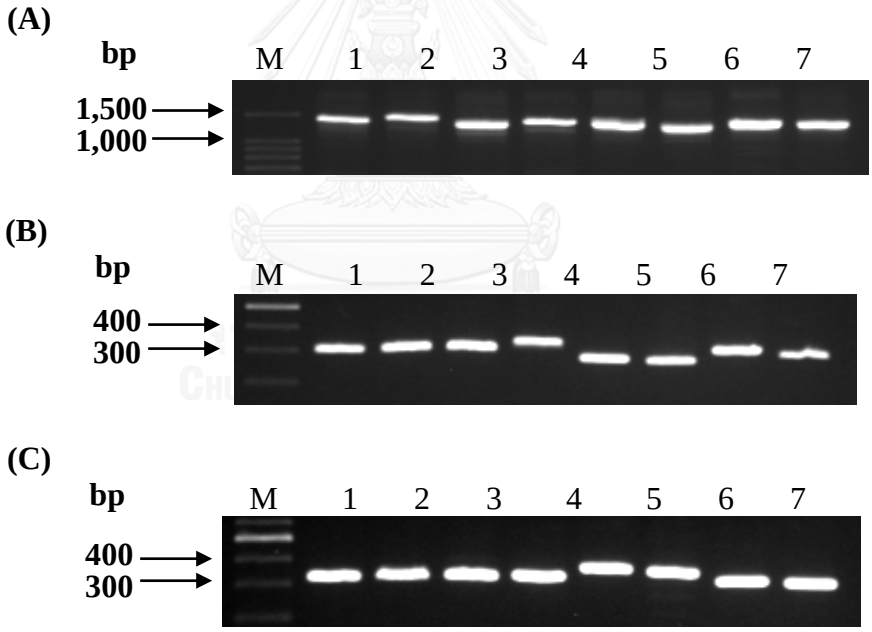


Figure 4.3 Amplified PCR products from various pairs of primers, F1/R1 (A); F1/R2 (B) and F2/R1 (C). Lane M contained 100 bp DNA ladder marker. Lanes 1-8 contained the PCR product from pupa and house bee DNA of *A. andreniformis*, *A. florea*, *A. cerana indica* and *A. dorsata*, respectively. The expected product size was 1,271 (A), 283 (B) and 356 bp (C), respectively.

4.1.4.2 PCR amplification of bisulfite-converted DNA

After bisulfite PCR with biotinylated primer, the expected product size of 158, 158, 141 and 146 bp was obtained from pupae, house bees and forager bees of *A. andreniformis*, *A. florea*, *A. cerana indica* and *A. dorsata*, respectively (Figure 4.4). Although there were some non-specific bands and primer dimers detected in pupa and house bee bisulfite-treated DNA of *A. andreniformis* and *A. dorsata*, respectively, the expected band was cut out of an agarose gel and purified before sequencing.

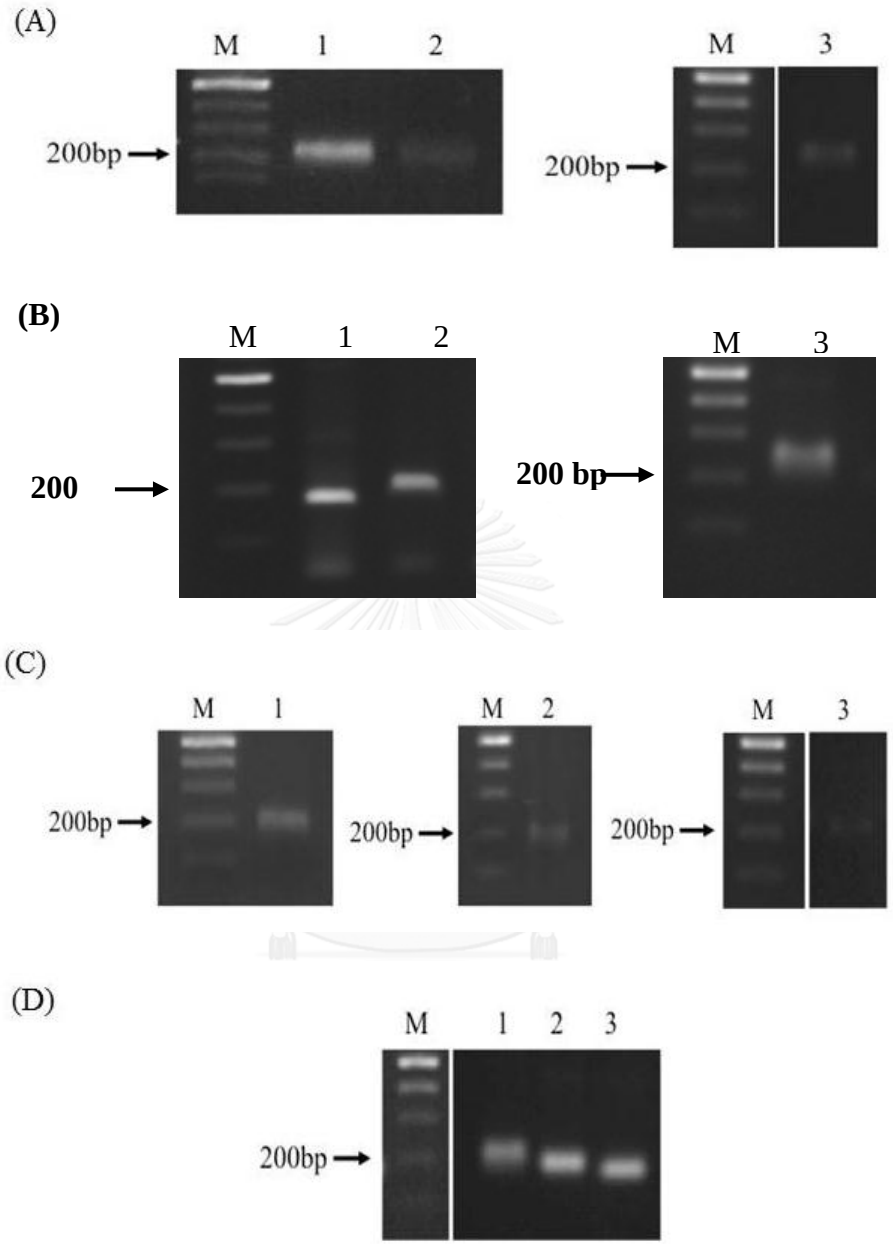


Figure 4.4 The methylation profile of *PLA2* in *A. andreniformis* (A), *A. florea*, (B) *A. cerana indica* (C) and *A. dorsata* (D). Lane M contained 100 bp ladder marker. Lane 1, 2 and 3 contained the PCR products from pupa bisulfite-treated DNA, house bee bisulfite-treated DNA and forager bisulfite-treated DNA, respectively. The expected size of product was 158 (A and B), 141 (C) and 146 bp (D).

4.1.4.3 Methylation level of *PLA2* among *Apis* spp.

DNA methylation level of *PLA2* was different among pupae, house bees and foragers of *A. andreniformis*, *A. florea* and *A. dorsata* but not *A. cerana indica* (Table 4.3). The level of DNA methylation in pupae was higher than that in house bees and forager bees of *A. florea* and *A. dorsata*. This was contrast to the DNA methylation level in *A. andreniformis* and *A. cerana indica*.

Table 4.3 DNA methylation level (%) of *PLA2* in the pupae, house bees and forager bees of the four native Thai *Apis* spp.

Apis spp.	Methylation level (%)		
	Pupae	House bees	Forager bees
A. andreniformis	7.8	10	13
A. florea	27.5	9.4	10
A. cerana indica	13	13	13
A. dorsata	12	7.0	5.0

4.2 DNA methylation in worker sterility of *A. mellifera*

4.2.1 Ovary activation assessment

The ovary activation was assessed by dissection. At 5 days old, workers had no active ovaries in both control and treatment cages. Active ovary was observed starting in workers at 7 days old in control cages while there was no individual with active ovary at 7 days old in CO_2-treated cages (Table 4.4). After 7 days old, workers in CO_2-treated cages started to die. Thus, no sample could be collected at 12 days old as the final collection time point. However, there were only ovary samples obtained from workers from control cages at this time point (Table 4.4).

Table 4.4 Number of examined samples which each sample represents pooled ovaries from four individuals within the same cage.

Age of bees	Control (No CO_2)		CO_2 treatment	
	Non-active ovary	Active ovary	Non-active ovary	Active ovary
5 days	9	0	9	0
7 days	9	8	9	0
12 days	8	8	0	0

4.2.2 Number of polymorphic loci and epigenetic diversity

To determine the difference in frequency of methylation to ovary activation and effect of CO_2 treatment to methylation, groups of comparison were analysed as shown in Table 4.5. Methylation-sensitive amplified fragment length polymorphism (MS-AFLP) assay from 60 pooled ovary samples with 18 primer combinations

revealed a total number of 306 loci. Among those 306 loci, 22 loci were sensitive to methylation (MSL) and suitable for analysis. Eighteen of them were polymorphic across treatment combinations (Table 4.5).

Shannon's index (*I*) measuring epigenetic diversity of polymorphic loci demonstrated that the mean diversity for each treatment comparison was in the same range between 0.512 – 0.619 (Table 4.5). The lowest mean of methylation diversity was detected from non-active ovary between control and CO_2 treatment cages of all ages of workers. The highest value of that was found between workers with non-active and active ovary at 12 days old.

Table 4.5 Epigenetic diversity of polymorphic loci between group comparison.

Comparison [Sample (number of samples), source]	Age	Number of loci	Shannon's diversity index (*I*)
Non-active (44) vs. active (16), all cages	all	18	0.524±0.042
Non-active (26) vs. active (16), control cages	all	18	0.541±0.024
Non-active (9) vs. active (8), control cages	7 days	18	0.536±0.035
Non-active (8) vs. active (8), control cages	12 days	16	0.619±0.019
Non-active (26), control vs. non-active (18), CO_2 treatment	all	18	0.512±0.027
Non-active (9), control vs. non-active (9) CO_2 treatment	5 days	15	0.516±0.040
Non-active (9), control vs. non-active (9), CO_2 treatment	7 days	18	0.541±0.033
Non-active (9), control at 5 days (9), 7 days (9) and 12 days (8) old	all	17	0.531±0.031

4.2.3 Methylation frequency in different states

The frequency (proportion) of loci from different methylation states of each treatment comparison was reported as percentage in Table 4.6. The majority of loci was unmethylated (39-48%), then, followed by internal cytosine methylation (25-34%) and hemimethylation (23-29%). However, the total frequency of methylated loci (internal cytosine methylation and hemimethylation) was higher than unmethylated loci. Uniformative state was found at the lowest frequency (0.5-2%).

Table 4.6 Proportion of polymorphic MSL loci (%) in each methylation state for comparison of workers with non-active ovaries (NAO), active ovaries (AO), CO_2 treatment ($+CO_2$) and control ($-CO_2$).

Methylation pattern	All ages pooled		All ages (control cages)		7 days old (control cages)		12 days old (control cages)		All ages (NAO)		5 days old (NAO)		7 days old (NAO)		NAO (control cages)		
	NAO	AO	NAO	AO	NAO	AO	NAO	AO	$-CO_2$	$+CO_2$	$-CO_2$	$+CO_2$	$-CO_2$	$+CO_2$	5 days	7 days	12 days
HPA+/MSP+ (Unmethylated)	45.0	38.7	46.2	41.5	43.4	42.0	42.9	38.1	43.8	45.8	47.6	46.0	40.7	45.0	47.6	40.7	42.9
HPA+/MSP- (Hemimethylated)	25.5	28.6	24.8	27.3	22.7	28.4	27.4	27.4	25.8	24.9	26.5	27.0	23.8	23.3	26.5	23.8	26.8
HPA-/MSP+ (Internal C methylation)	28.6	31.6	28.1	30.1	32.3	28.4	29.2	33.3	29.5	28.6	24.9	26.5	33.9	30.7	24.9	33.9	29.8
HPA-/MSP- (Uninformative)	0.9	1.2	0.9	1.1	1.5	1.1	0.6	1.2	0.9	0.8	1.1	0.5	1.6	1.1	1.1	1.6	0.6

4.2.4 Epigenetic differentiation

Principal coordinate analyses (PCoA) which depicted all sample groups between i) non-active and active ovary groups, ii) control and treatment group or iii) different ages could not separate each other along with the first two principal coordinates (Figure 4.5). This indicated that there was no epigenetic differentiation in any group comparisons. The first two axes explain a total of 43.7 – 57.3% of the variation.

Similarly, there was no statistically significant difference in genome-wide methylation pattern between any treatment comparison analysed by AMOVA ($P > 0.05$) (Table 4.7).

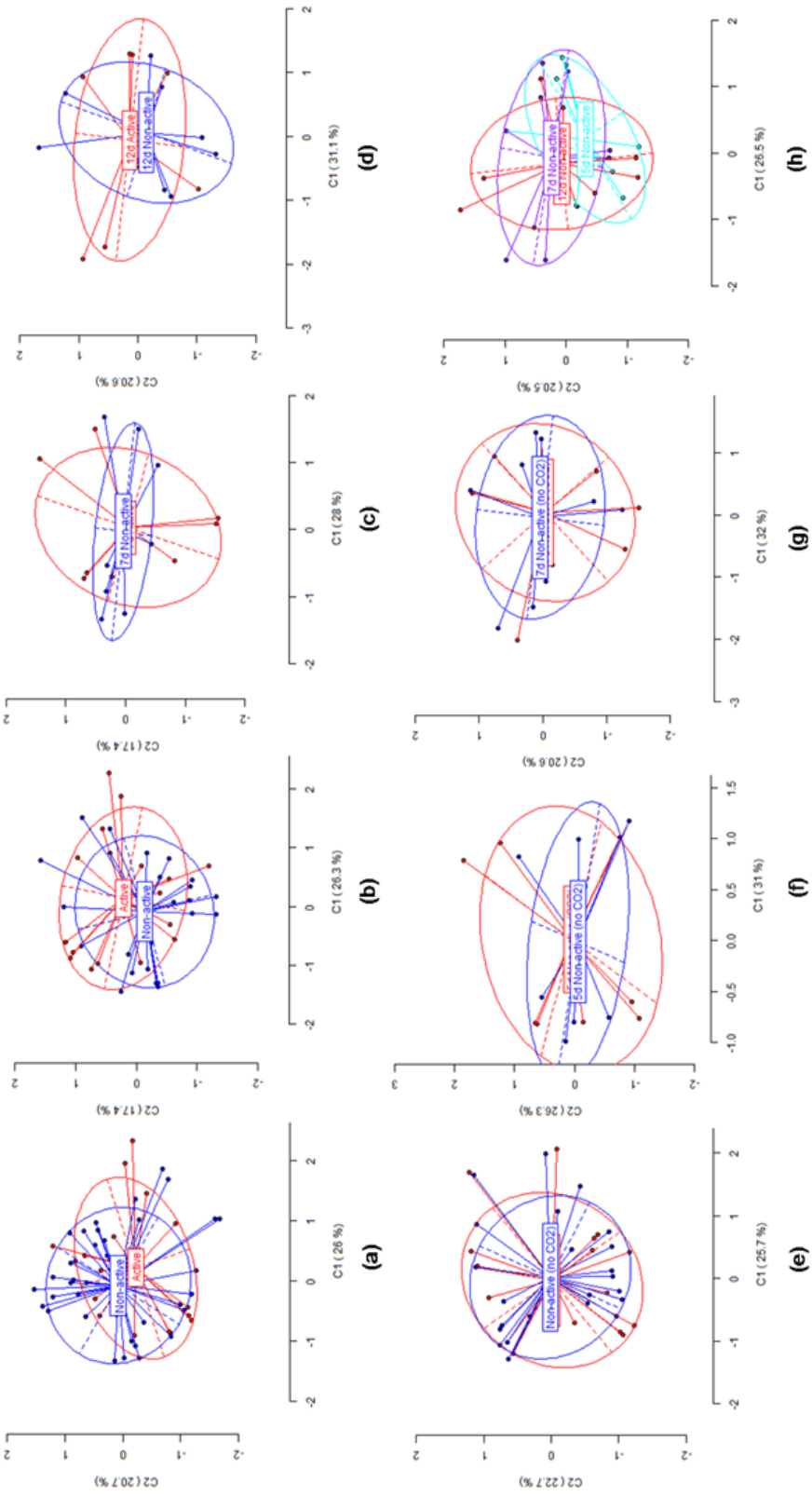


Figure 4.5 Principal coordinates analysis for epigenetic differentiation between workers with non-active (blue) and active (red) ovaries at a) all ages from both control and treatment cages; b) all ages from control cages; c) 7 days old from control cages; d) 12 days old from control cages and between workers with non-active ovaries from control (blue) and CO_2 treatment cages (red); e) at all ages; f) at 5 days old; g) at 7 days old and among workers with non-active ovaries from control cages; h) at 5 (light blue) , 7 (purple) and 12 (red) days old. The variance explained by the first principal component (C1) is given in brackets on the horizontal axis while the variance explained by the second principal component (C2) is given in brackets on the vertical axis. Each point represents a single sample. Group labels depict the centroids for each group. The ellipse displays the dispersion of each group with the long dashed axis of the ellipse indicating the direction of maximum dispersion and the short dashed axis displaying the direction of minimum dispersion

Table 4.7 Population differentiation statistics to determine genome-wide methylation pattern.

Comparison [Sample (number of samples), source]	Age	AMOVA test (Φ_{ST})	*P* value
Non-active (44) vs. active (16), all cages	all	0.022	0.133
Non-active (26) vs. active (16), control cages	all	0.020	0.168
Non-active (9) vs. active (8), control cages	7 days	-0.036	0.716
Non-active (8) vs. active (8), control cages	12 days	-0.082	0.966
Non-active (26), control vs. non-active (18), CO_2 treatment	all	-0.021	0.831
Non-active (9), control vs. non-active (9), CO_2 treatment	5 days	-0.025	0.610
Non-active (9), control vs. non-active (9), CO_2 treatment	7 days	-0.070	0.956
Non-active controlat 5 (9), 7 (9) and 12 (8) days old	all	-0.013	0.569

4.3 Methylation of *Kr-h1* in *A. mellifera*

4.3.1 Sample analysis and bisulfite PCR assay

Workers at 5 and 6 days old with non-active ovaries were obtained from control and CO_2-treated cages while workers at these ages with active ovaries were not observed at all. Active ovaries were initially developed in 7 days old workers in control cages but there were no active ovaries in 7 days old worker from the CO_2-treated cages. After 8 days old, worker mortality occurred highly in the CO_2 treatment cages. That led to the insufficiency of survival samples for ovary assessment. Thus, the comparison was limited to workers collected at 7 days old. Ovary samples were then obtained from workers at this age to examine CpG DNA methylation pattern between non-active and active ovaries and to investigate the effect of CO_2 narcosis to DNA methylation level.

Three replicates of individual ovaries with the same phenotype (non-active or active) at 7 days old within each cage were examined for DNA methylation. This provided total of 18 individual bee ovary samples across all control and treatment cages. They consisted of three non-active ovaries and three active ovaries from each of the two control cages and three non-active ovaries from each of the two CO_2-treated cages. After bisulfite modification, bisulfite-converted DNA of ovary was amplified for *Kr-h1* target region providing a product of 1,088 bp (Figure 4.6).

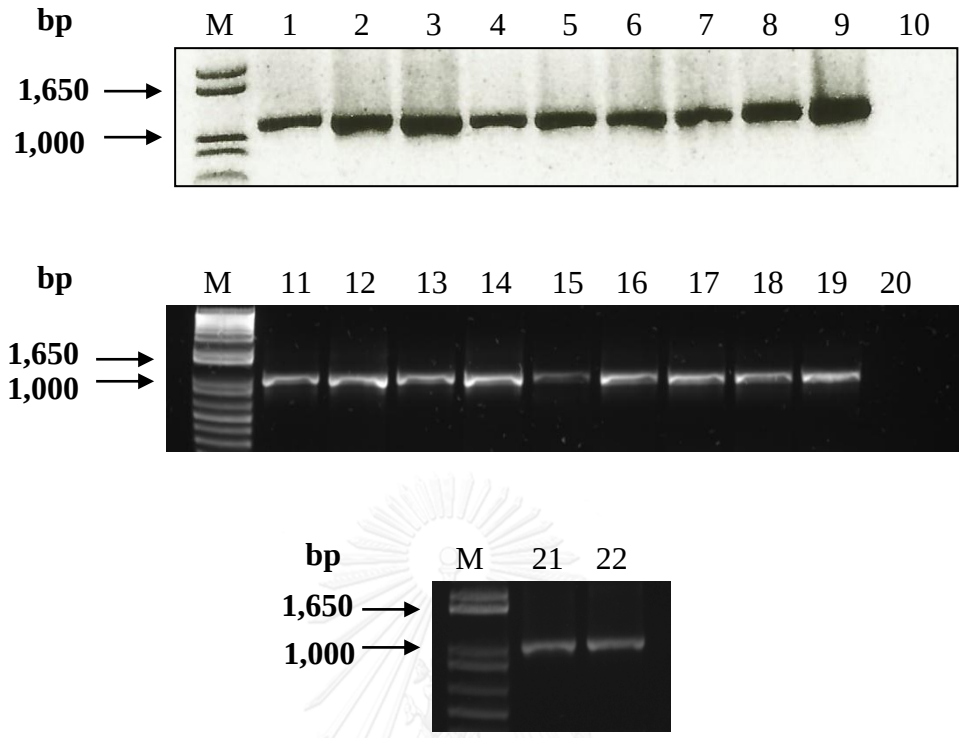


Figure 4.6 Amplified PCR products from primers for *Kr-h1* amplification. Bisulfite-treated ovary DNA was from cage 1 (lanes 1-3 and 11-13), cage 2 (lanes 4-7 and 14-15), cage 3 (lanes 8, 21-22) and cage 4 (lanes 16-18). Lane M contained 1 kb plus DNA ladder marker. Lanes 9 and 19 contained a positive reaction. Lanes 10 and 20 contained a negative reaction. The expected product size was 1,088 bp.

Bisulfite PCR products were cloned to pCR®4-TOPO® vector and a target colony was selected by colony PCR using M13 primers providing the product of ~ 1,250 bp (Figure 4.7). Ten to eighteen positive clones from each sample were sent for sequencing to determine methylation frequency.

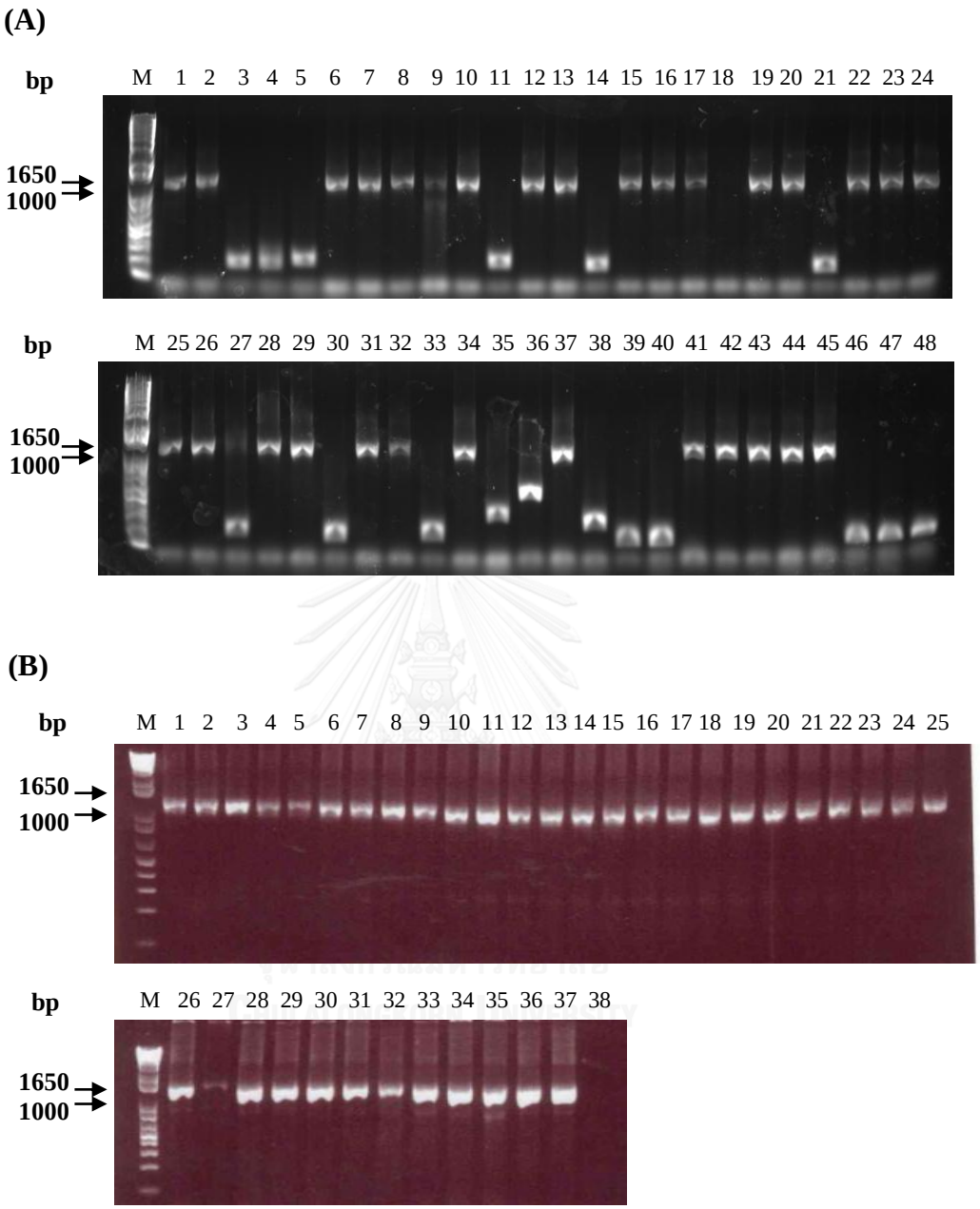


Figure 4.7 Colony PCR products by M13 primers to check *Kr-h1* fragment from cage 1 (A, lanes 1-48) and cage 2 (B, lanes 1-35). Lane M contained 1 kb plus DNA ladder marker. Lanes 36B and 37B contained a positive reaction. Lanes 38B contained a negative reaction. The expected product size was approximately 1,250 bp.

4.3.2 Methylation analysis

After sequencing, total of 239 bisulfite-treated DNA sequences at *Kr-h1* region was obtained from 18 worker ovary samples (Table 4.8). A different sequence was referred to a different clone.

Table 4.8 Number of clones (sequences) from non-active (NAO) and active (AO) ovary samples.

Treatment	Cage number	Ovary type	Bee number	Total clones (sequences)
Control	1	NAO	1	10
			2	12
			3	15
		AO	4	13
			5	14
			6	18
	2	NAO	1	11
			2	14
			3	12
		AO	4	15
			5	15
			6	14
CO_2-treated	3	NAO	1	11
			2	12
			3	13
	4	NAO	1	13
			2	12
			3	15

Each bisulfite-treated DNA sequence was examined for methylated CpG sites comparing to genome *Kr-h1* sequence. The result showed that there were 12 methylated CpG sites across amplified *Kr-h1* region where 11 sites were located on exon 2 and 1 site was located on the 3' UTR (Figure 4.8). The methylated CpG sites (# 1-12) correspond to the position of 128, 135, 220, 238, 251, 473, 484, 597, 618, 629, 659 and 894 bp of the amplified region, respectively. Interestingly, some sequences exhibited deletions of 8 bp (AAAAATAT) at the position of 839-846 bp which was 55 bp upstream to the methylated CpG site # 12 (Figure 4.8).

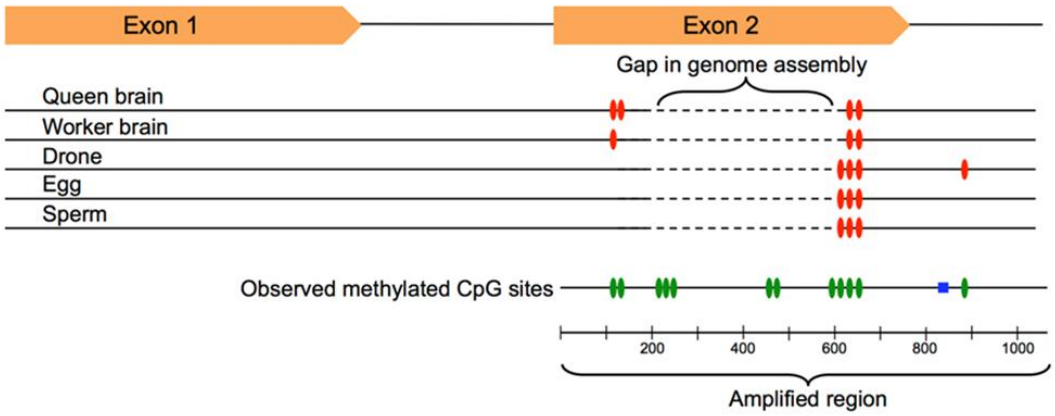
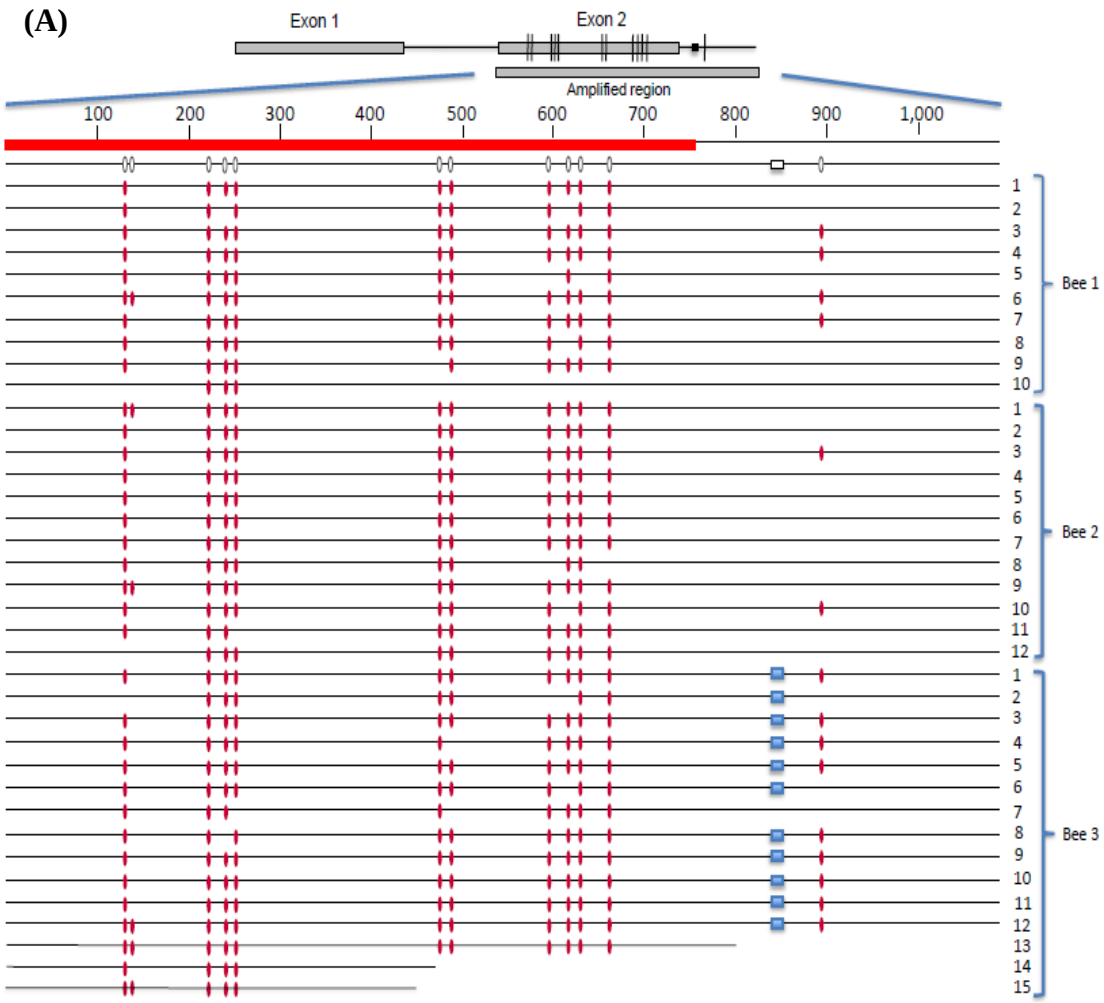


Figure 4.8 Methylated CpG sites in the examined *Kr-h1* (GB14867) region of 1,088 bp. Twelve methylated CpG sites are indicated in green. The position of the 8 bp deletion of some sequences is indicated by a blue box. In addition, the methylated CpG sites from five honey bee methylomes (queen and worker brain, drone tissues, egg and sperm) are indicated in red.

The methylation pattern of each methylated CpG site in workers with non-active and active ovaries from cage 1 and cage 2 and in workers with non-active ovaries from cage 3 and cage 4 were shown in Figures 4.9-4.12, respectively.

Cage 1 Non-active worker ovary



Cage 1 Active worker ovary

(B)

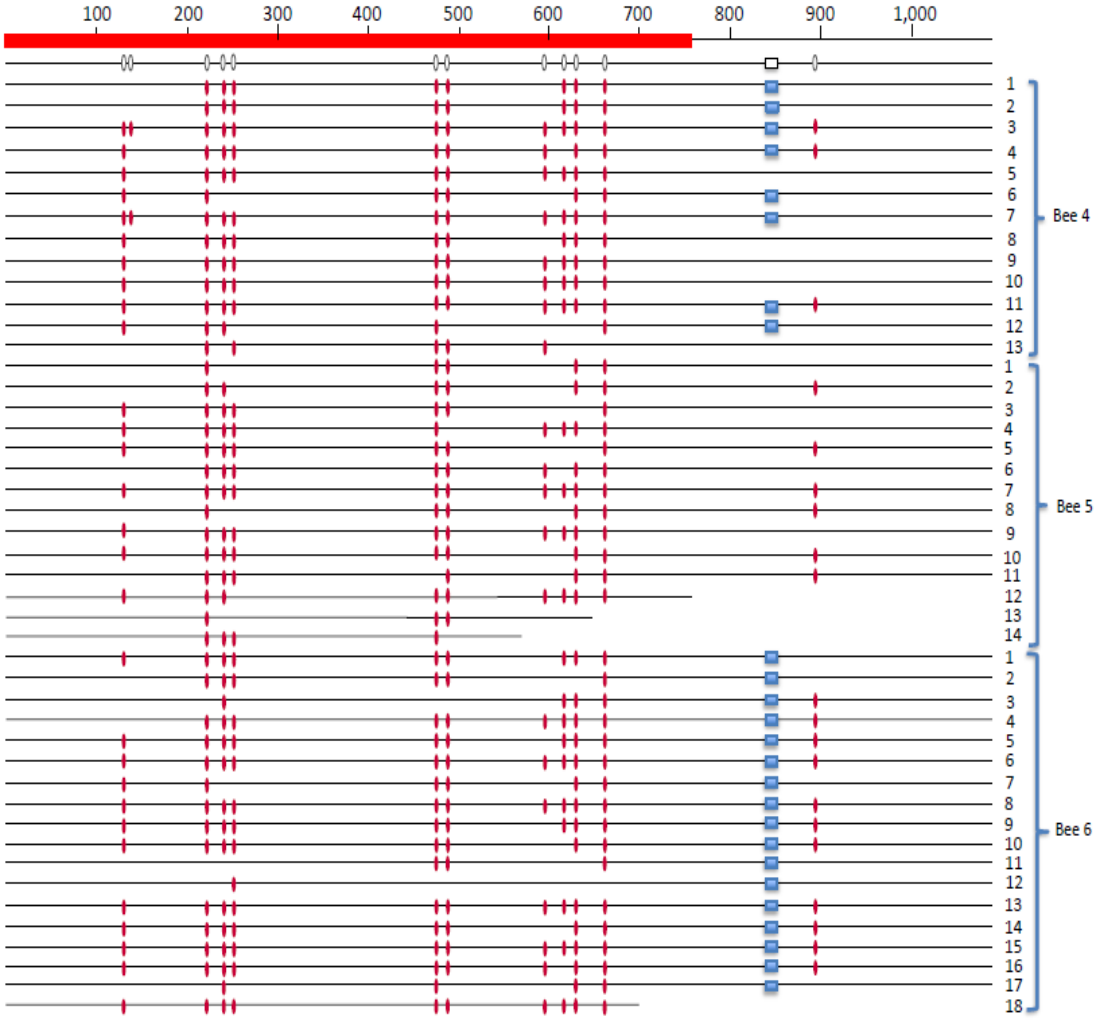
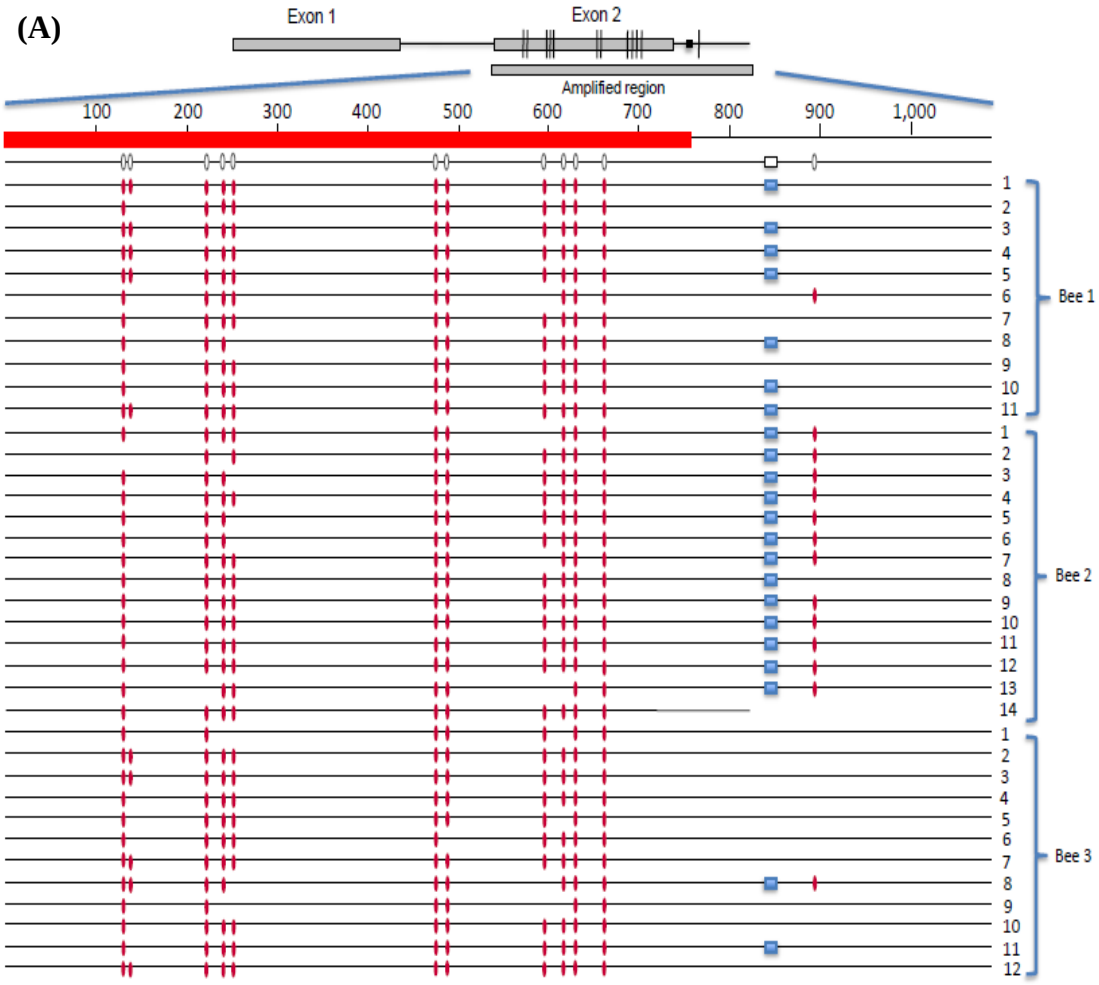


Figure 4.9 The methylation pattern at each of 12 CpG sites in the amplified *Kr-h1* region from workers in control cage 1. The pattern was from non-active (n = 3, A) and active (n = 3, B) ovaries. A blank ellipse indicates the CpG sites and a red filled ellipse indicates the methylated CpG sites. The 8 bp deletion is indicated by a blue box. Each line represents a different clone.

Cage 2 Non-active worker ovary



Cage 2 Active worker ovary

(B)

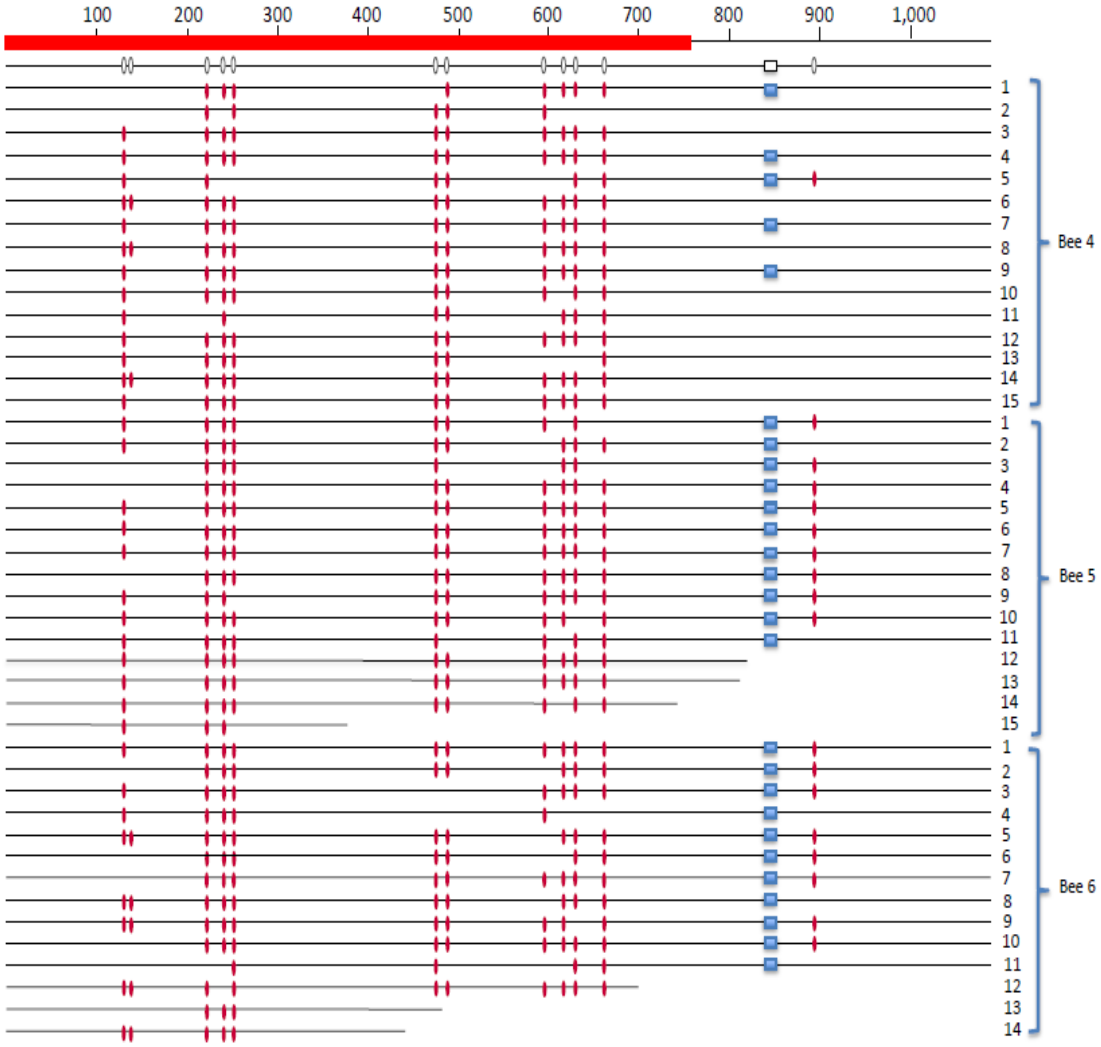


Figure 4.10 The methylation pattern at each of 12 CpG sites in the amplified *Kr-h1* region from workers in control cage 2. The pattern was from non-active (n = 3, A) and active (n = 3, B) ovaries. A blank ellipse indicates the CpG sites and a red filled ellipse indicates the methylated CpG sites. The 8 bp deletion is indicated by a blue box. Each line represents a different clone.

Cage 3 Non-active ovary worker

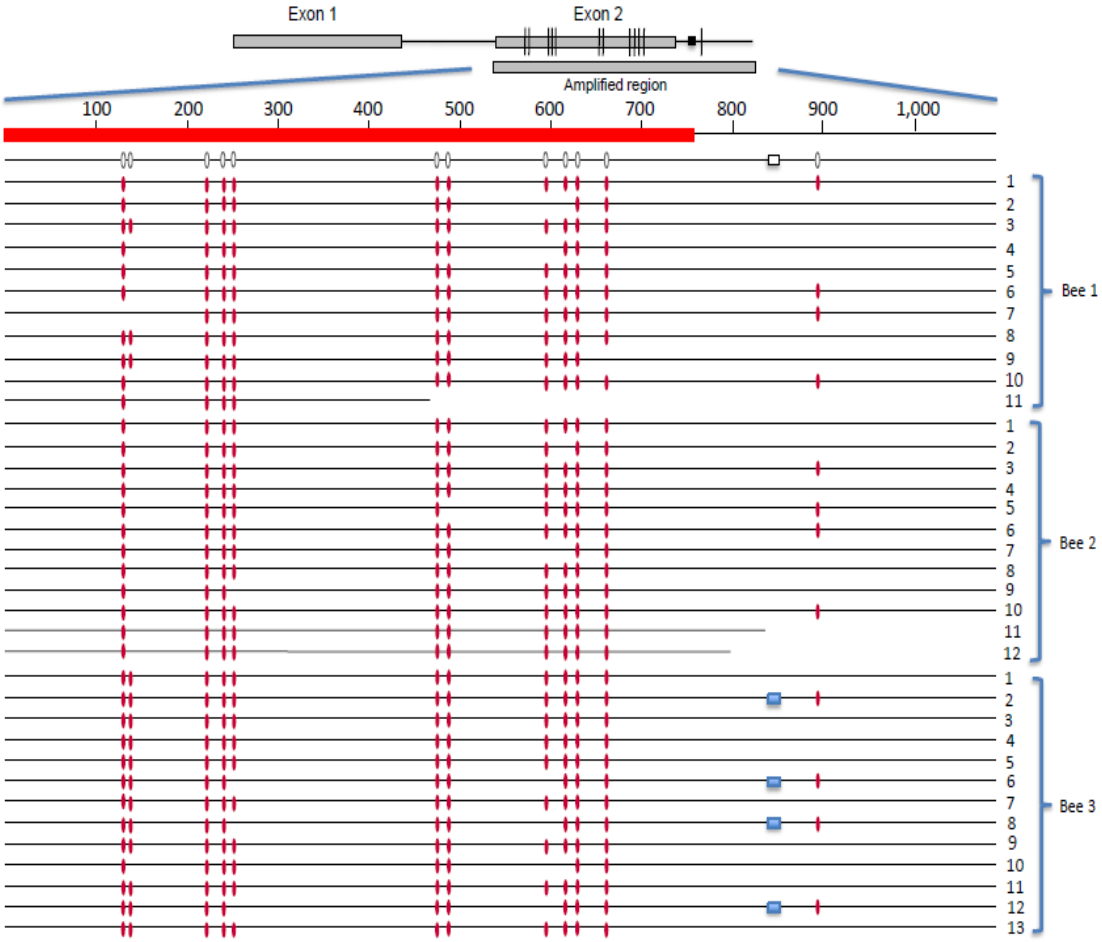


Figure 4.11 The methylation pattern at each of 12 CpG sites in the amplified *Kr-h1* region from CO_2 treated workers in cage 3. The pattern was from non-active (n = 3) ovaries. A blank ellipse indicates the CpG sites and a red filled ellipse indicates the methylated CpG sites. The 8 bp deletion is indicated by a blue box. Each line represents a different clone.

Cage 4 Non-active ovary worker

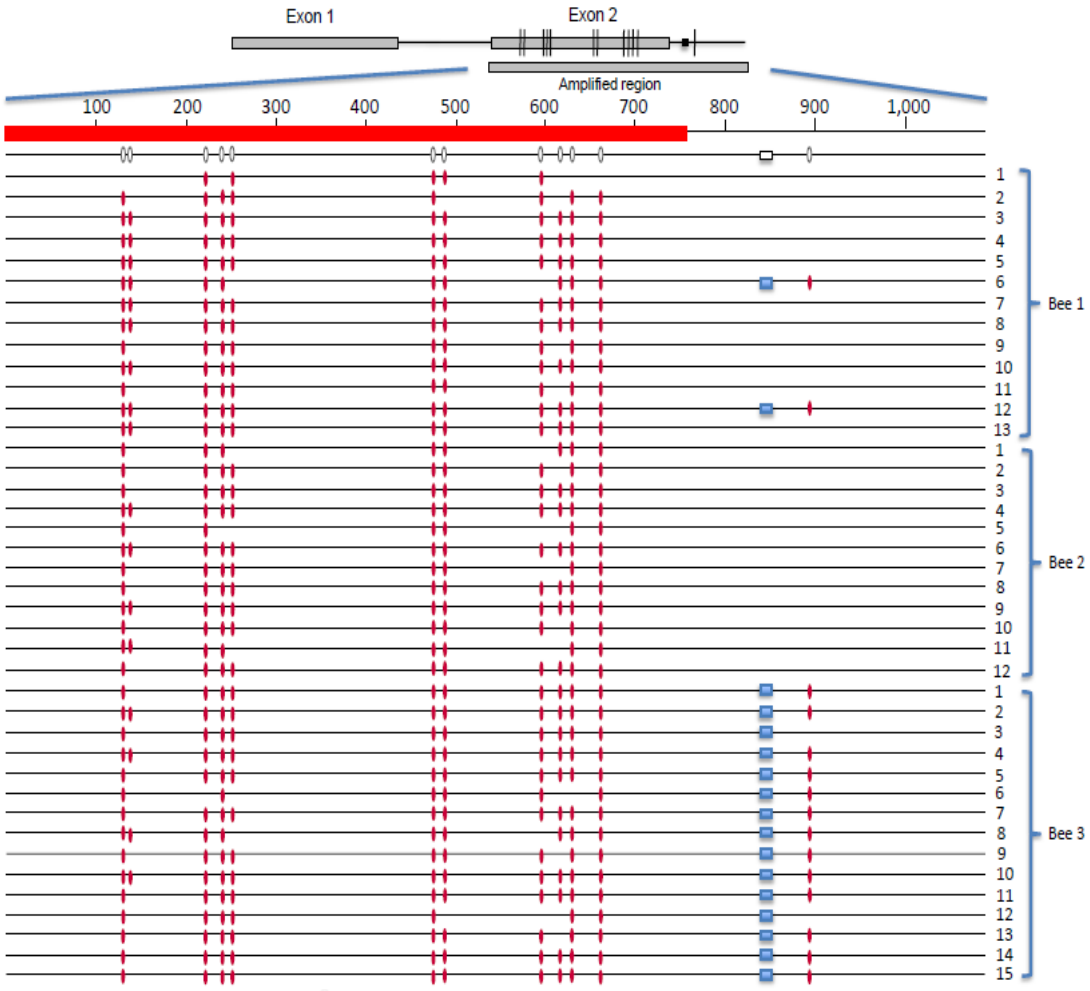


Figure 4.12 The methylation pattern at each of 12 CpG sites in the amplified *Kr-h1* region from CO_2 treated workers in cage 4. The pattern was from non-active (n = 3) ovaries. A blank ellipse indicates the CpG sites and a red filled ellipse indicates the methylated CpG sites. The 8 bp deletion is indicated by a blue box. Each line represents a different clone.

Difference in methylation pattern at individual and overall methylated CpG sites in an examined region between non-active and active worker ovaries were compared to each of two control cages (Figures 4.13 and 4.14). The result from two control cages were similar in term that methylation in non-active ovaries increased, comparing to active ovaries (cage 1: $\chi^2 = 22.57$; d.f. = 1; $P < 0.001$ and cage 2: $\chi^2 = 5.61$; d.f. = 1; $P = 0.02$). Also, the methylation pattern that is different between bees with non-active and active ovaries were similar across the two control cages (χ^2 heterogeneity = 1.64, d.f. = 1, $P = 0.20$).

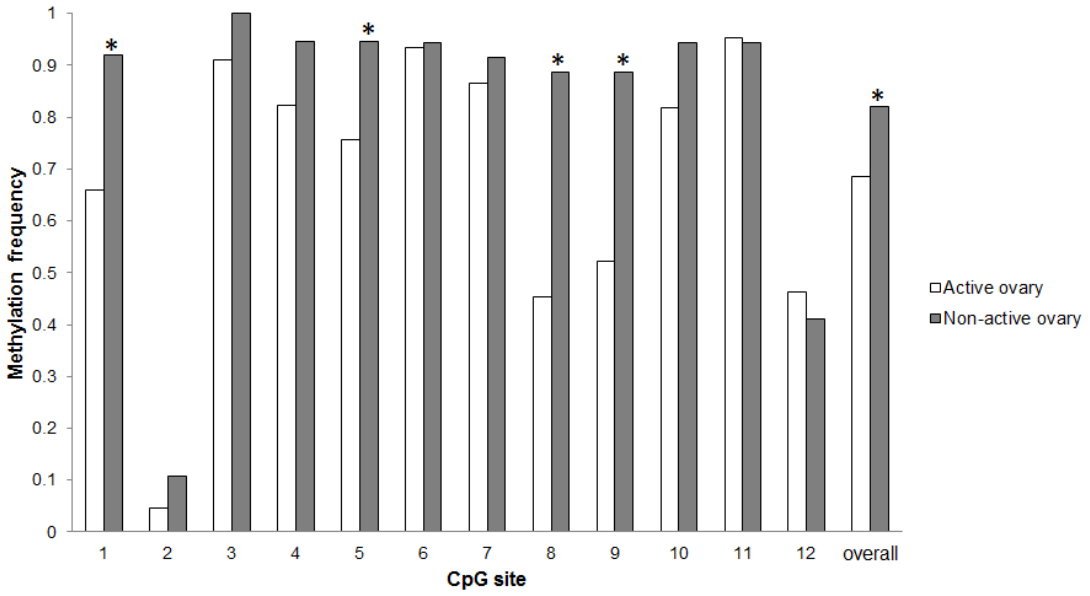


Figure 4.13 The methylation frequency at each of 12 CpG sites in the amplified *Kr-h1* region with non-active (n = 3) and active (n = 3) ovaries from control cage 1. The * indicates the significant difference between workers with non-active and active ovaries (* $P < 0.05$, Chi-square test).

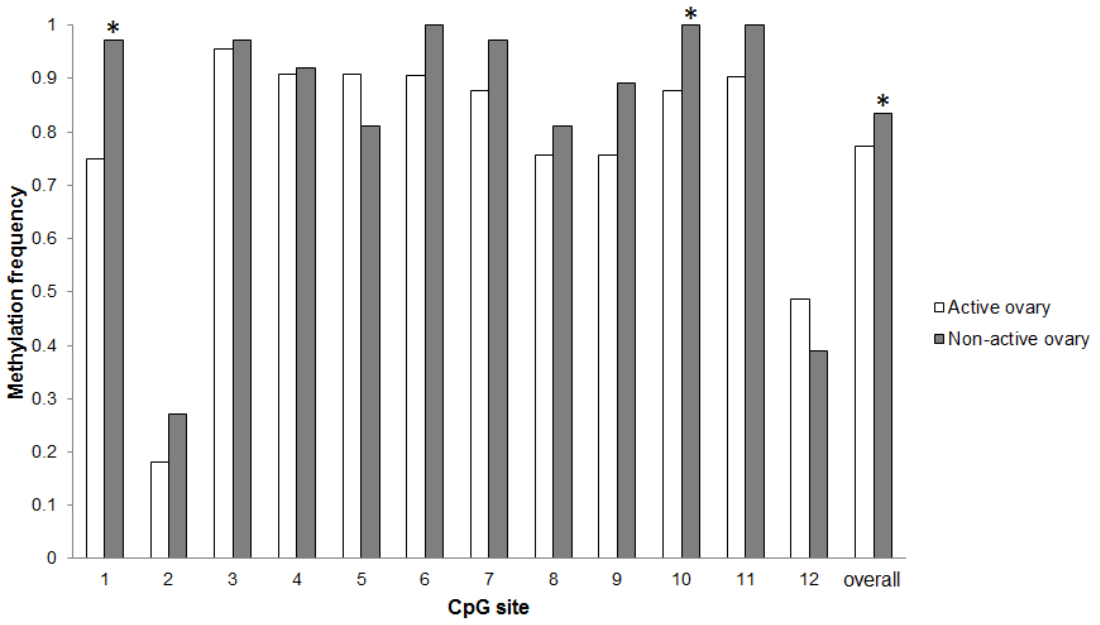


Figure 4.14 The methylation frequency at each of 12 CpG sites in the amplified *Kr-h1* region with non-active (n = 3) and active (n = 3) ovaries from control cage 2. The * indicates the significant difference between workers with non-active and active ovaries (* $P < 0.05$, Chi-square test).

Therefore, the data were pooled from the two control cages to compare the methylation level of *Kr-h1* between non-active and active worker ovaries (Figure 4.15). In overall, CpG methylation frequency was significantly higher in non-active ovaries, comparing to active ovaries (χ^2= 26.54, d.f. = 1, $P < 0.001$) (Figure 4.15). Significantly increased methylation of *Kr-h1* in non-active ovaries relative to active ovaries were found at CpG sites # 1 (χ^2 = 15.524, d.f. = 1, $P < 0.001$), # 8 (χ^2 = 11.652, d.f. = 1, P = 0.001), # 9 (χ^2 = 13.427, d.f. = 1, $P < 0.001$) and # 10 (χ^2 = 7.067, d.f. = 1, P = 0.008) (Figure 4.15).

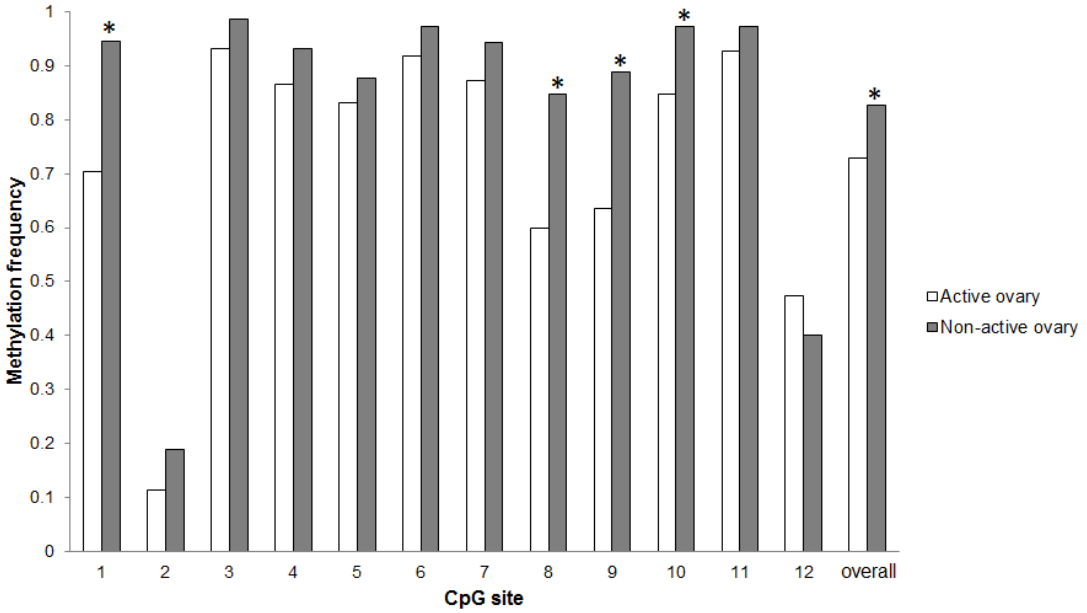


Figure 4.15 The methylation frequency at each of 12 CpG sites in the amplified *Kr-h1* region in workers with non-active (n = 6) and active (n = 6) ovaries from two control cages. The * indicates the significant difference between workers with non-active and active ovaries (* $P < 0.05$, Chi-square test).

The methylation pattern at each methylated CpG site of non-active ovary between cage 3 and cage 4 were compared. The result demonstrated that there was no significant difference in methylation pattern between worker ovaries from both cages (χ^2 = 2.311, d.f. = 1, *P* = 0.128). To determine the effect of CO_2 narcosis on methylation of *Kr-h1*, methylation levels were compared between workers with non-active ovaries from control against CO_2-treatment cages. The result showed that there was no significant difference in overall CpG methylation frequency between examined workers from both cages (χ^2 = 0.341, d.f. = 1, *P* = 0.559) (Figure 4.16) However, the significantly increased methylation can be detected at methylated CpG site # 2 (χ^2 = 9.48, d.f. = 1, *P* = 0.002) (Figure 4.16).

Interestingly, the deletion of 8 bp at the position of 839-846 bp in the examined region of *Kr-h1* upstream to the CpG site # 12 was found to be significantly correlated to the appearance of methylation at the CpG site # 12 (χ^2 = 57.795, d.f. = 1, *P* < 0.05) in overall cage comparison.

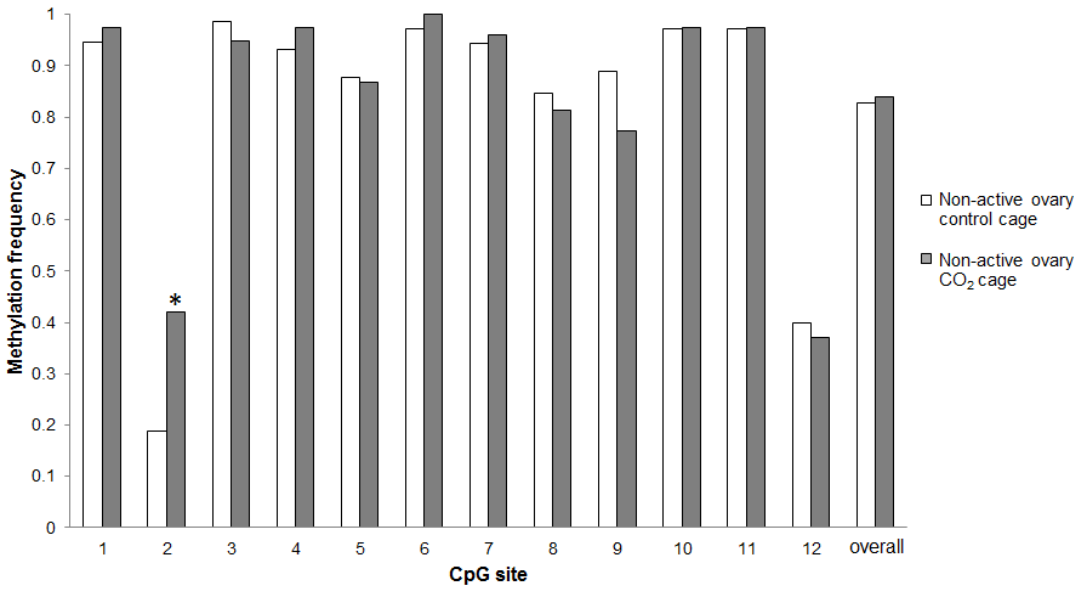


Figure 4.16 The methylation frequency at each of 12 CpG sites in the amplified *Kr-h1* region in workers with non-active ovaries from control cages (n = 6) and from CO_2 treatment cages (n = 6). The * indicates the significant difference between workers with non-active ovaries of both cages (* $P < 0.05$, Chi-square test).

CHAPTER V

DISCUSSION

5.1 Expression and methylation of *Phospholipase A_2* (*PLA2*) in Thai native honey bees

Many previous studies have been focused on characterization and properties of the PLA2 enzyme (Perumal Samy *et al.* 2007; Nomura *et al.* 2013) while the background of its expression and activity related to the developmental stadia of honey bees is a few. It is important to understand the transcriptional and translational profiles of PLA2 in honey bees because it can be beneficial to collect bees at an appropriate stage to fit a purpose of an experiment.

The expression of *PLA2* transcripts has been already reported in the Chinese honey bee (*A. cerana cerana*) (Li *et al.* 2005), but it is still unknown whether the expression control of *PLA2* and the properties of PLA2 are similar in all honey bee species. In case for application, the obtained *PLA2* sequences will be used for antigen production to reduce a symptom caused by stinging.

An expression profile using RT-PCR revealed the highest expression of *PLA2* transcript was observed in house bees of all four Thai *Apis* species. This corresponds to the expression profile of *PLA2* in *A. cerana cerana* using northern blot analysis where the highest level of expression is detected during the first 8 days of workers (Li *et al.* 2005). In this study, house bees are denoted as young workers performing in-hive tasks and can initiate off-hive tasks such as guarding. In general, guard bees are 7

to 22 days old after post-eclosure and usually play a guard role for less than a day or only a couple of days prior to foraging (Moore *et al.* 1987). Therefore, it is highly possible that house bees in this study can be guarders. Guard bees are engaged in patrolling the hive entrance and rejecting non-nestmates (Breed and Rogers 1991). If the colony is invaded by an intruder, it is possible that the guard bees will respond by stinging (Arechavaleta-Velasco and Hunt 2003; Oldroyd and Thompson 2006). Thus, the expression of *PLA2*, which encodes a major component of bee venom, is corresponded to the task performance.

Interestingly, the expression of *PLA2* transcript is highly observed only in foragers of *A. florea* and *A. dorsata*, but not in *A. andreniformis* and *A. cerana indica* even though the highest expression levels was found in house bees of all four species. This might be caused by the overlapping task between foragers and house bees (Winston 1987). Though workers engage in guarding prior to foraging, they, especially in younger bees, can switch a task between foraging and guarding (Butler and Free 1951). Thus, this could explain why the high expression level of *PLA2* was detected in foragers.

The obtained partial cDNA sequences of *PLA2* in this study were 393 bp which present a similarity in a nucleotide sequence to the same gene in *A. mellifera* (GenBank, accession# NM_001011614.1) at 89%, 90%, 95% and 94% for *A. andreniformis*, *A. florea*, *A. cerana indica* and *A. dorsata*, respectively. As expected, *PLA2* sequence from *A. cerana indica* showed the highest homology to the *PLA2* sequence from *A. mellifera*. Thus, it is supported that both species are very close in evolution (Oldroyd and Wongsiri 2006). However, the high similarity of *PLA2* cDNA

sequences among four *Apis* species was observed which indicated the close relationship among them also.

The venom content relates to the developmental stages of honey bees as well. In *A. mellifera*, the level of PLA2 content in the venom extract from workers at eclosion is very low but consistently increases through the first week after eclosion. In 7-10 days old adult, the PLA2 content reaches 40 µg which coincided to a task of being a guard bee (Owen *et al.* 1990). Likewise, in *A. cerana cerana*, PLA2 content is nearly undetectable in pupa. The low level of PLA2 content from venom extract is found in very young adults while the highest content of 12 µg is detected in adults at 7–10 days old (Li *et al.* 2005). Besides PLA2, the other main component in bee venom like melittin is found in the low level in newly emerged bees. In contrast, the level dramatically increases during the second to third weeks old of *A. mellifera* adults (Owen and Pfaff 1995). These data suggests that the highest level of PLA2 content is important for acting as a guard bee.

Considering the crude venom extract from all Thai native honey bees, house bees had higher specific activity of PLA2 while black-eyed pupae had undetectable specific activity of PLA2. The result was supported by (Li *et al.* 2005) which PLA2 content is nearly undetectable in pupae and highly to the highest detectable at around one week old adults. Considering venom organ, it is not well developed in pupae. Thus, synthesizing PLA2 and other proteins in this stage is not ready, comparing to adults. Although the differentiation of secretory cells in venom gland occurs during pupal development, the ability of their secretion begins at almost the end of pupa stage. That period is close to an eclosion of adults. The gradual change of those cells

corresponded to stages of adult development (Bridges and Owen 1984). That leads to the well-developed venom organ in workers.

The specific activity of PLA2 does not depend on a body size. Even though *A. dorsata* has the largest body size (Oldroyd and Wongsiri 2006), the specific activity of PLA2 from crude venom extract is not the highest. Similarly, the specific activity of PLA2 in the crude venom extract of *A. andreniformis* is slightly higher than that of *A. florea* although the body size of *A. andreniformis* is the smallest. This may relate to the stronger defensive behavior of *A. andreniformis* than that of *A. florea* (Oldroyd and Wongsiri 2006). The venom content in *A. andreniformis* therefore tends to be higher than that in *A. florea*. However, specific activity of purified PLA2 should be performed.

Previously, the activity of recombinant PLA2 from *A. cerana cerana* is reported to be lower than that of native PLA2 (Shen *et al.* 2010). This might be that synthesized recombinant venom is toxic to insect host cells (Shen *et al.* 2010). Alternatively, this recombinant venom requires some post-translational modification.

DNA methylation is one of the epigenetic mediators playing an essential role in regulating gene expression (Klose and Bird 2006). However, a functional role of DNA methylation depends on target regions which are different between in vertebrates and invertebrates (Suzuki *et al.* 2007). In this study, the observed region for DNA methylation in *PLA2* is at the gene body or a transcriptional region where is a primary target of DNA methylation in honey bee (Feng *et al.* 2010). DNA methylation in honey bee has been reported to be implicating in several biological processes, particularly in caste determination (Elango *et al.* 2009).

Although a mechanism of gene body methylation to gene expression has not been well understood, there are some clues of association between DNA methylation and gene expression illustrated in queen and worker castes. In both castes, mRNA of three genes [*target of rapamycin* (*TOR*), *DNA methyltransferase 3* (*Dnmt3*) and *S6K* (similar to RPS6-p70-protein kinase)], which involves in caste differentiation (Patel *et al.* 2007; Kucharski *et al.* 2008; Wheeler *et al.* 2014), is differentially and developmentally expressed (Shao *et al.* 2014). In the 1st instar larva, the expression level of these three mRNA is not significantly different in both castes. In contrast, the expression level of *TOR* mRNA is higher in the 3rd instar of queen larva than worker larva. The methylation level of all three genes is lower in queen larvae, comparing to worker larvae although the difference is not significant (Shao *et al.* 2014). This may link to the data found in this study that the expression level of *PLA2* in *A. florea* and *A. dorsata* are possibly associated with their methylation pattern. The absence of *PLA2* expression in pupae is affected by the high level of methylation while the high *PLA2* expression in adults of both species relates to the low methylation level. Although the obtained data in *A. andreniformis* and *A. cerana indica* are different from the data in *A. florea* and *A. dorsata*, it is possible that the selected methylated region in the DNA sequence may not be associated with the transcription of this gene. As known, only a subset of CpG context is involved with this event. Thus, more regions like promoter, intron should be investigated. In addition, it is interesting that there is correlation between the length of gene and the methylation status of gene in honey bees. Hypomethylated and hypermethylated genes are coherently different in length of intron and exon (Zeng and Soojin 2010).

5.2 DNA methylation in worker sterility of *A. mellifera*

Honey bee is an eusocial insect which the most notable characteristic is a reproductive division of female castes (Wilson 1971). A queen is reproductive while workers are normally non-reproductive (Ratnieks *et al.* 2006). Nonetheless, under a particular condition of colony such as the queen loss, workers can activate their own ovaries and lay male-developed eggs (Martin *et al.* 2004). This indicates that ovary activation in workers is plastic and is able to be manipulated. This raises an interest to study the regulation of worker sterility in several areas, particularly in an underlying mechanism at a molecular level.

An epigenetic process by DNA methylation is a prevalent and significant mechanism because it directly influences the gene expression and phenotype in plant and animal systems (Law and Jacobsen 2010). This mechanism has been established in social insects and implicating in several regulations including developmental and behavioral plasticity (Glastad *et al.* 2014; Yan *et al.* 2014).

In this study, worker's ovaries with different phenotypes from control and CO_2 treatment cages are determined for the genome-wide DNA methylation using MS-AFLP technique.

The result presents that, by MS-AFLP assessment, there is no significant difference in genome-wide DNA methylation pattern, between 1) workers with non-active and active ovaries and 2) control and CO_2-treated workers, in any comparison. The data appear to be contrary to expectation. It is possible that the control of the gene networks involving in the plasticity of the worker ovary may not be achieved by the mechanism of DNA methylation in this observation.

In honey bee, DNA methylation is majorly found at CpG dinucleotides despite a very low frequency observed in non-CpG context sequences (Feng *et al.* 2010). MS-AFLP merely detects methylated cytosine of recognition site which is 5'CCGG 3' (Fulneček and Kovařík 2014). Thus, the methylated cytosine sites that occur out of the target sequences or 5'CCGG 3' fail to be detected by this method (Weiner *et al.* 2013).

In this study, 22 methylated sites were identified across all samples and all primer combinations. This accounts for a small fraction of total number of methylated CpG sites demonstrated in genome of honey bee brain (~70,000 sites) (Lyko *et al.* 2010) and genomes of honey bee egg and sperm (~130,000 sites) (Drewell *et al.* 2014). In addition, according to AT rich appeared in honey bee genome (Honey Bee Genome Sequencing Consortium 2006) and low level of DNA methylaton in honey bee (Lyko and Maleszka 2011), only a proportion of methylated sites can be analysed by MS-AFLP. Moreover, DNA methylation causes gene expression change prior to phenotypic change. It may be further obscured to understand the association between reproductive phenotype and pattern of DNA methylation. As explained, it cannot rule out the possibility that DNA methylation is one of the mechanisms which regulate ovary activation state of workers. The existence of methylated site of a subset of gene network related to ovary activation in worker genome could generate a different pattern of methylation. This would emphasize the role of DNA methylation to facilitate a differential phenotype of worker ovaries.

The results in this study contrast with the result from the study in bumble-bee, *Bombus terrestris* (Amarasinghe *et al.* 2014). With MS-AFLP assay, the significant

differentiation of DNA methylation between non-reproductive and reproductive workers in a queenless condition is revealed. The contrasting result might be from the different number of methylated sites detected in bumble bees (38 sites). This could also be explained by a different set of primers used between experiments. They are different in an additionally selective base in pre-selective and selective primers. Also, the primer combination number is different. This can produce the different DNA fragment profile leading to a variation in detecting methylated fragments.

However, the difference in result indicates the variation in epigenetic control of worker reproduction in social insects. The finding in this study is also similar to the result of Libbrecht *et al.* (2016) that they cannot detect the significant differences in DNA methylation between reproductive (queen-like) and brood care (worker-like) phases in the clonal raider ant, *Cerapachys biroi*, with the whole-genome bisulfite sequencing.

CO_2 narcosis is known to suppress an ovary activation in a queenless worker of *A. mellfera* (Harris *et al.* 1996) although its mechanism remains unclear. The previous study showed that CO_2 narcosis affects the expression of gene relating to an ovary activation in a queenless CO_2-treated worker (Koywiwattrakul *et al.* 2005). This is intriguing to examine the ability of CO_2 narcosis to DNA methylation change in a worker ovary.

The finding in this study is consistent with the previous report that the CO_2 treatment is able to inhibit an ovary activation in workers (Berger *et al.* 2015). Although how CO_2 narcosis work is unknown, one possibility is that CO_2 narcosis can induce the onset of foraging behavior relating to decrease a reproductive potential in

workers (Brito *et al.* 2010). However, workers exposed to CO_2-treatment will die at 7 days post treatment in this study. Thus, the other effect of CO_2 narcosis is more noticed. This can explain that the exposure to CO_2 can reduce the length of life of honey bees (Czekońska 2009).

Focusing on the effect of CO_2 narcosis to methylation, it is found that CO_2 does not alter DNA methylation pattern between workers with non-active ovaries that had and had not been treated with CO_2. The data are therefore pooled for other comparisons. Even though the effect of CO_2 narcosis to DNA methylation pattern in worker ovaries are not detectable in this study, at least the effect of CO_2 narcosis to decrease an ovary activation in workers are confirmed. The ability of CO_2 narcosis to alter the reproductive phenotype and expression of genes involving in an ovary activation in CO_2-treated workers may not associate with the regulatory process of DNA methylation.

At present, MS-AFLP has been established to be the most widespread and cost efficient method for analysing DNA methylation pattern across genome which is suitable for a high number of samples. In this case, a whole genome sequencing is not required (Alonso *et al.* 2016; Gombeau *et al.* 2016; Verhoeven *et al.* 2016). Nevertheless, the number of methylated sites obtained in this study is quite low and this restricts the analysis. Therefore, it cannot definitely conclude that DNA methylation is not responsible for worker sterility in honey bee. Further study should be done by comparing whole methylomes between workers with and without active ovaries using whole-genome bisulfite sequencing which is able to provide the high-

resolution of cytosine methylation data. This will be useful for illustrating the functional role of DNA methylation to worker sterility in honey bee.

5.3 DNA methylation of *Kr-h1* in regulating worker sterility of honey bee *A. mellifera*

Worker sterility is an important characteristic in eusocial insect including honey bee (Michener 1974; Wilson and Hölldobler 2005). The regulation of worker ovary activation is involved in several factors such as queen and brood pheromone (Slessor *et al.* 2005) and policing from other colony members (Chapman *et al.* 2010) including the effect of CO_2 narcosis (Harris *et al.* 1996). However, a worker ovary is flexible as it can be activated in certain condition like a queen loss from its colony (Makert *et al.* 2006). Although some difference in an expression profile of genes between sterile and reproductive workers can be detected (Grozinger *et al.* 2007), a mechanism under the differentially expressed genes which is thought to be related to worker ovary activation remains unclear.

DNA methylation, an important epigenetic signal, has an ability to alter a gene expression resulting in a phenotypic change (He *et al.* 2011). At present, there is an increasing interest to study the role of DNA methylation in plasticity regulation in eusocial insects (Yan *et al.* 2015). *Krüppel homolog 1 (Kr-h1)* is thought to be involved in an ovary activation of workers according to a modulation by queen pheromone (Grozinger *et al.* 2003) and presents the high number of methylated CpG sites relative to other candidate genes in previous honey bee methylome. This is therefore intriguing to investigate the association of DNA methylation of the top most

candidate gene, *Kr-h1*, to the variation of a reproductive phenotype in workers and the influence of CO_2 narcosis to methylation of this gene.

In this study, the CpG methylation pattern of *Kr-h1* between workers with non-active and active ovaries is significantly different. More significant methylation of *Kr-h1* is found in non-active than in active worker ovaries. This indicates that the CpG methylation of *Kr-h1* can play a significant role in controlling differential phenotype of ovary activation in workers via a differential gene expression. An increasing level of DNA methylation suggests that inactivation of *Kr-h1* occurs in workers with non-active ovaries. On the other hand, a decreasing level of DNA methylation of *Kr-h1* in workers with active ovaries may switch on transcription of genes those are normally deactivated in workers with non-active ovaries in the queenright condition. Nonetheless, more mechanisms are reported to be involved in worker sterility. Ronai *et al.* (2016) presented that increased expression of *Anarchy* is strongly associated with suppression of oogenesis. Down regulated *Anarchy* affected the expression of *Buffy* which regulates programmed cell death. Since *Anarchy* transcript is found in degenerating oocytes, *Anarchy* should involve in the regulation of oogenesis through programmed cell death.

Apparently, the observation of differential methylation of genes associated with ovary activation in workers is parallel to the previous study of Kucharski *et al.* (2008). They demonstrate the differentiation between non-reproductive (workers) and reproductive (a queen) castes is influenced by DNA methylation. Larval females having the suppressed *de novo* methylation will develop to be an adult with a queen-like phenotype at the high frequency. In concordance with this, the overall

methylation in queen larvae is significantly lower than in worker larvae (Shi *et al.* 2013). Similarly, an adult queen and a worker show a difference in DNA methylation pattern of brains (Lyko *et al.* 2010). This suggests that DNA methylation not only plays an essential role in caste developmental plasticity as illustrated, but also it is crucial in regulating ovary activation of workers as presented in this study.

In this study, 12 methylated CpG sites within amplified region of *Kr-h1* are observed. Eleven sites are located on exon 2 and one site is located on the 3' UTR. This shows that the methylation is majorly accumulated in the coding sequence of *Kr-h1* amplified region. This finding is in agreement with a previous observation that DNA methylation in honey bee is preferentially found in gene body and particularly in exonic region (Zemach *et al.* 2010; Hunt *et al.* 2013; Remnant *et al.* 2016). Methylation in gene body can lead to transcriptional silencing even though a mechanism of this event is unclear (Brenet *et al.* 2011; Rangani *et al.* 2012). Gene body methylation in honey bee is correlated with regulating alternative splicing of genes (Foret *et al.* 2012; Li-Byarlay *et al.* 2013). For instance, a hypermethylated exon tends to be frequently included in transcript variants (Flores *et al.* 2012). This demonstrates the critical role of CpG methylation of gene body may involve in gene regulation at transcriptional level to generate the distinct phenotype among honey bee castes and behavioral phenotypes in workers.

As genic methylation is obviously noticed in honey bee, the association between differential methylation of genes and differential phenotype in worker are reported. *Dynactin p62*, a gene that is influenced by dietary change in honey bee larvae, is one of those genes. Significant decrease in level of CpG methylation of

dynactin p62 is correlated with higher frequency of adult developing as queen (Shi *et al.* 2011). In addition, hexamerin 110 (*Hex 110*), which is associated with ovary development in worker, shows a differential methylation at specific CpG sites between queen and worker larvae (Ikeda *et al.* 2011). Furthermore, methylation of *PKCbp1* is related to controlling task behavior of worker caste. Forager demonstrates the higher level of methyaltion of *PKCbp1* than nurses did (Lockett *et al.* 2012).

The association between the upstream deletion of 8 bp and methylation at CpG site # 12 of *Kr-h1* are observed (Figures 4.9-4.12). This indicates that a certain methylated CpG site is affected by the adjacent DNA sequence and the state of methylation is transmitted as a Mendelian manner (Wedd *et al.* 2015; Maleszka 2016; Remnant *et al.* 2016). The interaction between genotype and methylation state could lead to variation of phenotypes in honey bee.

In this experiment, CO_2 narcosis is found to decrease ovary activation in workers as the previous study (Harris and Harbo 1990; Berger *et al*. 2015). Workers those have and have not been treated with CO_2 narcosis are not significantly different in a methylation pattern. This suggests that CO_2 narcosis barely affects the CpG methylation pattern of *Kr-h1*. In previous report, CO_2 treatment increases the expression of *Kr-h1* in CO_2-treated workers, comparing to untreated workers. Also, this is associated with a transition to foraging behavior (Brito *et al*. 2010). Unlike Thompson *et al*. (2007), it showed the expression level of *Kr-h1* between both worker groups is not significantly different. This suggests that CO_2 narcosis does not alter the methylation of *Kr-h1* although it is possible to affect the expression of *Kr-h1*. However, DNA methylation affected by an environmental factor like CO_2 may be too

susceptible and rapidly to detect. The correlation between methylation and gene expression changing by external factors might be monitored closely.

Epigenetic regulation mediated by DNA methylation in *Kr-h1* appears to have a significant effect on the regulation of worker sterility. This finding indicates DNA methylation should be a potential mechanism to control reproduction in honey bee workers. *Kr-h1* is thus the promising candidate gene in a regulatory gene network of ovary activation in workers. Also, it may be used as molecular signal for worker sterility.

CHAPTER VI
CONCLUSIONS

1. The expression of phospholipase A_2 (*PLA2*) is the highest in house bees of *A. andreniformis*, *A. florea*, *A. cerana indica* and *A. dorsata* while the high expression level of the gene is observed in foragers of *A. florea* and *A. dorsata*. Nonetheless, the expression of *PLA2* transcript is undetectable in red-eyed and black-eyed pupae of all four Thai native honey bee species.

2. The partial cDNA sequence of *PLA2* is obtained at 393 bp from all Thai *Apis* spp. The nucleotide sequences from *A. andreniformis*, *A. florea*, *A. cerana indica* and *A. dorsata* show the similarity to the homolog in Western honey bee *A. mellifera* at 89 %, 90 %, 95 % and 94 %, respectively. Among those honey bee species, *A. cerana indica* is mostly close to *A. mellifera* reflecting the close relationship of the same subgenus *Apis*.

3. The higher enzymatic activity of PLA2 from crude venom extract is found in house bees (0.29 – 5.64 µmol/min/ml), comparing to black-eye pupae (0.01-0.05 µmol/min/ml) in all four native honey bee species. Similarly, the specific activity revealed the same pattern as PLA2 activity. The higher specific activity is from house bees (0.72-2.26 U/mg) while it is nearly undetectable in black-eyed pupae (0 U/mg) among all Thai *Apis* spp.

4. Bisulfite sequencing is used to determine the DNA methylation level of examined region of *PLA2*. The result demonstrates that DNA methylation level of *PLA2* in pupae (27.5 % and 12 %) is higher than in house bees (9.4 % and 7 %) and foragers (10 % and 5 %) for *A. florea* and *A. dorsata*. In contrast, for *A. andreniformis* and *A. cerana indica*, the methylation level is not quite different among these developmental stages.

5. Under the queenless condition, the genome-wide DNA methylation pattern between workers with and without active ovaries and workers those have and have not been narcotized with CO_2 is determined by using methylation-sensitive amplified fragment length polymorphism (MS-AFLP) assay. The number of methylated loci found is 22, 18 of which are polymorphic across all samples. Epigenetic diversity from polymorphic loci evaluated by Shannon's diversity index (*I*) is between 0.512 – 0.619.

6. There is no significant difference in DNA methylation pattern detected between workers with non-active and active ovaries at both particular and all age comparisons. Similarly, genome-wide DNA methylation pattern between non-narcotized and CO_2-narcotized workers are not significantly different in any comparisons.

7. DNA methylation at particular genes involving in the regulation of ovary activation of workers is investigated in queenless workers at 7 days old using bisulfite sequencing. The result reveals that DNA methylation assay of candidate gene *Krüppel*

homolog-1 (*Kr-h1*) at target region exhibits 12 sites of CpG methylation with 11 sites found on exon and 1 site found on the 3' UTR.

8. The overall DNA methylation level of *Kr-h1* in studied region is significantly higher in workers with non-active ovaries, comparing to workers with active ovaries. However, there is no significant effect of CO_2 narcosis to DNA methylation change in *Kr-h1* of workers with non-active ovaries.

9. Furthermore, in some bees, there is an observation of the previous deletion of 8 bp adjacent to CpG methylation site # 12. Statistical analysis showed there is correlation between the existence of upstream deletion of 8 bp and the CpG methylation site # 12 on the amplified region of *Kr-h1*.

Perspective

In this study, the expression pattern of venom *PLA2* at both transcriptional and translational levels are obtained from different developmental stages of native honey bee species in Thailand. The regulation of *PLA2* expression is associated with DNA methylation. In addition, the regulation of honey bee worker sterility is not significantly involved with genome-wide DNA methylation but modulated by DNA methylation of *Kr-h1*. Furthermore, CO_2 narcosis does not affect the methylation change.

This suggests that DNA methylation is an important mechanism that controls the reproductive physiology in honey bee workers. The results provide the better

understanding of a regulatory process of venom organ, ovary activation and a gene action relating to reproductive physiology of workers. This finding of fundamental information of methylation related to reproductive physiology in honey bee workers could be used for comparative study to investigate whether the DNA methylation mechanism regulates reproductive physiology in other invertebrates or vertebrates including human. Further study would explore methylation of other specific genes directly involved in venom gland and reproductive status of workers. Also, it requires performing the whole-genome bisulfite sequencing of venom gland and between inactive and active ovaries from workers to provide a promising candidate gene for methylation study.

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APPENDIX

VITA

Miss Manlika Kilaso was born on September 25th, 1984 in Chainat province. She obtained a B.Sc. degree in Biology in 2007 and M.Sc. degree in Biotechnology in 2010 from Faculty of Science, Chulalongkorn University. At present, she is a Ph.D. candidate in Biotechnology Program, Faculty of Science, Chulalongkorn University.

Publication

1. Kilaso, M., Chapman, N. C., Remnant, E. J., Oldroyd, B. P. and Chanchao, C. 2016. No evidence that DNA methylation is associated with the regulation of fertility in the adult honey bee Apis mellifera (Hymenoptera: Apidae) worker ovary. Austral Entomology. Accepted

2. Kilaso, M., Tipgomut, C., Sanguankiattichai, N., Teerapakpinyo, C. and Chanchao, C. 2016. Expression and DNA methylation of phospholipase A2 in Thai native honeybees (Hymenoptera: Apidae). Russian Journal of Developmental Biology. Accepted

3. Kilaso, M., Remnant, E. J., Chapman, N. C., Oldroyd, B. P. and Chanchao, C. 2016. DNA methylation of Kr-h1 is involved in regulating ovary activation in worker honey bees (Apis mellifera). Insectes Sociaux. Submitted

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5. Kilaso, M., Kaewmuangmoon, J., Karnchanatat, A., Sangvanich, P. and Chanchao, C. 2011. Expression and characterization of Apis dorsata alpha-glucosidase III. Journal of Asia-Pacific Entomology. 14: 479-488.