การแสดงออกที่แตกต่างของยีนจากต่อมขากรรไกรของผึ้งโพรง *Apis cerana* ระยะผึ้งพยาบาลและผึ้งหาน้ำหวาน

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DIFFERENTIAL EXPRESSION OF GENES IN MANDIBULAR GLAND OF *Apis cerana* NURSE AND FORAGER HONEYBEES

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ได้ใช้เทคนิค differential display PCR (DD-PCR) ในการศึกษาการเปลี่ยนแปลงการแสดงออกของยีนในต่อม mandibular ของผึ้งงาน 2 ระยะ คือผึ้งพยาบาล (อายุ 5-15 วัน) และผึ้งหาน้ำหวาน (อายุ 21 วัน) โดยใช้ไพรเมอร์ oligo-dT และ arbitrary จำนวน 36 คู่ ได้เก็บแถบ cDNA จากผึ้งพยาบาลที่มีการแสดงออกต่างจากผึ้งหาน้ำหวาน 70 แถบ จากไพรเมอร์ 19 คู่ ประกอบด้วยยีนที่มีการแสดงออกสูงกว่าระยะหาน้ำหวาน 50 แถบ และยีนที่มีการแสดงออกเฉพาะผึ้งพยาบาล 20 แถบ นอกจากนี้ยังเก็บแถบ cDNA ของผึ้งหาน้ำหวานที่มีการแสดงออกแตกต่างจากระยะผึ้งพยาบาล 11 แถบที่ได้จากไพรเมอร์ 7 คู่ ประกอบด้วยยีนที่มีการแสดงออกสูงกว่าผึ้งพยาบาล 1 แถบ และยีนที่มีการแสดงออกเฉพาะในระยะหาน้ำหวาน 10 แถบ หลังนำ cDNA ทุกแถบที่เก็บมาเพิ่มปริมาณด้วยพีซีอาร์แล้ว จึงทำการโคลน ได้โคลนของ cDNA ของผึ้งพยาบาลและผึ้งหาน้ำหวาน 52 และ 11 แถบ ตามลำดับ สุ่มเลือกโคลนจากแต่ละแถบไปวิเคราะห์ลำดับเบส พบว่า cDNA 27 แถบ (43%) ประกอบด้วยลำดับเบสชนิดเดียวกัน หลังเปรียบเทียบข้อมูลใน GenBank พบว่าประกอบด้วยยีนที่แตกต่าง 11 ยีน โดยเป็นยีนที่พบแสดงออกเฉพาะระยะผึ้งพยาบาล 2 ยีน ได้คัดเลือกยีน ATP synthase และ Thioesterase ทดสอบเพื่อยืนยันการแสดงออกที่แตกต่าง โดยการทำ RT-PCR พบว่า ATP synthase และ Thioesterase แสดงออกในต่อม mandibular ของผึ้งพยาบาลสูงกว่าผึ้งหาน้ำหวาน 1.78 และ 1.40 เท่าตามลำดับ

โคลนของ cDNA อีก 36 แถบเมื่อวิเคราะห์ลำดับเบสพบว่าประกอบด้วย cDNA มากกว่า 1 ชนิด เมื่อนำลำดับเบสไปเปรียบกับข้อมูลใน GenBank พบว่าเป็นยีนที่คล้ายคลึงกับยีนของผึ้ง (*Apis spp.*) 21 ยีน ในจำนวนนี้เป็นยีนที่มีการแสดงออกเฉพาะระยะผึ้งพยาบาล 8 ชนิด

ภาควิชา.....ชีวเคมี…….....ลายมือชื่อนิสิต.....

สาขาวิชา.....ชีวเคมี.....ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา.....2547.....

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KEY WORD: nurse bee/ forager bee/ mandibular gland/ Differential Display Polymerase Chain Reaction (DD-PCR)

PUTTARAT SECHANA: DIFFERENTIAL EXPRESSION OF GENES IN MANDIBULAR GLAND OF *Apis cerana* NURSE AND FORAGER HONEYBEES. THESIS ADVISOR: ASSOCIATE PROFESSOR SIRIPORN SITTIPRANEED, Ph.D.,138 pp.

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Differential display PCR (DD-PCR) technique was used for detecting the differences in gene expression in mandibular glands of nurse (5-15 days old) and forager (more than 21 days old). The DD-PCR reaction was performed with 36 combinations of oligo-dT and arbitrary primers. Seventy differentially expressed bands of nurse from forager using 19 combinations of primer were selected. Fifty (72%) out of seventy bands had higher intensity than those of forager and twenty bands (28%) were nurse specific genes. Moreover, in forager stages, eleven differentially expressed bands from 7 primer combinations were selected. Only one band (9%) showed higher intensity than those in nurse and ten bands (91%) were forager specific genes. Each band was reamplified and cloned into pGEM-Teasy vector. Fifty two of nurse and eleven of forager DD-PCR fragments could be successfully cloned, respectively. These cloned were selected and sequenced. The sequence analysis showed that 27 bands out of 63 contained unique sequence. Eleven genes were similar to the GenBank analysis. Two genes were found to be nurse specific genes. ATP synthase and Thioesterase were employed in order to confirm the different expression of nurse and forager using RT-PCR. The expression level of ATP synthase and Thioesterase in nurse was approximately 1.78 and 1.40 times higher than those of forager, respectively.

After the nucleotide sequences were analyzed, it was found that from each 36 DD-PCR bands contained more than one cDNA type. When compared the nucleotide sequences with GenBank database, 21 genes were found similar to *Apis spp*. Eight of them were nurse specific genes.

Department……...Biochemistry……..Student's signature…….

Field of study…… Biochemistry…….Advisor's signature…….

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CONTENTS

pages

pages

LIST OF TABLES

pages

pages

LIST OF FIGURES

pages

LIST OF ABBREVIATIONS

AcMRJP	=	*Apis cerana* major royal jelly protein
AmMRJP	=	*Apis mellifera* major royal jelly protein
A,T,C,G	=	nucleotide containing the bases adenine, thymine, cytosine and guanine, respectively
bp	=	base pair
°C	=	degree celcius
cDNA	=	complementary deoxyribonucleic acid
DD	=	differential display
DD-PCR	=	differential display polymerase chain reaction
DNA	=	deoxyribonucleic acid
dNTPs	=	deoxyribonucleotide triphosphates (dATP, dTTP, dCTP, dGTP)
EDTA	=	ethylenediamine tetraacetic acid
HCl	=	hydrochloric acid
9-HDA	=	(E)-9 hydroxydec-2-enoic acid
10-HDA	=	(E)-10 hydroxydec-2-enoic acid
10-HDAA	=	10-hydroxydecanoic acid
kb	=	kilobase
$MgCl_2$	=	magnesium chloride
ml	=	millilitre
mM	=	millimolar
MRJP	=	major royal jelly protein
total RNA	=	total ribonucleic acid
ng	=	nanogram
9-ODA	=	(E)-9-oxodec-2-enoic acid
PCR	=	polymerase chain reaction
RJ	=	royal jelly
SDS	=	sodium dodecyl sulfate

Tris	=	tris(hydroxy methyl)aminomethane
μg	=	microgram
μl	=	microlitre
μM	=	micromolar
UV	=	ultraviolet
V	=	volt
W	=	watt

CHAPTER I

INTRODUCTION

Honeybees are an economically important insect. They help pollination the crops that increase their yield and improve the seed and fruitqualities. In addition the products from honeybees (honey, beewax, royal jelly and pollen) are many valuable that use as supplement food, ingredient in foods, cosmetics and medicine like products.

Honeybees are highly social insects in Genus *Apis*, which live in colony. Their colony consisted of 3 caste honeybees, that a single queen, approximately 10-30 thousand sterile female workers, and from zero to few thousand drones (depending on the time of year). Workers perform all of the tasks associate with colonial living while drones fly daily from the nest-seeking mates. Queens mate with many males while in flight, soon after they develop into adults. They store the sperm from these many mates in a specialized structure, the spermatheca, for the rest of their egg-laying life (Carey, 2001).

Honeybee Queen

The queen is female, heterozygotes (diploid 2n:32) grown from fertilized egg. Queen destined larvae receive royal jelly (RJ, rich mixture of food) throughout development. RJ is secretion from both the hypopharyngeal and mandibular glands by young worker bee (nurse bee). Therefore, the queen is usually anatomically adapter to high output egg production, is larger in size, and has a longer length of life than her non-reproductive worker offspring. She secretes queen pheromones to control her offspring and suppress development of worker's ovaries (Liadlaw, 1992).

Honeybee worker

The worker bees are also female, heterozygotes (diploid 2n: 32). The different between the worker bees and queen are not due to genetic differences, but is regulated by the differential nourishment that the female larvae receive from the nurse bees. Worker larvae are nourished with RJ for 3 days following by worker jelly. RJ is a secretion from hypopharyngeal and mandibular glands of the nurse bees, whereas the worker jelly is the mixture of RJ diluted with honey and pollen. The developments of theirs ovaries are suppressed by queen pheromone. As a result they can not lay egg (Page and Peng, 2001). Worker bees have all tasks in the colony. A division of task among workers depends on their age.

The young workers are called nurse bees, generally ages less than 15 days of posteclosion. They take care of their brood by synthesizing and secreting RJ to young larvae and the queen. RJ is a secretion from the hypopharyngeal and mandibular glands between 5 and 15 days old nurse bee (Lercker, 1981).

The older workers are called foragers, ages more than 21 days after eclosion, forage outside the nest for nest construction materials, food and water for process it into honey (Robinson, 1991; Page and Peng, 2001).

Honeybees in Thailand

Honeybees are distributed in all parts of the world. They could be allocated to three different lineager based primarily on morphology and behavior; 1) the dwarf and the small dwarf honey bees are *Apis florae* and *Apis andrenifermis*, respectively, 2) the gaint or rock honey bees, *Apis dorsata* and 3) the cavity-nesting bees composing

of *Apis cerana* (the eastern honeybees) and *Apis mellifera* (the western honeybees) (Smith, 1991).

For *Apis mellifera*, this species is not native to Thailand. They were introduced from Europe for a bee keeping purpose. *Apis mellifera* and *Apis cerana* could be kept and managed in hive for commercial beekeeping due to non-aggressive behavior and simple management. Commercial beekeeping with *Apis mellifera* was well studied than those for *Apis cerana.* However, *Apis cerana* was suitable for beekeeping in Thailand because it shows more resistance to bee mite, by having an ability to detect and remove bee mites from the colonies. Moreover, it does not require sugar feeding and exhibits better climate adaptability than does *Apis mellifera* (Wongsiri *et al*., 1990).

Taxonomic identification of *Apis cerana* is as follow (Borrue *et al*., 1976, Gojmerac, 1980).

Phylum Arthropoda

Class Insecta

Order Hymenoptera

Super-family Apoidea

Family Apidae

Genus *Apis*

Specter *cerana*

Scientific name : *Apis cerana*

Royal jelly (RJ)

Royal jelly (RJ, also called bee-milk) is a creamy product that is one of the essential and high valuable widely produced in beekeeping. It can be sold in various forms such as the fresh RJ, freeze-dried RJ and mixed with other product such as various juices. It was used as supplement food and used in cosmetics industry. The largest production and exportion of RJ in the world is come from China. Moreover, Japan has the highest domestic consumption of RJ, a large part of which was imported from other Asian countries included Thailand. In Thailand, business originally based on cosmetics with RJ and other related bee products were successful and consistently grew into a multimillion dollar enterprise (Krell, 1996).

RJ is a product secreted from the hypopharyngeal and mandibular glands of the nurse bees mainly between five and fifteen days of their life to feed young larvae and the adult queen bees (Lercker, 1981). Hypopharyngeal and mandibular glands are located in the head of nurse bees (Figure 1.1). RJ is always fed directly to the queen over their life span and first three days of worker and drone larvae as it is secreted. Subsequently, a mixture of honey and pollen was supplied as worker and drone larvae diets for the remaining time (Johansson, 1955; Iannuzzi, 1990 and Cordiff, 1994). RJ is a thick yellow creamy, has a slightly pungent phenolic odor and a characteristic of sour flavour.

The differentiation between queen and worker bees is related to feeding during the larval stages, queen bee is particular rich for RJ feeding. Indeed, all female eggs can develop to queen depending on RJ feeding. Queen attains a larger size than worker and the reproductive organ is well developed to mature stage and is able to lay several thousand eggs a day. In contrast, workers are smaller in size. The reproductive

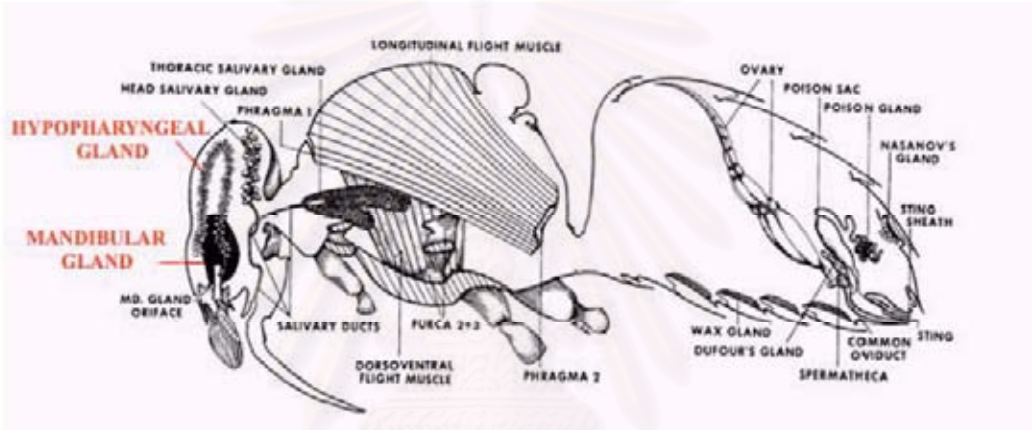

Figure 1.1 Diagram showing the organ systems of an adult honeybee

organ is not well developed but organs that related with their tasks such as pollen baskets, mandibular, hypopharyngeal and wax glands are fully developed. Occasionally, when the queen is absent in the hive, workers can lay eggs instead. Basically, the time required for development of the queen larvae to the adult stage is about 15.5 days. The life span of the adult queen was several years, While worker requires 21 days for growing up with only a few months of life span (Krell, 1996).

Several studies have been examined the advantageous effects of RJ. For example, RJ might inhibit mild and slow growing tumors, but not rapid-growing tumor (Tamara *et al*., 1985). Moreover, RJ exhibited antibiotic activity against a variety of microorganisms including some bacteria and fungi (Fujiwara *et al*., 1990; Sanguandeekul and Nimachaikool, 1993). Cho (1997) reported that RJ could control cholesterol and triglyceride levels in blood. Consumption in amounts of 50-100 mg per day could reduce total cholesterol levels by about 14 % in people with moderately high cholesterol levels (Vittek, 1995). Anti-flammatory actions of RJ through inhibiting proinflammatory cytokine production by activated macrophages were reported (Kohno *et al*., 2004). However, the allergic reaction was found to be the common side effect for people who extremely allergic to bee products when using RJ. Allergic reactions from inter-muscular injection were the cutomatic imbalance symptoms such as malaise, caumesthesia and hypersensitive responsibility. To more severe reactions, including mild gastrointestinal upset, asthma, anaphylaxis (shock), intestinal bleeding, and even death when RJ was ingested (Thien *et al*., 1996; Leung *et al*., 1997 and Yonci *et al*., 1997).

Composition of Royal jelly

Numerous chemical analysis of RJ has been published over the years. RJ is acidic substance with pH between 3.6 to 4.2. The principal constituents of RJ of *Apis mellifera* are water (60-70 %), proteins (12-17 %), sugars (11-12.5 %), lipids (3-5.5 %) and trace amount of mineral salts, respectively. The composition of RJ remains relatively constant when comparing between different colonies, bee races and time. In addition, a protein in RJ was also investigated. All free amino acids essential for humans are present, a total of 29 free amino acids and derivatives have been identified. The lipids fraction consist of five fatty acids with unusual and uncommon structures, they are mostly short chain hydroxyl fatty acids or dicarboxylic acids (10-HDA, 10-HDAA, 3-HOAA, C10:0DA, C10-1DA). The major fatty acids in RJ is 10-hydroxy-2-decanoic acid (10-HDA) at an average concentration of 50.3 % of the total fatty acids content. The sugars consist mostly of fructose and glucose, however fructose was prevalent in all RJ samples. In many case fructose and glucose together account for 90 % of the total sugars (Howe *et al*., 1995; Palma, 1992 and Krell, 1996).

Recently, compositions of fresh RJ from *Apis cerana indica* and *Apis cerana japonica* were also examined compared to that of *Apis mellifera*. The chemical composition of RJ produced by the species is show in the Table 1.1. Fresh RJ of *Apis cerana* consist of 52.1-65.3% water, 16.4-19.5% crude proteins, 9.4-23.0 % carbohydrates, 3.9-7.4 % lipids and 1.5 % ash, respectively.

Interestingly, water content of *Apis cerana japonica* from Japan and *Apis mellifera* was higher than that of *Apis cerana indica* from Thailand, whereas crude protein content, carbohydrates content and acidity of RJ of *Apis cerana japonica* and

Table 1.1 chemical compositions of royal jellies from *Apis cerana indica*, *Apis cerana japonica* and *Apis mellifera*.

composition	*Apis cerana indica*	*Apis cerana japonica*	*Apis mellifera*
Water (%)	52.1	65.3	68.3
Crude proteins (%)	19.5	16.4	12.7
Carbohydrates (%)	23.0	9.4	11.9
Lipids (%)	3.9	7.4	6.1
Ash (%)	1.5	1.5	1.0
Acidicity *	56.2	39.3	42.2
reference	Kevinseksan (1994)	Takenaka and Takenaka (1996)	Takenaka and Takenaka (1996)

*Acidicity: Volume of 1 N NaOH (ml) / 100 g of fresh royal jelly

Apis mellifera were lower than those of *Apis cerana indica* (Kavinseksan, 1994; Takennaka and Takenaka, 1996).

Hypopharyngeal gland secretions

Hypopharyngeal or food glands are pains acinous glands (secretory glands) each of which are composed of about a dozen of secretary cells. A protein rich-substances that are component of RJ were synthesized from this glands (Brouwers, 1982). The ultrastructural changes of hypopharyngeal gland in different development bees were analyzed. The number of rough endoplasmic reticulum (RER) in hypopharyngeal cells increased within a few days after bee emerged, reached to maximum number during the nursing phase and decreased in foragers (Knecht and Kaatz, 1990). In addition, hypopharyngeal glands are well developed in the nurse bee, but it shrink in the forager bee (Kubo *et al*., 1997).

Royalisin was the first RJ protein which the complete amino acid sequence was characterized. Royalisin of *Apis mellifera* bee is composed of 51 amino acid residues, with the calculated molecular weight of 5.5 kDa. It is found to have potent antibacterial activity against Gram-positive bacteria (Fujiwara *et al*., 1990). The proteins of RJ which were characterized by cloning and sequencing of their complementry DNAs (cDNAs) were RJP 57-1 (MRJP3), RJP 57-2 (MRJP4) (Klaudiny *et al*., 1994), α-glucosidase (Ohashi *et al*., 1996) and the dominant 56 kDa protein (MRJP1) (Ohashi *et al*., 1997).

Subsequently, Schmitzova (1998) isolated cDNA clones coding for RJ proteins from uni-ZAP XR expression cDNA library, which prepared from the head of 8 days old nurse honeybees (*Apis mellifera*). It was done in parallel with

electrophoretic analysed and N-terminal sequencing of RJ proteins. The results of N-terminal sequences of these proteins and cDNA sequence data from cDNA library, indicated that RJ contained major proteins and that all the proteins belong to one protein family designated MRJP (Major Royal Jelly Protein). The family consists of eight main members (MRJP1, MRJP2, MRJP3, MRJP4, MRJP5, MRJP6, MRJP7 and MRJP8) represents 82-90% of the total protein content of RJ. All members of MRJP are glycoprotein. In addition, The MRJP gene family encodes a group of closely related proteins that share a common evolutionary origin with the yellow protein of *Drosophila melanogaster*. Yellow protein has functions in cuticle pigmentation in *Drosophila melanogaster* (Albert *et al*., 1999). Excluding MRJPs, cDNA coding for orthologues of *Drosophila* yellow protein was reported. From its homology with the yellow-f gene product of *Drosophila*, the cDNA for MRJPs was also designated as am-yellow-f (Albert and Klaudiny, 2004).

MRJPs contain high amount of essential amino acids (39.3-51.4%), presumably that MRJPs have nutritional function in honeybee larval food. Amino acid compositions of *Apis mellifera* MRJPs are illustrated in Table 1.2.

MRJPs family (MRJP1, MRJP2, MRJP3, MRJP4, MRJP5 and MRJP6) in *Apis cerana* (AcMRJPs) were studied. The full length of AcMRJP1 and AcMRJP3 cDNA were identified from the hypopharyngeal cDNA library and RT-PCR. They were 1302 bp and 1824 bp encoding for 433, and 608 amino acids, respectively. Complete nucleotide sequence of AcMRJP2, AcMRJP4, AcMRJP5 and AcMRJP6 cDNA were obtained from the RT-PCR cloning of hypopharyngeal glands mRNA. The complete nucleotide sequence of AcMRJP2, AcMRJP4, AcMRJP5 and AcMRJP6 mRNA were 1302, 1608, 1881 and 1450 bp encoding for 463, 485, 579

Table 1.2 Amino acid composition of *Apis mellifera* MRJPs.

	MRJP1	MRJP2	MRJP3	MRJP4	MRJP5	MRJP6*	MRJP7*	MRJP8*
Ala	3.9	6.2	4.9	4.3	3.8	5.8	4.3	4.6
Arg	**3.4**	**3.8**	**4.9**	**4.1**	**9.0**	**3.1**	**3.8**	**3.6**
Asn	6.9	11.3	15.9	13.8	8.7	11.0	9.5	9.1
Asp	8.6	7.1	7.5	7.5	12.0	6.5	8.1	5.5
Cys	2.5	1.5	1.1	1.3	1.0	1.2	1.4	1.7
Gln	3.9	5.1	7.1	6.3	3.8	5.3	5.0	4.3
Glu	3.9	3.8	3.8	3.9	2.5	4.1	4.3	3.6
Gly	5.6	6.0	6.4	4.1	4.0	5.0	5.2	6.2
His	**2.3**	**2.4**	**2.2**	**3.9**	**1.8**	**2.6**	**1.4**	**1.2**
Ile	**6.0**	**5.1**	**4.0**	**3.2**	**4.8**	**7.4**	**7.5**	**7.7**
Leu	**9.5**	**8.2**	**6.8**	**9.7**	**5.2**	**7.9**	**8.6**	**10.8**
Lys	**5.1**	**6.9**	**5.8**	**5.0**	**4.3**	**6.0**	**5.2**	**4.8**
Met	**3.5**	**2.4**	**2.2**	**2.4**	**11.4**	**3.6**	**2.7**	**1.4**
Phe	**4.2**	**4.4**	**1.7**	**2.2**	**2.6**	**3.8**	**4.1**	**4.3**
Pro	3.7	3.1	2.5	2.2	2.6	2.9	2.7	2.6
Ser	8.1	5.8	5.9	8.4	6.2	8.2	6.8	7.7
Thr	**6.3**	**4.6**	**4.0**	**4.7**	**5.6**	**3.4**	**6.6**	**7.0**
Trp	**1.2**	**1.3**	**0.9**	**1.3**	**1.1**	**1.4**	**1.6**	**2.0**
Tyr	4.4	3.5	3.1	3.9	3.3	5.0	4.5	4.8
Val	**6.5**	**7.5**	**6.8**	**8.0**	**5.6**	**5.8**	**7.0**	**6.6**
Ess. aa.	48 %	47 %	39.3 %	44.5 %	51.4 %	45 %	48.5%	49.4%

Percent content of amino acid in native protein was obtained by computer analysis of its sequence (Schmitzova *et al.*, 1998). Essential amino acids are marked in boldface.

* Amino acid composition of AmMRJP6 was obtained by computer analysis employing the program ProtParam (Albert and Klaudiny, 2004).

and 435 amino acids, respectively (Srisuparbh, 2002; Imjongjairak, 2003; Cenpakdee, 2003).

Mandibular gland secretions

The mandibular glands of 5-15 days old worker bees produce the secretion rich in lipid and then mixed with the secretion (rich in protein) from the hypopharnygeal glands to form RJ. The functions of lipid components from worker mandibular glands are attributed to food preservation and larval nutrition. The mandibular gland of female castes, queen and worker, of *Apis mellifera* have been so well characterized (Winson, 1987).

Moreover, the mandibular gland has been coined to be the social signal to control a variety of key functions such as pheromone massages, food preservation and larvae nutrition. It's compounds may cut together as bouquet signal like the queen mandibular complex (QMP) or alternatively the various components can be individually involved in separate function (Slessor *et al*., 1988; Winston and Slessor, 1998).

Queen mandibular glands produce the compounds, which were functionalization at the penultimate (ω-1) position of the chain such as (E)-9-oxodec-2-enoic acid (ODA) and the two enantiomers of (E)-9-hydroxydec-2-enoic acid (9-HDA). Furthermore, two aromatic compounds, methyl *p*-hydroxybenzoate (HOB) and 4-hydroxy-3-methoxyphenylethanole (HVA) are released as well as primer pheromone qualities, e.g. it attracts nearly workers to the queen, given rise to a routine of workers around the queens or inhibits queen rearing by worker. The major compound, 9-ODA has been claimed to inhibit the ovarian development of the worker

(Velthvis, 1970, Hepburn *et al*., 1991.), and inhibits juvenile hormone III biosynthesis in workers (Winston *et al*., 1990; Kaatz *et al*.,1992; Winston and Slessor, 1998).

In worker mandibular glands, functionalization of compounds is usually occurred at the terminal (ω) position, 10-hydroxydecamoic acid (10-HDAA) and (E)-10-hydroxydec-2-enoic acid (10-HDA), predominate. When a worker emerges, the development of her glandular system is extremely dynamic. The complex pattern effects the changes in the bees behaviour over the life time, related to the tasks that is performing (Seeley, 1982; Robinson and Page, 1989). The mandibular secretion of *Apis mellifera* workers appear to be involved in food preservation and larval nutrition. The hydroxyl acids and the corresponding diacids are found in RJ, when these compounds act as antiseptics (Blurn *et al*., 1959). The 10-HDA can inhibit the germination of pollen that is important for pollen storage (Winston, 1987), it is an important larval nutrient that prevents larvae from pupating precociously and is most abundant in workers of foraging age (Plettner *et al*., 1997). Basically the amount of volatiles per gland is found to increase with age (Engels *et al*., 1997).

The lipid components in mandibular gland of worker bee were identified using Gas chromatography (Table 1.3) (Lercker *et al*., 1982).

Recently, the chemical compositions of mandibular glands of closely related species, *Apis cerana* and *Apis nigrocineta*, workers have been analyzed. *Apis nigrocineta* was distinct population of cavity-nesting honey bees found in Sulawesi, Indonesia. The components of mandibular gland were extracted and analyzed using Gas chromatography-mass spectrometry (GC-MS) (Table 1.4). Compounds found in the workers of these two species were significantly different. *Apis nigrocineta* workers had greater quanlities of all the ω-functionalized acids (10-HDAA, 10-HDA,

Table 1.3 Organic acids, lipid components present in royal jelly (Lercker *et al*., 1982).

Compounds
Diester
Hydroxyester
Methyl hexenadioate
7-hydroxydecanoate
3-hydroxydecanoate
6-hydroxydecanoate
Methyl-octanedioate
n-nonanedioate
8-hydroxyoctanoate
p-hydroxybenzoate
Methyl-octenedioate
9-hydroxynonaoate
Isophthalate
9-hydroxydecanoate
n-decanedioate
10-hydroxydecanoate
9-hydroxy-2-decenoate
Palmitate
Hydroxyl-ester
n-decenedioate
hydroxyester
10-hydroxy-2-decenoate
Methyl-tridecenedioate
11-defroxyundecanoate
Octadeconoate
3, 10-dihydroxy decanoate

Table 1.4 Quantitative analysis of mandibular gland components in worker head extracts of *Apis cerana* and *Apis nigrocineta* (Keeling *et al*., 2001).

compounds	*Apis cerana* (µg/worker)	*Apis nigrocineta* (µg/worker)
3-hydroxyoctanoic acid (3-HOAA)	5.76	8.81
4-hydroxyacetophenone	0.09	0.12
8-hydroxyoctanoic acid (8-HOAA)	6.19	7.97
4-hydroxybenzoic acid (HOB)	0.04	0.07
3-hydroxydecanoic acid (3-HDAA)	0.33	0.62
(E)-9-oxodec-2-enoic acid (9-ODA)	0.21	0.16
(E)-9-hydroxydec-2-enoic acid (9-HDA)	1.00	0.75
10-hydroxydecanoic acid (10-HDAA)	2.64	4.95
(E)-10 hydroxydec-2-enoic acid (10-HDA)	3.55	9.42
decanedioic acid (C10:0DA)	1.92	3.86
(E)-dec-2-enedioic acid (C10-1DA)	3.22	6.48

10-ODA and 10-1DA) than those in *Apis cerana* workers (Keeling *et al*., 2001).

The quantity and composition of the six major mandibular gland components (HOB, HVA, 9-ODA, 9-HDA, 10-HDAA and 10-HDA) of 1-4 days old *Apis mellifera* worker bee, were determined in both queenright and queenbees colonies. In queenright colonies the content of some mandibular gland components was found to coincide with the task the workers performed and with age. Two days old worker bee which they functions as nurse bee to feed the brood, hydroxy acids of 10-HDAA and 10-HDA were found to present at large amounts in RJ. In another hand, mandibular gland of 4 days old worker bee lower content of 10-HDAA, 10-HDA and HOB than those of 2 days old workers were found, whereas the production of ODA and 9-HDA was increased in mandibular gland of 4 days old worker.

The total lipid and 10-HDA contents of *Apis cerana* RJ from northern and southern population of Thailand were determined by Trongnipatt (2002) as shown in Table 1.5 and Table 1.6. The quantity of *Apis cerana* RJ lipid and 10-HDA was lower than those in *Apis mellifera*.

The biosynthesis of fatty acid in the mandibular gland

All of fatty acids biosynthetic routes consist of three processes. The first process is the synthesis of precursor fatty acid. Acetate is usually used as the precursor for biosynthetic pathways in most species. The second process, functionalization of the precursor fatty acid, composes of the introduction of the second functionality such as a double bond or a hydroxyl group. In many cases, the chain length of the precursor fatty acid does not correspond to that of the final product

Table 1.5 The lipid content (%) of these (A, B, C) commercial *Apis mellifera* RJ, northern (N) and southern (S) *Apis cerana* RJ in Thailand.

% lipid in *Apis mellifera* royal jelly			% lipid in *Apis cerana* royal jelly	
A	B	C	N	S
7.6	6.7	6.0	4.6	6.9

Table 1.6 Quantitaties of 10-HDA (%) of these (A, B, C) commercial *Apis mellifera* RJ, northern (N) and southern (S) *Apis cerana* RJ in Thailand.

% 10-HDA in *Apis mellifera* royal jelly			% 10-HDA in *Apis cerana* royal jelly	
A	B	C	N	S
2.6	2.6	2.5	0.9	1.3

therefore shortened or elongated of precursor fatty acid before or after functionalization is required. The last process is the modification of the carboxyl group. Biosynthesis of fatty acids found in mandibular gland of worker and queen honeybee have been studied by incubated the worker mandibular glands with 1-^{13}C acetate. The results showed incorporation of 1-^{13}C acetate into 9-HDA, 8-HOAA, 9-HDAA, 10-HDAA, ODA and 10-HDA, which suggested their intermediate precursors as showed in Figure 1.2. The biosynthesis of these compounds from 1-^{13}C acetate composes of three steps. The first process is the synthesis of precursor fatty acid (stearic acid, C18:0). The second process is composed of hydroxylation at ω and ω-1 position for worker and queen, respectively. Following by shortened of precursor fatty acid by β-oxidation.

Many insects show polyphenisms which are based on differential gene expression rather than genetic polymorphism. The differentiation between the reproductive organs of developing workers and queen has been studied. The suppressive-subtraction of mRNA from queen and worker of the same development stage (larvae) found that seven genes appear to be differentially expressed between the two castes, including insect storage protein (hexamerins and arylphorins) was expressed at quantitatively higher levels in queen than in workers, in contrast λ crystalline was expressed strongly in workers. Fatty-acid binding proteins, hexamerin storage protein, oxidoreductase and transcription factor (Ets-family member ELK-3) were expressed exclusively in workers (Evan and Wheeler, 1999).

RNA-differential display of mitochondrial gene of *Apis mellifera*, including a gene homologous to the nuclear-encoded mitochondrial translation initiation factor 2 (AmlF2m), cytochrome C oxidase subunit I (COX-I; mitochondrial-encoded) and

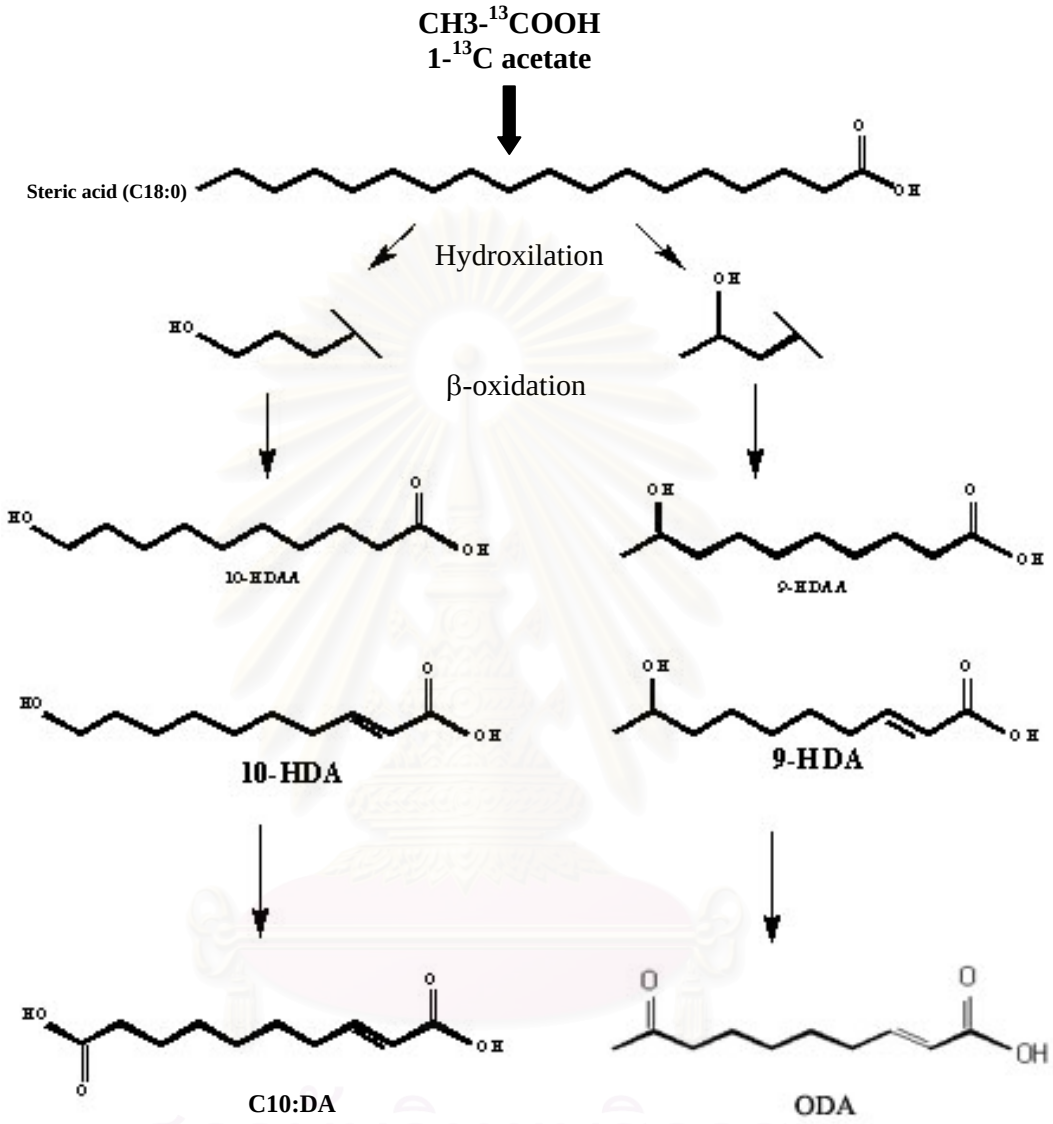

Figure 1.2 Biosynthesis of ω- and (ω-1)-fuctionalized 10-carbon acids from 1-^{13} C acetate in worker and queen honeybees (Plettner *et al.*, 1998).

cytochrome C (cyt C; nuclear-encoded) have been reported. These genes revealed greater expression in queen larvae than did worker larvae. That the higher respiratory rate previously documented in queen larvae (Corona *et al*., 1999).

In many publishes, the differential expression of gene between queen and worker has been characterized whereas the change in gene expression of workers (between nurse and forager) is not known. The level of mRNA in the brain of labor in honeybee colonies oscillated in all ages, in foragers the level of mRNA were higher at all time (Toma *et al*., 2000).

Age-dependent role change of labor, the most common from of division of labor among workers in insects societies, is based on a pattern of behavioral development by individual workers. Kubo *et al*. (1995) studied age-dependent role change in the hypopharyngeal gland of honeybee *Apis mellfera* found that three major proteins with molecular masses of 50, 56 and 64 kDa were synthesized in this gland. Immunoblotting analysis using affinity-purified antibodies against those proteins showed that they localized in the nurse bee. They also found the major 70 kDa protein which is an α–glucosidase in the hypopharyngeal glands. The content of RJ proteins and α–glucosidase were very high, accounting for more than 50% of total proteins in the nurse and forager bee hypopharyngeal glands, respectively. In addition cDNA for 56 and 64 kDa proteins were isolated and analyzed the expression of the genes for those RJ proteins and α–glucosidase. The mRNA for 56 and 64 kDa proteins were detected by *in situ* hybridization in the nurse bee gland, whereas mRNA for the 56 kDa protein and α–glucosidase were detected in that of forager bee gland (Ohashi *et al*., 1997).

The evidence of the different substances were produced in mandibular glands of nurse and forager bees. In young workers (nurse) the gland produced a lipid-rich white substance that is a component in RJ. In old workers (forager), 2-alkanones, 2-heptanone, 2-nonanone and 3-hydroxy fatty acids (C8 and C10) were found to be present in these glands as common compounds. The 2-heptanone is known as one of the alarm pheromone in *Apis* spp. The component in mandibular glands of *Apis cerana japonica* were analyzed using GC-MS, 3-hydroxy fatty acids was found as a forager specific major compound with a small amount of 2-heptanone whereas 2-heptanone and trace mount of 3- hydroxyl fatty acid were found in *Apis mellifera* (Sasagawa, 2003).

Objectives of this research

Since age-dependent role change in the mandibular gland of honey bee is not well understood. Beside that the expression of genes in this gland is not presently known. Moreover, the report on the different lipids substances were found in mandibular glands of nurse and forager bee showed that the biosynthesis of lipids in this gland was different. Therefore, identification of genes controlling different function of mandibular gland between nurse and forager bee would be performed using differential display PCR. Then the differential expressed genes are cloned and finally these genes will be sequenced.

CHAPTER II

MATERIAL AND METHODS

2.1 Chemicals

Absolute ethanol (Merck, Germany)

Acrylamide (Merck, Germany)

Agarose: Seakem LE Agarose (FMC Bioproducts, USA)

Ammonium persulfate (Promega, USA)

Ampicillin (Sigma, USA)

Bacto-agar (DIFCO, USA)

100 Base pair DNA ladder (Promega Coperation, USA)

Boric acid (Merck, Germany)

5-Bromo-4-chloro-3-indole-beta-D-galactopyranoside; X-gal (Sigma, USA)

Bromophenol blue (Merck, Germany)

Chloroform (Merck, Germany)

Delta Differential Display Kit (Clontech, USA)

Deoxynucleotide triphosphate:dNTPs (Promega Coperation, USA)

Diethyl pyrocarbonate:DEPC (Sigma, USA)

Ethidium bromide (Sigma Chemical Company, USA)

Ethylene diamine tetra-acetic acid di-sodium; Na_2EDTA(Fluka, Switzerland)

Ficoll type 400 (Sigma, USA)

Formaldehyde (CARLO ERBA Reagenti, Italy)

Formamide (Bio Basic Inc., Canada)

Hydrochloric acid (Merck, Germany)

Isoamyl alcohol (Merck, Germany)

N,N-methylene-bis-acrylamide (Sigma Chemical Company, USA)

N,N,N',N'-tetramethylenediamine (Sigma Chemical company, USA)

NucleoSpin® Extract kit (Macherey-Nagel, Germany)

Phenol crystal (BDH, England)

QIAprep Miniprep plasmid DNA purification kit (QIAGEN, Germany)

QIAquick Gel Extraction kit (QIAGEN, Germany)

Sodium acetate (Merck, Germany)

Sodium chloride (BDH, England)

Sodium dodecyl sulfate:SDS (Sigma, USA)

Sodium hydroxide (Carlo Erba Reagenti, Italy)

Tris-(hydroxy methyl)-aminomethane (Fluka, Switzerland)

TriZol Reagent (Invitrogen life Technologies, UK)

Tryptone (DIFCO, USA)

Urea (Fluka, Switzerland)

Xylene cyanol FF (Sigma, USA)

Yeast extract (DIFCO, USA)

2.2 Equipments

Autoclave: H-88LL (Kokusan Ensinki Co. Ltd., Japan)

Automatic micropipette:pipetman P2, P20, P100, P200, P1000 (Gilson Medical Electronics S.A., France)

Camera: Pentax K1000 (Asahi Opt. Co., Japan)

Centrifuge:J2-21 (Beckman Instrument Inc.,USA)

Electronic balance: Alsep EY220A (A&D Co. Ltd., Japan)

-20° C Freezer (Krungthai Ltd., Thailand)

-80° C Freezer (Bara laboratory Co. LTD., Thailand)

Ultrasonic bath: 28H (Ney Dental Inc., USA)

Hydrotech vacuum pump ((BioRad Laboratories, USA)

Incubator: BM-600 (Memmert Gambh, Germany)

Incubator shaker (Gallenkamp, UK)

Gel dryer Model 583 (BioRad Laboratories, USA)

Magnetic stirrer and heater (Fisher Scientific, USA)

Microwave Oven: TRX1500 (Turbora International Co. Ltd., Korea)

Power supply: POWERPAC 300 (BioRad Laboratories, USA)

Vertical gel electrophoresis apparatus: SQ_3 sequencer (Hoefer Inc, England)

Thermocycler:GeneAmp PCR system 2400 (Perkin Elmer Cetus, USA)

UV transilluminator: 2001microwave (San Gabriel California, USA)

Vortex: K-550-GE (Scientific Industries, USA)

2.3 Inventory supplies

Microcentrifuge tubes: 0.5, 1.5 ml (Axygen Hayward, USA)

Glass plate for vertical gel electrophoresis: 30x40 cm (Hoefer Inc, England)

Pipette tips: 10, 20, 100 ul (Axygen Hayward, USA)

Thin-wall microcentrifuge tubes: 0.2 ml (Axygen Hayward, USA)

Cassette with intensifying screen: 35x43 cm (Cokamuto, Japan)

Sharkstooth comb: 64 well (Pharmacia Biotech, USA)

Spacer set: 0.2 mm (Pharmacia Biotech, USA)

X-ray film (Kodak, USA)

2.4 Enzymes

The Advantage polymerase mix (Clontech, USA)

DNase I (Promaga, USA)

MMLV Reverse transcriptase (Clontech, USA)

Restriction endonucleases

: *Eco*RI (Amershem Pharmacia Biotech Inc., USA)

T4 DNA ligase (New England Biolab, England)

Taq DNA Polymerase (Fermantus, USA)

2.5 Radioisotope

[α^{33}-P] dATP (Amershem Pharmacia Biotech Inc., USA) specific activity 1000-3000 Ci/mmole; 3.3 µM

2.6 Bacterial strains

Escherichia coli JM109, genotype: F' *tra*D36 *pro*AB^{+} *lac*I^{q} *lac* ZΔM15 *rec*A1 *sup*E44 *end*A1 *hsd*R17 *gyr*A96 *rel*A1 *thi* Δ (lac-proAB)

2.7 Plasmids

pGEM®-T Easy vector (Appendix C)

2.8 Sample preparation

2.8.1 Honeybee samples

Honeybee samples, *Apis cerana*, were collected from manage beekeeping colonies at bee research center (Chumporn province). Newly emerged bees were marked on their thorax with color marker (Figure 2.1), and were collected when they were 5-15 days old, these samples were called “nurse bee sample”. Forager bee were captured near the hive entrance they were identified as returning bee with pollen on their pollen baskets or with on extended abdomen that estimate their age to vary from 20-30 days old. Honeybee samples immediately preserved in liquid nitrogen and then stored at -75^{o}C for later used.

2.8.2 Mandibular gland samples

Mandibular glands were dissected out from the head of each frozen nurse and forager bees under a binocular microscope at 4^{o}C. A knife was used to cut through the wall of the mask, across the vertex, a round the margins of the compound eyes, and a round the edges of the mask. The mask was then taken off. A mandibular gland joined with mandible was removed and placed into the tube containing pre-chilled buffer constituting of guanidium thiocyanate and N-lauroyl sarcosine (supply with TriZol Reagent) and stored in liquid nitrogen.

2.9 Preparation of RNase-free solution, glassware and plasticware (Sambrook and Russell., 2001)

This step is a most important consideration for RNA research. The 1.0% diethyl pyrocarbonate; DEPC in water was used for ribonuclease (RNase) inactivation

Figure 2.1 The marked honeybee in the hive

All aqueous solutions were prepared under RNase-free condition by autoclaving the stand-overnight 0.1% DEPC-treated water for 15 minutes at 15 psi (pound per square inch). RNase-free glassware and plasticware were prepared by washing them with detergent and dried in a hot air oven at 240^{o}C for 4 hours or overnight. Then, they were filled with water containing 0.1% DEPC at 37^{o}C for 1 hour or overnight at room temperature. After that, they were rinsed several times with 0.1% DEPC-treated water and were then autoclaved for 15 minutes at 15 psi. The new microcentrifuge tubes and tips were autoclaved 2 times with the same condition mention above.

2.10 Differential expression of the genes in mandibular gland of nurse and forager bees

Differential expression analysis of the genes in mandibular gland of nurse and forager bees, the total RNA was isolated from mandibular glands of nurse and forager bee using TriZol Reagent (Invitrogen life Technologies, UK). First-stranded cDNA was synthesized by the reverse transcription (RT) method. In the RT reaction, the oligo(dT) primers were used to transcribe all the mRNA species into cDNA. They annealed to the 3' poly (A) tails of mRNA molecules, and then MMLV reverse transcriptase (Clontech, USA) synthesized the first-standed cDNAs. The first-stranded cDNAs were used as template in differential display PCR reaction that containing arbitrary primers, [α^{33}-P] dATP and hot start *tag* DNA polymerase (Clontech, USA). The amplified PCR products were analyzed by electrophoresed through a denaturing 5% polyacrylamide/8 M urea gel and following by autoradiography. The differential expressed bands of nurse and forager bees were collected by eluted each different band from the gel and reamplified using the primers used in the original PCR. The

reamplified PCR products (double stranded cDNA) were electrophoretically analyzed through a 1.2% agarose gel and were purified from the gel by QIAquick spin column. They were ligated with pGEM-T easy vector. The ligation product was electro-transformed to *E. coli* JM 109. The recombinant clones contained the cDNA inserts were screened by blue-white colony screening on selective plate. Positive clones were selected and cultured in LB broth (1% tryptone, 0.5% yeast extract, 1.0% NaCl; pH 7.5) containing 50 µg/ml of ampicillin. The recombinant plasmids were extracted using on alkaline lysis method. The extracted plasmid was linearlized by digested with restriction enzyme *Eco*RI. The size of digested product was electrophoretically analyzed through 2% agarose gel. The clones that contained insert cDNAs were selected for sequencing. The nucleotide sequences were identified by Blasted against the nucleotide sequence that deposited in GenBank database. The nucleotide sequences were further characterized by various computer program.

2.10.1 Total RNA extraction

The total RNA was extracted from 400 mandibular glands (2 glands/bee) of nurse and forager bees using TriZol Reagent (Invitrogen life Technologies, UK). Mandibular glands were homogenized in 1.0 ml of TriZol reagent containing guanidium thiocyanate and phenol. The homogenate was stored for 5 minutes at room temperature. Extraction of the homogenate with 0.2 ml was performed chloroform by cover the samples tightly and shakes vigorously for 15 seconds. The mixture was stored at room temperature for 15 minutes after that centrifuged at 12,000 xg for 15 minutes. Then the aqueous phase was transferred to a new tube and total RNA was precipitated from the aqueous phase by mixing with 0.5 ml of isoamyl alcohol. The

mixture was stored at room temperature for 10 minutes and centrifuged at 12,000 xg, 4^{o}C for 8 minutes. The isoamyl alcohol was removed and the RNA pellet was briefly air-dried the for 5 minutes. RNA pellets were dissolved in DEPC-treated water and then incubated the solution for 10-15 minutes at 60^{o}C.

Quantity and qualify of total RNA were then spectrophometrically determined and electrophoretically analyzed through denaturing agarose gel electrophoresis. The concentration of total RNA was calculated using the following formula: [total RNA] = A_{260} x 40 x dilution factor.

2.10.2 DNase I treatment of total RNA

Twenty five µg of each total RNA sample was incubated in the reaction mixture of 50 µl containing 10 unit of RNase-free DNase I (Clontech, USA), 0.5 M Tris-HCl; pH 7.5, 0.5 M $MgCl_2$ and 10 units of RNase inhibitor. After gently mixed and incubated at 37^{o}C for 1 hour, the reaction was stopped by addition of 2.5 µl of 0.2 M EDTA and 2 µl of 3 M sodium acetate pH 5.2. The total RNA was extracted from the reaction mixture using TriZol reagent as mention in 2.9.1.

2.10.3 Differential expression of gene by reverse transcriptase-polymerase chain reaction (RT-PCR)

2.10.3.1 First-stranded cDNA synthesis

The first-stranded cDNA was synthesized from total RNA using MMLV reverse transcriptase. Approximately 2 µg of nurse or forager total RNA was mixed with 1 µl of cDNA synthesis primer (oligi(dT)primer), then the reaction volume was adjusted to 5 µl with DEPC-treated water. The mixture was gently mixed and

denatured by incubate at 70^{o}C for 3 minutes. The mixture was quickly chilled on ice for at least 2 minutes and spun down. Subsequently, 2 µl of 5x First buffer, 2 µl of 5 mM dNTP mix and 200 units of MMLV reverse transcriptase were added into the mixture. The contents were mixed by gently pipetting and spun down. The reaction was incubated at 42^{o}C for 60 minutes. At the end of the incubation period, the reaction was terminated by heating at 70^{o}C for 10 minutes before chilled on ice. The single stranded cDNAs were diluted to 2 various conditions. The dilution A (1:10), 8 µl of first-strand cDNA sample were diluted with 72 µl of sterile water and the dilution B (1:40), 2 µl of first-standed cDNA sample were diluted with 78 µl of sterile water. Both of dilution mixtures were used for constructing the second-strand cDNA.

2.10.3.2 Differential Display Polymerase Chain Reaction (DD-PCR)

Differential display PCR (DD-PCR) was performed using Delta Differential Display kit. Arbitrary primers and oligo (dT) primers used in the second-stranded cDNA construction were listed in Table 2.1. Thirty six DD-PCRs were performed using different pair of primers as indicated in Table 2.2.

The reaction was preformed in 20 µl total volume containing 1 µl of first-stranded cDNA template, 1 µM of each primer (arbitrary and oligo(dT) primers), 1x PCR reaction buffer containing 1.5 mM Mg^{2+} (400 mM Tricine-KOH; pH 9.2, 150 mM potassium acetate, 35 mM magnesium acetate and 37.5 µg/ml Bovine serum albumin), 50 µM each of dNTPs (dATP, dCTP, dGTP and dTTP), 0.4 µl of 50x advantage *KlenTaq* Polymerase mix and 0.2 µl [α^{33}-P] dATP. The PCR was separated to 3 steps of cycles. First, the reaction was respectively preheated at 94^{o}C, 40^{o}C and 68^{o}C for 5 minutes per each temperature, follow by two cycles of 94^{o}C for 2 minutes,

Table 2.1 Arbitrary primers and oligo (dT) primers used in DD-PCR

Primer name	Primer sequence
Arbitrary primer	
P1	5’- ATT AAC CCT CAC TAA A TG C TG G GG A -3’
P2	5’- ATT AAC CCT CAC TAA A TC GGT CAT AG -3’
P3	5’- ATT AAC CCT CAC TAA A TG C TG GTG G -3’
P4	5’- ATT AAC CCT CAC TAA A TG C TG GTA G -3’
P5	5’- ATT AAC CCT CAC TAA AGA TCT GAC TG -3’
P6	5’- ATT AAC CCT CAC TAA A TG C TG GGT G -3’
P7	5’- ATT AAC CCT CAC TAA A TG C TG TAT G -3’
P8	5’- ATT AAC CCT CAC TAA A TG GAG CTG G -3’
P9	5’- ATT AAC CCT CAC TAA A TG TGG CAG G -3’
P10	5’- ATT AAC CCT CAC TAA AGC ACC GTC C -3’
Oligo (dT) primer	
T1	5’- CAT TAT GCT GAG TGA TAT CTT TTT TTT TAA -3’
T2	5’- CAT TAT GCT GAG TGA TAT CTT TTT TTT TAC -3’
T3	5’- CAT TAT GCT GAG TGA TAT CTT TTT TTT TAG -3’
T4	5’- CAT TAT GCT GAG TGA TAT CTT TTT TTT TCA -3’
T5	5’- CAT TAT GCT GAG TGA TAT CTT TTT TTT TCC -3’
T6	5’- CAT TAT GCT GAG TGA TAT CTT TTT TTT TCG -3’
T7	5’- CAT TAT GCT GAG TGA TAT CTT TTT TTT TGA -3’
T8	5’- CAT TAT GCT GAG TGA TAT CTT TTT TTT TGC -3’
T9	5’- CAT TAT GCT GAG TGA TAT CTT TTT TTT TGG -3’

Table 2.2 Pairs of primers used in the DD-PCR

Pair name of primers	Arbitrary primer	Oligo (dT) primer
D1	P1	T1
D2	P1	T2
D3	P2	T1
D4	P2	T2
D5	P3	T3
D6	P10	T8
D7	P3	T4
D8	P5	T4
D9	P5	T5
D10	P6	T5
D11	P6	T6
D12	P7	T6
D13	P7	T7
D14	P8	T7
D15	P8	T8
D16	P9	T8
D17	P1	T9
D18	P2	T8
D19	P3	T7
D20	P4	T5

D21	P5	T6
Pair name of primer	Arbitrary primer	Oligo (dT) primer
D22	P6	T4
D23	P7	T3
D24	P8	T2
D25	P9	T1
D26	P9	T9
D27	P1 : P9	
D28	P2 : P8	
D29	P3 : P4	
D30	P4 : P5	
D30	P5 : P9	
D32	P6 : P8	
D33	P7 : P5	
D34	P8 : P4	
D35	P9 : P8	
D36	P10 : P9	

40°C for 5 minutes and 68°C for 5 minutes. Then, 25 cycles of 94°C for 1 minute, 60°C for 1 minute and 68°C for 2 minutes were performed. Finally extension was performed at 68°C for 7 minutes. The PCR products were electrophoretically analyzed in denaturing 5% polyacrylamide gel (30x40 cm).

2.10.3.3 Electrophoresis and Autoradiography

Denaturing polyacrylamide gel was used for size fractionation and purification of DNA fragments from DD-PCR products. The experiments used denaturing 5% polyacrylamide/8 M urea gel, approximately 70 ml of 5% polyacylamimd/8 M urea was prepared in 0.5x TBE buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA; pH 8.3). Then added 250 µl of freshly prepared 2% ammonium persulfate and swirled the gel solution gently to mix the reagents. After that, added 40 µl of TEMED to the gel solution, and swirled the gel solution gently to mix the reagents. The gel solution was poured into the mold that prepared using glass plates and 0.2 mm spacers (thin gels). The flat slide of a sharkstooth comb (0.2 cm) was immediately inserted into the gel solution. After the gel was completely set, the flat slide of a sharkstooth comb was placed into the open end of the gel mold so that it fit snugly. An enough volume of 1x TBE was poured to cover the gel and rinsed wells of the gel prior to loading. The gel was prerun at 33 mA (constant current) for at least 30 minutes. Five µl of the DD-PCR product was mixed with 5 µl of loading buffer (50% glycerol, 10 mM EDTA; pH 8.0, 0.25% (W/V) bromophenol blue and 0.25% (W/V) xylene cyanol FF). The DD-PCR samples were denatured by incubating at 94°C for 2 minutes and then placed on ice immediately. Two µl of the samples were loaded into the gel. Electrophoresis was usually operated at 70 W for 5 hours until the xylene cyanol dye

had migrated through the entire gel. After the glass plates were cool down to room temperature the gel was laid on Whatman paper. Finally, plastic wrap was placed carefully over the gel and the gel was dried under vacuum at 75^{o}C for 60 minutes. X-ray film was exposed to the gel at -70^{o}C for 48 hours with an intensifying screen.

2.10.3.4 Purification of interested DNA fragments

A differentially expressed bands between nurse and forager bees appeared on autoradiograph were picked from the gel. The autoradiograph was aligned on top of the dried gel. Using a sharp pins, the differentially expressed bands were marked via poking holes through the film and the gel beneath. Then the differentially expressed bands were excised from the gel using a cleaned scalpel. Each cDNA fragments (differential expressed band) were placed into fresh tubes. Forty µl of sterile water was added into the tubes and heated at 100^{o}C for 5 minutes. The supernatant was carefully removed into a new tube. The solutions of eluted cDNA bands were stored at -20^{o}C.

2.10.3.5 Reamplification of the eluted bands

Each differentially expressed band was reamplified using the same primer pair as in the differential display PCR. Amplification reaction was carried out in a 20 µl reaction volume containing 2 µl of eluted DNA, 1x PCR buffer with $MgCl_2$ (400 mM Tricine-KOH; pH 9.2, 150 mM potassium acetate, 35 mM magnesium acetate and 37.5 µg/ml Bovine serum albumin), 500 µM of dNTP mix, 1 µM of each the primer and 0.4 µl of 50x advantage *KlenTaq* polymerase mixture. The reaction was denaturing at 94^{o}C for 1 minute, annealing at 50^{o}C for 1 minute and extension at 68^{o}C

for 2 minutes. After amplification, 5 µl of reaction mixture was electrophoretically analyzed using a 1.2% agarose gel.

2.10.3.6 Analysis of cDNA products by agarose gel electrophoresis

Agarose gel electrophoresis was standard method used to size fractionate and purify of DNA fragments. The concentration of agarose gel was for separation are depended on DNA fragments size. In this study, 1.2% agarose gel was used for analyzed the PCR products. An appropriate amount of agarose was weighted out and dissolved in the appropriate volume of 1x TBE buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA; pH 8.3). The gel slurry was heated until completed solubilization in microwave oven. The agarose solution was incubated at 65^{o}C and further left to 50^{o}C before poured into the electrophoretic gel mould. The comb was inserted. After the gel was completely set, the comb was carefully removed. The gel was placed in the electrophoresis chamber. An enough volume of 1x TBE was poured to cover the gel 2-3 cm. One-fifth volume of loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF and 15% ficoll 400) was added into the sample and loading into the gel. Electrophoresis was usually run at 100 volts (10 volts per cm) until bromophenol blue reached approximately 1 cm from the bottom of the gel. The gel was stained with a 2.5 µg/ml ethidium bromide solution for 5 minutes and destained in deionized water for 15 minutes. The DNA was visualized under a long wavelength UV light (approximately 325 nm) and photographed with gel documentation. The concentration or molecular weight of DNA sample was analyzed by compared the intensity and relative mobility with the standard DNA fragments, respectively.

2.10.4 Purification of cDNA product by recovery from agarose gel using QIAgen Kit

An approximate amount of reamplified of the eluted cDNA bands was electrophoretically analyzed through 1.2% agarose gel. The DNA bands on agorose gel were visualized under a long wavelength UV light. The desired cDNA band was excised from the gel and placed into the preweigth microcentrifuge tube. The gel slice was weighted. Three volumes of QC buffer were added to one volume of gel (estimated 100 mg per 100 µl**).** The gel mixture was incubated at 50^{o}C for 10 minutes or until the gel slice was completely dissolved (the color of gel mixture would turn to be yellow).

The gel mixture was applied to a QIAquick spin column, which was placed into a provided 2 ml collection tube and centrifuged at 10,000 xg for 1 minute at room temperature. The offluent was discarded. Optionally, 0.5 ml of QC buffer was added to remove all traces of agarose from the column and centrifuged at 10,000 xg for 1 minute. After that, 0.75 ml of the PE buffer was added, left for 2-3 minutes and centrifuged at 10,000 xg for 1 minute. The column was placed into a new microcentrifuge tube. Finally, DNA was eluted by adding 50 µl of the EB buffer (10mM Tris-HCl; pH 8.5) and centrifuged at 10,000 xg for 1 minute.

2.10.5 Ligation of differentially expressed cDNA to pGEM-T easy vector

The pGEM-T easy vector (Appendix C) was used for cloning of differentially expressed cDNA. The ligation reaction was performed in the total volume of 10 µl containing 250 ng of purified cDNAs (from 2.9.4) 50 ng pGEM-T easy vector, 5 µl of 2x rapid ligation buffer (60 mM Tris-HCl; pH 7.8, 20 mM $MgCl_2$, 20 mM DTT, 2

mM ATP and 10% PEG 8000), 3 units of T_4 DNA ligase. The ligation mixture was mixed and incubated at 4^{o}C overnight. The ligation product was electro-transformed to *E. coli* JM 109.

2.10.6 Electro-transformation of recombinant DNA into *E.coli* JM 109

2.10.6.1 Preparation of host cells for electro-transformation

Five ml overnight culture of *E. coli* JM 109 was inoculated to 500 ml of LB broth (1% tryptone, 0.5% yeast extract and 1.0% NaCl; pH 7.5). The cuture was incubated at 37^{o}C with shaking at 250 rpm for 2-3 hours until the optical density at 600 nm of culture reached 0.5-0.7. The cuture was chilled on ice for 20-30 minutes and harvested by centrifugation at 8,000 xg for 15 minutes at 4^{o}C. The supernatant was carefully decanted. The cell pellet was washed two times with 500 ml and 250 ml of cold water, and then washed with 20 ml of ice-cold 10% glycerol. The cells were collected by centrifugation at 8,000 xg for 15 minutes at 4^{o}C. Finally, the cell pellet was resuspended in a total volume of 1.0 ml of ice-cold 10% glycerol and divided into 40 µl aliquots and stored at -80^{o}C until used.

2.10.6.2 Electro-transformation

An aliquot of 40 µl of concentrated cell (2.9.6.1) was thawed on ice and mixed with 1 µl of ligation product (2.9.5). The mixture was transferred into the narrow gap of cold electroporation cuvette (0.2 cm) and tapped to the bottom. The cuvette was then placed in the chamber slice, pushed into the chamber until the cuvette was seated between the contacts in the base of the chamber and pulsed once. The condition of

electroporation was set as follows; 25 μF, 200Ω and 2.50 kV of the pulse controller unit.

After one pulse was applied, 1 ml of the LB broth (1% tryptone, 0.5% yeast extract and 1.0% NaCl; pH 7.5) was immediately added to the cuvette and the cells were immediately resuspended with a Pasture pipette. The cell suspension was transferred to the tube and incubated at 37^{o}C for 1 hour. Aliquots of the cells were spread on the selective plates and incubated for 16 hours as describe in 2.9.7

2.10.7 Blue-White colony screening for recombinant plasmid

The LB selective plate (1% tryptone, 0.5% yeast extract, 1.0% NaCl and 1.5% Bacto-agar) containing 50 μl/ml ampicillin and coating with 4 μl of 20% IPTG, 40 μl of 20 mg/ml X-gal was used for screening. Cell suspension (250 μl) from electro-transformation were spread onto LB selected plates and incubated at 37^{o}C for 16-18 hours. White colonies were selected for further analysis.

2.10.8 Characterization of the insert DNA of recombinant plasmid

For characterization of the insert DNA of recombinant plasmid, the recombinant plasmids were extracted by alkaline lysis method. Subsequently, the recombinant plasmids were characterized by digested with *Eco*RI. The digested products of the recombinant plasmid must have at least two DNA fragment of the DNA insert and the linear pGEM-T easy vector. The recombinant plasmids containing the DNA insert were selected for nucleotide sequencing. The nucleotide sequences obtained were blasted against those deposited in the GenBank database to identify the DNA insert.

2.10.8.1 Plasmid extraction

Three recombinant clones of each differential expressed band were randomly picked for plasmid extraction. Each recombinant clone (white colony on selected plate 2.9.7) was inoculated into 3 ml of LB broth containing 50 µl/ml ampicillin and incubated at 37^{o}C with constant shaking at 250 rpm for 16-18 hours. The culture was transferred into 1.5 ml microcentrifuge tube and centrifuged at 15,000 xg for 5 minutes. The cell pellet was collected and resuspended with 100 µl of solution I (25 mM Tris-HCl; pH 8.0, 10 mM EDTA and 50 mM glucose). The mixture was completely dispersed by vortexing, and then placed on ice. The mixture was then added with 200 µl of freshly prepare solution II (0.2 N NaOH and 1% SDS), gently mixed and placed on ice. Additionally, 200 µl of solution III (3 M sodium acetate; pH 4.8) was added, gently mixed and placed on ice for 10 minutes. To eliminate the insoluble fraction, the mixture was centrifuged at 12,000 xg for 10 minutes. The supernatant was transferred into the new microcentrifuge tube and extracted with an equal volume of phonol : chloroform : isoamyl alcohol (25:24:1). The mixture was centrifuged at 8,000 xg for 5 minutes. The supernatant was removed to a new microcentrifuge tube. Plasmid DNA was precipitated with the addition of 2 volumes of ice-cold absolute ethanol for 15 minutes at -80^{o}C and recovered by centrifugation at 10,000 xg for 10 minutes at 4^{o}C. The DNA pellet was washed with 70% ethanol, centrifuged at 10,000 xg for 10 minutes, air died and dissolved in 30 µl TE buffer (10 mM Tris-HCl; pH 7.5 and 1 mM EDTA) containing 20 µg/ml RNase A. Then plasmid mixture was incubated at 37^{o}C for 1 hour and stored at -20^{o}C until used.

2.10.8.2 Size of insert DNA in the recombinant plasmid

The extracted recombinant plasmid from positive clone was separately digested with *Eco*RI (Amershem Pharmacia Biotech Inc., USA). The reaction was carried out in 20 µl containing approximately 500 ng of the recombinant plasmid, 10 unit of each restriction enzyme and 1x reaction buffer. The reaction mixture was incubated at 37^{o}C for 1 hour. The size of digested products was electrophoretically analyzed through 2.0% agarose gel.

2.10.8.3 Nucleotide sequencing and data analysis

Insert DNA from two-three recombinant clones were sequenced. Recombinant plasmid containing DNA insert was extracted from recombinant clone and sequenced at Bioservice unit (BSU), Thailand. The universal M13 forward primer was used for sequencing by an automated DNA sequencer (Applied Biosystems 373 A DNA sequencer), using the dideoxynucleotide chain termination of PRISM kit (Perkin Elmer, USA.). Nucleotide sequences obtained were blasted against those deposited in the GenBank database using the BlastN and BlastX programs (Http://www.ncbi.nlm.nih.gov).

2.11 Semiquantitative PCR assay

Comparisons of the expression level of some genes (ATP synthase gene (N13), Thioesterase gene (N60), Phosphoglycerate gene (N107/sequence1), Apolipophorin III gene (N139/sequence1), Transketolase C gene (N141/sequence1) which showed differential expression in nurse and forager mandibular glands were

done by semiquantitative PCR assay, using ribosomal protein (RpS8) gene as house keeping gene.

The specific primer of ATP synthase gene (N13), Thioesterase gene (N60), Phosphoglycerate gene (N107/sequence1), Apolipophorin III gene (N139/sequence1), Transketolase C gene (N141/sequence1) were designed from their sequence, using Oligo 4.0 (Table 2.3). The primers of the control house keeping gene, ribosomal protein (RpS8) gene were listed in Table 2.3.

The PCR condition was followed by amplification of differential display PCR. Briefly the reaction was carried out in a 25 µl reaction volume containing approximately 50 ng of cDNA template, 1x PCR buffer (20 mM Tris-HCl; pH 8.4, 50 mM KCl), 1.2 $MgCl_2$, 500 µM of each dNTP, 1 µM of each the primer and 1 unit of *Taq* DNA polymerase (Fermentus, USA). The reaction was denaturing at 94^{o}C for 30 seconds, annealing at 50^{o}C for 30 seconds and extension at 72^{o}C for 1 minute. After amplification, 5 µl of reaction mixture was electrophoretically analyzed using a 2.5% agarose gel.

Table 2.3 Sequence of gene-specific primers used for semiquantitive RT-PCR assay

Templates	Primer sequence (5' to 3')
ATP syntase (N13)	For: CTG ATG AAA TGG TGG AAT Rev: TTG ACG AGA ACG ATA ACT
Thioesterase (N60)	For: TAT TCA CTA CTG TTG GT Rev: TAT CAG GTC TGT CTT CT
Phosphoglycerate (N107/sequence1)	For: CAC CAG CGG TAC GGC AC Rev: AGA TTC GTT CAG TTA CA
Apolipophorin III (N139/sequence1)	For: TGT TGT TGT TGT GGT GG Rev: AAT GGA GAC GAG GAA GA
Transketolase C (N141/sequence1)	For: ATC CAG TTG TTG TGT TTA GAA A Rev: TAA TCT CCG CAC CGA TAC CAC
Ribosomal protein (RpS8)	For: ACG AGG AGC GAA ACT GAC TGA Rev: GCA CTG TCC AGG TCT ACT CGA

CHAPTER III

RESULTS

3.1 Total RNA extraction

The nurse and forager worker bees from Chumporn province, Thailand, were shock-frozen in liquid nitrogen. The mandibular glands were dissected from two hundreds of both nurse and forager worker bees head under microscope on ice cold tray. Then the total RNAs of pooled gland samples were extracted using TriZol reagent (Invitrogen life Technologies, UK). The concentration of total RNA were determined by spectrophotometry at the wavelength of 260 nm and calculated following the formula: [total RNA] = A260 x 40 µg/ml. The extracted RNA of 20.33 and 24.38 µg was obtained from 200 nurse and 200 forager bees, respectively. The quality of RNA was analyzed by subjected to agarose gel electrophoresis (Figure 3.1) and spectrophotometrically measured of OD_{260}/OD_{280}. The result from agarose gel showed that extracted RNA did not contained high molecular weight DNA. The OD_{260}/OD_{280} ratio of about 2 indicated that acceptable quality of extracted RNA was obtained. The RNA solution was kept at -70^{o}C until used.

3.2 Selection of the differential expression of genes in mandibular gland of nurse and forager bee

Preparation of a first stranded cDNA was performed by using 2 µg of extracted total RNAs from nurse and forager mandibular glands as a template for cDNA synthesis. The total RNA was treated with DNase I before used. The first stranded cDNAs were constructed in the reverse transcription reaction using oligo(dT) primer. After that, the seconded stranded cDNA constructions were performed by

Figure 3.1 The total RNA extracted from the mandibular glands of nurse and forager of *Apis cerana* analyzed by 1% denaturing agarose gel electrophoresis

Lane N total RNA from nurse

Lane F total RNA from forager

Delta Differential Display kit (Clontech, USA). Differential display polymerase chain reaction (DD-PCR) of 36 different primer combinations using first stranded cDNA from mandibular glands of nurse and forager bees as DNA template were performed (Table 2.2). The differential expression of the transcripts between nurse and forager mandibular gland were analyzed by denaturing 5% acrylamide/8M urea gels. For example, the result for 14 different primer combinations, is showed in Figure 3.2 (the another different primer combinations are showed in Appendix A). As can be seen, each primer combinations led to a distinct and reproducible band pattern. The code number of DD-PCR bands was F for forager stage and N for nurse stage. The number followed each code was the band number. Those bands were indicated in the red box as shown in the Figure 3.2 and Appendix A. From total of approximately 300 amplified transcripts that could be visualized. Seventy differentially expressed bands of nurse stage from 19 primer combinations were selected and excised from polyacrylamide gel. Of these cDNA bands, fifty bands (72%) showed higher intensity than those of forager and twenty bands (28%) showed as nurse specific genes. Moreover, eleven differentially expressed bands from 7 primer combinations were selected and excised from forager stage. Only one (9%) were showed higher intensity than those in nurse bees and ten (91%) showed as forager specific genes.

These selected 81 DD-PCR bands were reamplified by PCR with the same set of primer used for the differential display reaction. In forager, all DD-PCR bands could be successfully reamplified and cloned (100%). Only fifty five (78%) of DD-PCR bands from nurse could be successfully reamplified. The reamplified cDNA bands from forager and nurse were shown in Figure 3.3 and Appendix B. The size of

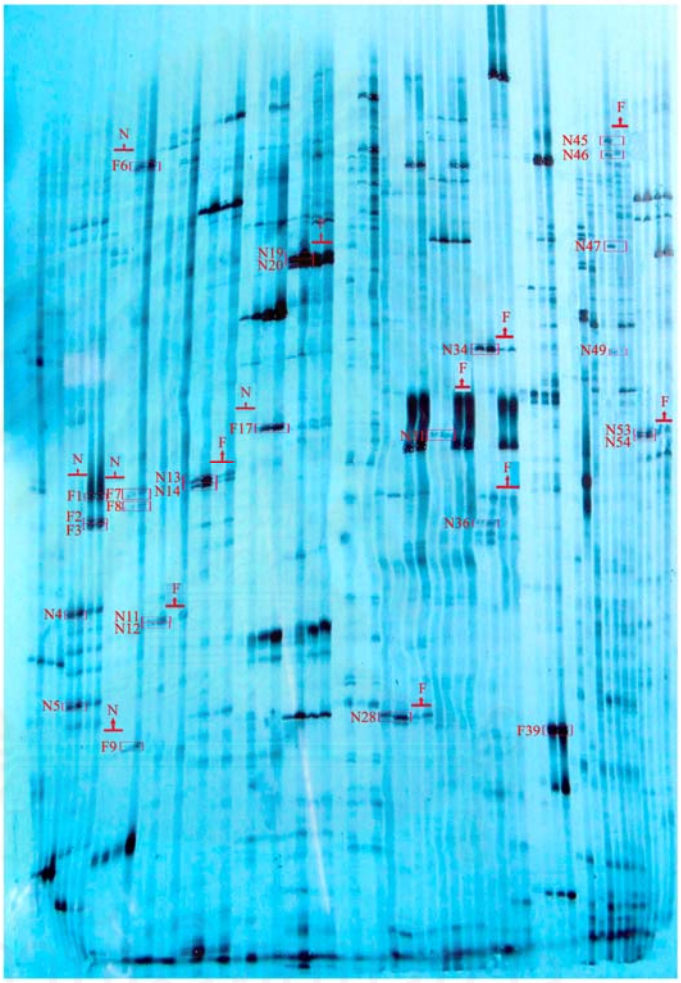

Figure 3.2 Differential expressed RNA identified by differential display method as described in Chapter II. The red latter and box indicate bands that were purified and used to make a template for reamplification.

these 66 reamplified cDNA bands were found in the range of 400 to 1500 bp (Table 3.1).

Among these, cDNA band of F1-F9 and F39, F55, F57 was forager specific genes which expression were found only in forager. The cDNA band of N36, N45, N46, N47, N49, N74, N78, N81 and were nurse specific genes. The other cDNA bands showed differential expression between nurse and forager stage. The different expressed level of each cDNA band between these two stages was determined by measuring the relative intensity of cDNA band from DD-PCR display (Figure 3.2) using Gel document. For example, differential cDNA band N14 of 550 bp was cDNA band selected from nurse stage. It was amplified by primers P6 and T5 with the intensity in nurse 3.36 times higher than that in forager. The different of the intensity of all cDNAs between these two stages was ranging of 1.4-25.82 times.

The reamplified cDNA bands on agarose gel were recovered using QIAquick gel extraction kit (Qiagen, Germany) and ligated to pGEM-Teasy plasmid vector (Promega Co., USA.). The ligated plasmids were then transformed to bacteria *E. coli* JM 109 by electrophoretion. Blue/white colony screening was performed for each PCR products. Fifty two (94%) of reamplified PCR products of nurse could be successfully cloned. After that recombinant plasmid of differentially expressed bands were extracted from recombinant clones by alkaline lysis method. The size of cDNAs insert was determined after cleaved out from the recombinant plasmid with restriction *Eco*RI (Figure 3.4). The restriction pattern of digested products comprised of one cDNA insert and one linear pGEM-Teasy vector fragment.

After cloning, 63 set of recombinant clones of 63 DD-PCR bands (52 bands from nurse mandibular glands and 11 bands from forager mandibular glands) were

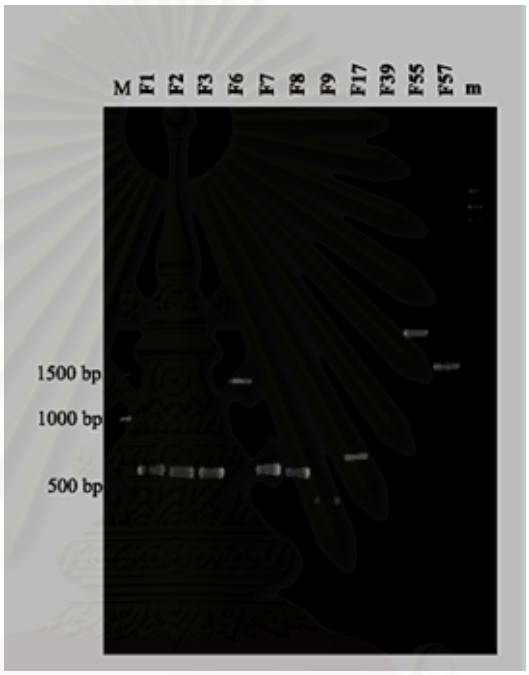

Figure 3.3 The analysis of reamplified differential expressed bands from forager stage

Lane M A 100 bp DNA ladder

Lane m A λ/*Hin*dIII standard DNA marker

F1-F54 reamplified differential expressed bands from different pair of primer

Table 3.1 Differentially expression of gene between nurse and forager mandibular gland

Differential bands	Primer pairs	Size (bp)	Relative intensities	
			Nurse	Forager
F1	P3 : T4	550	-	++
F2	P3 : T4	550	-	++
F3	P3 : T4	550	-	++
F6	P5 : T4	1300	-	++
F7	P5 : T4	550	-	++
F8	P5 : T4	550	-	++
F9	P5 : T5	450	-	++
F17	P6 : T6	600	1	5.88
F39	P1 : T1	450	-	++
F55	P1 : T9	1400	-	++
F57	P2 : T8	1100	-	++
N4	P3 : T4	500	1.67	1
N5	P3 : T4	450	1.94	1
N11	P5 : T5	500	2.86	1
N12	P5 : T5	500	2.64	1
N13	P6 : T5	550	3.04	1
N14	P6 : T5	550	3.36	1
N19	P7 : T6	800	1.97	1
N20	P7 : T6	800	1.79	1
N36	P9 : T8	500	++	-
N45	P10 : T8	1500	++	-
N46	P10 : T8	1300	++	-
N47	P10 : T8	800	++	-
N49	P10 : T8	700	++	-
N53	P4 : T5	650	2.53	1
N54	P4 : T5	650	3.27	1
N60	P4 : T5	650	3.94	1
N66	P8 : T2	500	3.53	1
N67	P8 : T2	400	3.63	1
N72	P5 : T6	850	3.73	1
N74	P9 : T1	800	++	-

N75	P9 : T1	750	++	-
N76	P9 : T1	700	++	-
N77	P9 : T1	650	++	-
N78	P9 : T1	650	++	-
N81	P9 : T9	550	++	-
N90	P1 : P9	500	++	-
N91	P1 : P9	450	6.33	1
N92	P1 : P9	450	4.17	1
N100	P3 : P4	950	5.39	1
N101	P3 : P4	750	1.49	1
N103	P3 : P4	700	10.46	1
N104	P3 : P4	550	1.46	1
N106	P3 : P4	500	6.06	1
N107	P3 : P4	500	7.81	1
N108	P3 : P4	500	4.48	1
N109	P3 : P4	500	6.04	1
N118	P4 : P5	500	3.05	1
N119	P4 : P5	500	4.69	1
N120	P4 : P5	400	10.83	1
N124	P5 : P9	700	++	-
N125	P5 : P9	700	2.65	1
N126	P5 : P9	700	2.81	1
N127	P5 : P9	500	++	-
N128	P5 : P9	450	++	-
N135	P8 : P4	650	9.89	1
N136	P8 : P4	650	7.67	1
N139	P9 : P8	800	++	-
N140	P9 : P8	700	25.82	1
N141	P9 : P8	600	++	-
N142	P10 : P9	++		-
N143	P10 : P9	900	++	-
N144	P10 : P9	900	++	-
N145	P10 : P9	550	1.97	1
N146	P10 : P9	500	5.91	1
N147	P10 : P9	500	ND	

++ = specific band found only in one stage.

ND = not determined

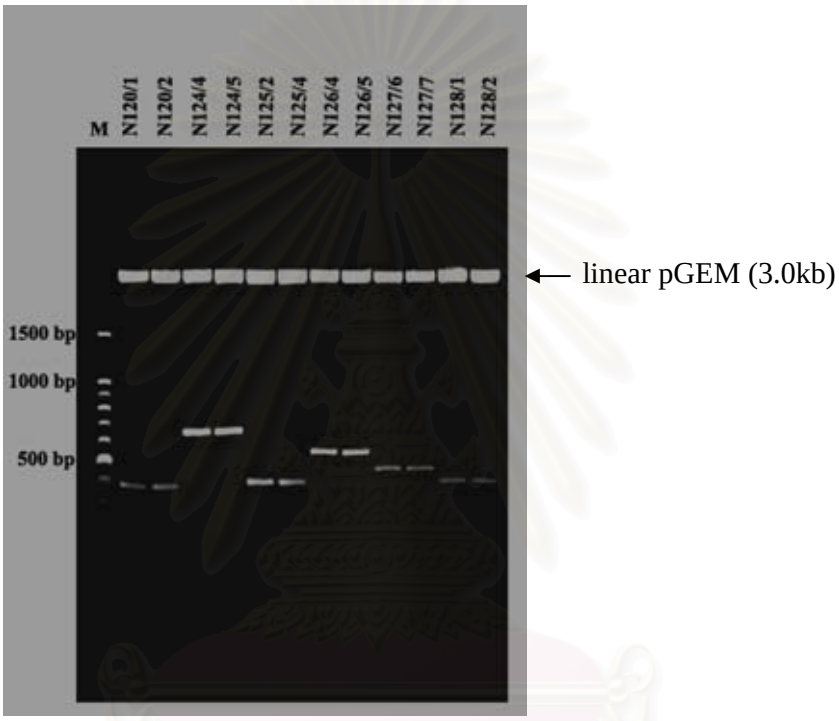

Figure 3.4 Restriction analysis of recombinant plasmid digest with *Eco*RI containing various size of cDNAs insert and linear pGEM-Teasy vector

Lane M A 100 bp DNA ladder

Lane m A λ/*Hin*dIII standard DNA marker

N120-N128/2 Digestion of recombinant plasmids from each DD-PCR fragment with *Eco*RI

obtained. Recombinant plasmid was separately extracted from two-three recombinant clones of each DD-PCR bands and sequenced. Fortunately, the first set of sequencing result of DD-PCR band (N13, N14, N54 and F39), exactly the same nucleotide sequence were obtained from three recombinant clones. Therefore, the number of recombinant plasmids selected for DNA sequencing analysis was sometime reduced from 3 to 2. From sequencing result, only 27 DD-PCR bands (43% out of 63 DD-PCR bands) (Table 3.2) gave single type of nucleotide sequence from 2-3 different recombinant clones. The nucleotide sequences obtained were analyzed for the homology by searching from GenBank DNA and Protein database using Blastn and tBlastx programs through the National Center for Biotechnology Information (NCBI).

The result of Blastn analysis of these 27 DD-PCR bands was shown in Table 3.2. Twenty one DD-PCR bands (N5, N12, N13, N14, N20, N36, N53, N54, N60, N66, N72, N77, N78, N81, N91, N109, N119, N120, N124, N125, N135, N136, N145) was similar to honeybee (*Apis spp*.) genes, among these genes only 18 (N5, N12, N13, N14, N20, N36, N53, N54, N60, N66, N72, N77, N78, N81, N120, N124, N125, and N145) were structural genes (protein coding genes). These DD-PCR bands (N109, N119, N135 and N136) were identified as 18S ribosomal RNA gene of *Apis mellifera*. Three DD-PCR bands (N72, N120 and N124) were fragments of cDNA for *Apis cerana* Major Royal jelly Protein 1(MRJP1), which was the most abundant protein in royal jelly (RJ). N36 was identified as cDNA of MRJP2. N125 showed the sequence similarity to *Apis mellifera* Zinc finger protein 39. DD-PCR bands of N13 and N14 were similar to cDNA of *Apis mellifera* ATP synthetase. Three of DD-PCR band selected from forager stage (F2, F6 and F39) were identified as genomic RNA of Kakugo viruses, while F9 was classified as cDNA from human. Sequences obtained

from four DD-PCR bands of N12, N53, N54 and N60 were similar to cDNA of Thioesterase domain 3 of *Apis mellifera* fatty acid synthetase.

As much as thirty six sets of recombinant clones harbouring recombinant plasmid of single DD-PCR band showed 2-3 types of nucleotide sequence from 2-3 clones. All sequences data were shown in Appendix E. Seventy two different sequences were obtained. However, only forty seven sequences were similar to known DNA sequences in GenBank data with Blastn: $< 10^{-6}$. As showed in Table 3.3. For example F1/sequence was identified as *Mus musculus* BAC clone RP24-360K9 which was classified as unknown function gene (Blastn; 5e-28). Whereas F1/sequence 2 was eliminated because *E*-value higher than 1e-06.

In order to confirm the nucleotide sequence obtained from Blastn, the amino acid sequence data from translated protein vs translated database (tBlastx) were analzed. The nucleotide sequences with different in blastn and tBlastx were eliminated. There was only one bands, F9 were eliminated since Blastn identified as *Homo sapiens* cig64 mRNA whereas tBlastx identified as cloning vector pSilentGene Hygromycin. The result of tBlastx analysis of these 27 DD-PCR bands was shown in Appendix F.

Moreover, thirty six sets of recombinant clones harbouring recombinant plasmid of single DD-PCR band showed 2-3 types of nucleotide sequence from 2-3 clones. Seventy two different sequences were obtained. However, only 57 sequences were similar to known DNA sequences in GenBank data with tBlastx: $< 10^{-6}$. Forty one bands (F3/sequence1,2, F3/sequence3, F7/sequence1, F8/sequence1, F8/sequence2, F17/sequence1, F17/sequence2, F55/sequence1, F55/sequence2, N4/sequence1, N11/sequence1, N11/sequence2, N74/sequence1, N75/sequence2,

Table 3.2 Differentially expression DD-PCR bands in mandibular gland from nurse and forager stages (Blastn)

DD-PCR bands	*E*-value	DNA
F2	5e-90	(AB070959) Kakugo virus genomic RNA
F6	0.0	(AB070959) Kakugo virus genomic RNA
F9	1e-o6	(AF026945) *Homo sapiens* cig64 mRNA
F39	4e-90	(AB070959) Kakugo virus genomic RNA
N5	2e-21	(XM_392933) *Apis mellifera* similar heat shock cognate 70 protein
N12	e-141	(XM_396268) *Apis mellifera* similar to ENSANGP00000016695, Thioesterase domain
N13	0.0	(XM_392639) *Apis mellifera* similar to ENSANGP00000009989, ATP syntase
N14	0.0	(XM_392639) *Apis mellifera* similar to ENSANGP00000009989, ATP syntase
N20	0.0	(XM_392962) *Apis mellifera* similar to putative activated protein kinase C receptor
N36	1e-78	(AF000632) *Apis mellifera* major royal jelly protein MRJP2 mRNA
N53	0.0	(XM_396268) *Apis mellifera* similar to ENSANGP00000016695, Thioesterase domain
N54	0.0	(XM_396268) *Apis mellifera* similar to ENSANGP00000016695, Thioesterase domain
N60	0.0	(XM_396268) *Apis mellifera* similar to ENSANGP00000016695, Thioesterase domain
N66	e-127	(XM_392691) *Apis mellifera* similar to translation elongation factor 2
N72	0.0	(AF525776) *Apis cerana* major royal jelly protein MRJP1 mRNA
N77	0.0	(XM_394434) *Apis mellifera* similar to CG1782-PA, Ubiquitin activating protein
N78	0.0	(XM_394434) *Apis mellifera* similar to CG1782-PA, Ubiquitin activating protein
N81	e-138	(XM_392035) *Apis mellifera* similar to ENSANGP00000020019, Peptidase family M16

Table 3.2 Differentially expression DD-PCR bands in mandibular gland from nurse and forager stages (Blastn) (continued)

DD-PCR bands	***E*-value**	**DNA**
N91	e-113	(X83495) *A. ervi* 28S rRNA
N109	0.0	(AY703484) *Apis mellifera* 18 S ribosomal RNA gene
N119	0.0	(AY703484) *Apis mellifera* 18 S ribosomal RNA gene
N120	0.0	(AF525776) *Apis cerana* major royal jelly protein MRJP1 mRNA
N124	e-115	(AF525776) *Apis cerana* major royal jelly protein MRJP1 mRNA
N125	e-162	(XM_397263) *Apis mellifera* similar to Zinc finger protein 39
N135	0.0	(AY703484) *Apis mellifera* 18 S ribosomal RNA gene
N136	0.0	(AY703484) *Apis mellifera* 18 S ribosomal RNA gene
N145	e-123	(XM_393410) *Apis mellifera* similar to high-affinity Na^{+}-dependent glutamate transporter

Table 3.3 Differentially expression of genes (*E*, < 10^{-6}) in mandibular gland from nurse and forager stages (Blastn)

Sequence no. of DD-PCR bands	*E*-value	DNA
F1/sequence 1	5e-28	(AC132102) *Mus musculas* BAC clone RP24-360K9 from chromosome 9
F3/ sequence1,2	1e-75	(AY292384) Deformed wing virus isolate PA
F3/sequence 3	0.0	(XM_392331) *Apis mellifera* similar to pDJA1 chapharone
F7/sequence 1	e-138	(AJ489744) Deformed wing virus genomic RNA
F8/sequence 1	2e-09	(AC132226) *Mus musculas* chromosome 1 clone RP24-571 A14
F8/sequence 2	e-152	(AB070959) Kakugo virus genomic RNA
F17/sequence 1	7e-18	(AB070959) Kakugo virus genomic RNA
F17/sequence 2	2e-25	(AJ489744) Deformed wing virus genomic RNA
F55/sequence 1	0.0	(AC154814) *Mus musculas* chromosome 16 clone RP24-532L22
F55/sequence 2	2e-59	(XM_394418) *Apis mellifera* similar to ENSANGP00000004035, transmembrane receptor
N4/sequence 1	3e-55	(XM_392933) *Apis mellifera* similar to heat shock cognate 70 protein
N11/sequence 1	0.0	(AC108399) *Mus musculus* chromosome 19 clone RP24-37507
N11/sequence 2	e-105	(XM_396268) *Apis mellifera* similar to ENSANGP00000016695, Thioesterase domain
N46/sequence 1	3e-09	(U18676) *Bacteroides fragilis* catalase (kat B) gene
N74/sequence 1	0.0	(XM_395455) *Apis mellifera* similar to eukaryotic translation initiation factor 4
N75/sequence 2	0.0	(XM_395158) *Apis mellifera* similar to CG33113-PA, neuroendocrine-specific protein
N76/sequence 1	e-110	(AL713960) Mouse DNA sequence from clone RP23-44819 on chromosome 11
N76/sequence 2	e-155	(XM_393632) *Apis mellifera* simila to Pgcp protein, peptidase
N90/sequence 1	1e-13	(U38230) *Pseudomonas aeruginosa* plasmid pSCH884
N92/sequence 1	0.0	(AF525776) *Apis cerana* major royal jelly protein MRJP1 mRNA
N92/sequence 2	e-170	(XM_393220) *Apis mellifera* similar to EG:BACR25B3.1, Laminir-type epidermal growth factor-like domain

Table 3.3 Differentially expression of genes (*E*, < 10^{-6}) in mandibular gland from nurse and forager stages (Blastn) (continued)

Sequence no. of DD-PCR bands	*E*-value	DNA
N100/sequence 1	1e-25	(AY251269) Varroa destructor virus 1
N100/sequence 2	0.0	(XM_394390) *Apis mellifera* similar to ENSANGP00000016661, Transcriptional repressor
N104/sequence 2	1e-60	(AY485644) *Triticum monococcum* phosphatidylserine decarboxylase
N106/sequence 1	0.0	(AY703484) *Apis mellifera* 18 S ribosomal RNA gene
N107/sequence 1	0.0	(XM_393453) *Apis mellifera* similar to ENSANGP00000015691, phosphoglycerate mutase family
N107/sequence 2	0.0	(XM_392469) *Apis mellifera* similar to CG5735-PB, RNA-recognition motif
N108/sequence 1	0.0	(XM_395948) *Apis mellifera* similar to 00000011664, Glutamate-cysteine ligase
N108/sequence 2	0.0	(AY703484) *Apis mellifera* 18 S ribosomal RNA gene
N118/sequence 2	0.0	(XM_395162) *Apis mellifera* similar to potassium channel modulatory factor1
N126/sequence 1	0.0	(XM_396647) *Apis mellifera* similar to G protein-coupled receptor kinase type-2
N126/sequence 2	e-123	(XM_395871) *Apis mellifera* similar to protein expressed in T-cells and eosinophils
N127/sequence 1	e-173	(XM_392236) *Apis mellifera* inositol 1,4,5-triphosphate receptor (ipr1)
N127/sequence 2	0.0	(XM_394406) *Apis mellifera* similar to ENSANGP00000010230, Gamma-glutamyltranspeptidase
N128/sequence 1	0.0	(XM_397263) *Apis mellifera* similar to zinc finger protein 39
N139/sequence 1	e-174	(XM_392675) *Apis mellifera* similar to ENSANGP00000015140, Apolipophorin III
N139/sequence 2	0.0	(XM_392741) *Apis mellifera* similar to ENSANGP00000009256, lipid binding protein

Table 3.3 Differentially expression of genes (*E*, < 10^{-6}) in mandibular gland from nurse and forager stages (Blastn) (continued)

Sequence no. of DD-PCR bands	***E*-value**	**DNA**
N140/sequence 1	0.0	(U00096) *Escherichia coli* K-12 MG 1665
N140/sequence 2	0.0	(XM_394657) *Apis mellifera* similar to Ubiquinol-cytochrome C reductase
N141/sequence 2	0.0	(XM_392193) *Apis mellifera* similar to ENSANGP00000010075, Transketolase C; pyridine binding domain
N142/sequence 2	1e-44	(AL591983) *Listeria monocytogenes* strain EGD segment 11/12
N144/sequence 1	5e-07	(BA000028) *Oceanbacillus iheyensis* HTE831 DNA
N146/sequence 1	0.0	(XM_396062) *Apis mellifera* similar to CG2247-PA
N146/sequence 2	0.0	(XM_395712) *Apis mellifera* similar to ENSANGP00000015136, short chain alcohol dehydrogenase
N147/sequence 2	e-111	(AY251269) Varroa destructor virus 1

N76/sequence1, N76/sequence2, N92/sequence1, N92/sequence2, N100/sequence1, N100/sequence2, N104/ sequence2, N106/sequence1, N107/sequence1, N107/sequence2, N108/sequence1, N108/sequence2, N118/sequence2, N126/sequence1, N126/sequence2, N127/sequence1, N127/sequence2, N128/sequence1, N139/sequence1, N139/sequence2, N140/sequence1, N140/sequence2, N141/sequence2, N144/sequence1, N146/sequence1, N146/sequence2, N147/sequence2) gene nucleotides of tBlantx analysis identical to Blastn analysis (Table 3.4). For example, when compared the sequence identify between Blastn (Table 3.3) and tBlastx (Table 3.4), it was found that 26 sequence* were similar to honeybee (*Apis spp*.) genes. Among of these genes, the result of N74/sequence 1 from Blastn and tBlastx was identified as (XM_395455) *Apis mellifera* similar to eukaryotic translation initiation factor 4 (Table 3.4).

Twenty six DD-PCR bands (F2, F6, F39, N5, N12, N13, N14, N20, N36, N53, N54, N60, N66, N72, N77, N78, N81, N91, N109, N119, N120, N124, N125, N135, N136, N145) which gave unique nucleotide sequence in the same cloning set were sorted into five different function categories (Table 3.6). Out of this 26 DD-PCR bands, 35% (9 DD-PCR bands) involved in metabolism. Five DD-PCR bands (19%) were classified as ribosomal RNA genes, 24% (6 DD-PCR bands) involved in regulatory role and 15% (4 DD-PCR bands) were encoded food storage protein. Moreover, twelve percent was related to pathogens (Kakugo virus).

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*F3/sequence3, F55/sequence2, N4/sequence1, N11/sequence2, N74/sequence1, N75/sequence2, N76/sequence2, N92/sequence1, N92/sequence2, N100/sequence2, N107/sequence1, N107/sequence2, N108/sequence1, N108/sequence2, N118/sequence2, N126/sequence1, N126/sequence2, N127/sequence1, N127/sequence2, N128/sequence1, N139/sequence1, N139/sequence2, N140/sequence2, N141/sequence2, N146/sequence1, N146/sequence2

Table 3.4 Differentially expression of proteins in mandibular gland from nurse and forager stages (tBlastx)

Sequence no. of DD-PCR bands	E-value	DNA
F3/sequence1,2	2e-71	(AJ489744) Deformed wing virus isolate PA
F3/sequence 3	e-107	(XM_392331) *Apis mellifera* similar to pDJA1 chapharone
F7/sequence 1	e-120	(AJ489744) Deformed wing virus isolate PA
F8/sequence 1	1e-38	(AC132226) *Mus musculus* chromosome 1 clone 24-571 A14
F8/sequence 2	e-116	(AB070959) Kakugo virus genomic RNA
F17/sequence 1	6e-31	(AB070959) Kakugo virus genomic RNA
F17/sequence 2	5e-42	(AJ489744) Deformed wing virus genomic RNA
F55/sequence 1	e-178	(AC154814) *Mus musculus* chromosome 16 clone RP24-532L22
F55/sequence 2	2e-05	(XM_394418) *Apis mellifera* similar to ENSANGP00000004035, Transmembrane receptor
N4/sequence 1	5e-08	(XM_392933) *Apis mellifera* similar heat shock cognate 70 protein
N11/sequence 1	5e-94	(AC108399) *Mus musculus* chromosome 19 clone RP24-37507
N11/sequence 2	1e-55	(XM_396268) *Apis mellifera* similar to ENSANGP00000016695, Thioesterase domain
N74/sequence 1	6e-90	(XM_395455) *Apis mellifera* similar to eukaryotic translation initiation factor 4
N75/sequence 2	9e-92	(XM_395158) *Apis mellifera* similar to CG33113-PA, neuroendocrine-specific protein
N76/sequence 2	1e-92	(XM_393632) *Apis mellifera* simila to Pgcp protein, peptidase
N92/sequence 1	e-117	(AF525776) *Apis cerana* major royal jelly protein MRJP1 mRNA
N92/sequence 2	8e-75	(XM_393220) *Apis mellifera* similar to EG:BACR25B3.1, Laminin-type epidermal growth factor like domain
N100/sequence 1	2e-96	(AY251269) Varroa destructor virus 1
N100/sequence 2	e-161	(XM_394390) *Apis mellifera* similar to ENSANGP00000016661, Transcriptional repressor
N104/sequence 2	1e-60	(AY485644) *Triticum monococcum* phosphatidylserine decarboxylase
N106/sequence 1	5e-87	(AY703484) *Apis mellifera* 18 S ribosomal RNA gene
N107/sequence 1	2e-93	(XM_393453) *Apis mellifera* similar to ENSANGP00000015691, phosphoglycerate mutase family
N107/sequence 2	4e-79	(XM_392469) *Apis mellifera* similar to CG5735-PB, RNA-binding protein

Table 3.4 Differentially expression of proteins in mandibular gland from nurse and forager stages (tBlastx) (Continued)

Sequence no. of DD-PCR bands	E-value	DNA
N108/sequence 1	1e-90	(XM_395648) *Apis mellifera* similar to 00000011664, Glutamate-cyteine ligase
N108/sequence 2	6e-86	(AY703484) *Apis mellifera* 18 S ribosomal RNA gene
N118/sequence 1	3e-54	(NM_137616) *Drosophila melanogaster* CG11208-PA, Transketolase
N118/sequence 2	4e-58	(XM_395162) *Apis mellifera* similar to potassium channel modulatory factor1
N126/sequence 1	1e-84	(XM_396647) *Apis mellifera* similar to G protein-coupled receptor kinase type-2
N126/sequence 2	3e-84	(XM_395871) *Apis mellifera* similar to protein expressed in T-cells and eosinophils
N127/sequence 1	4e-76	(XM_392236) *Apis mellifera* inositol 1,4,5-triphosphate receptor (ipr1)
N127/sequence 2	4e-93	(XM_394406) *Apis mellifera* similar to ENSANGP00000010230, Gamma-glutamyltransferase
N128/sequence 1	8e-73	(XM_397263) *Apis mellifera* similar to zinc finger protein 39
N139/sequence 1	1e-85	(XM_392675) *Apis mellifera* similar to ENSANGP00000015140, Apolipophorin III
N139/sequence2	e-127	(XM_392741) *Apis mellifera* similar to ENSANGP00000009256, lipid-binding protein
N140/sequence 1	e-141	(U00096) *Escherichia coli* K-12 MG 1665
N140/sequence 2	e-111	(XM_394657) *Apis mellifera* similar to Ubiquinol-cytochromeC reductase
N141/sequence 2	e-105	(XM_392193) *Apis mellifera* similar to ENSANGP00000010075, Transketolase C ; pyridine binding domain
N146/sequence1	e-105	(XM_396062) *Apis mellifera* similar to CG2247-PA
N146/sequence2	9e-96	(XM_395712) Apis mellifera similar to ENSANGP00000015136, short chain alcohol dehydrogenase
N147/sequence2	e-105	(AY251269) Varroa destructor virus 1

From DD-PCR bands that showed many nucleotide sequences, nucleotide sequences analyzed showed that forty one nucleotides of 11 sequences involved in metabolism and regulatory roles they were represented for 27%. A roughly equivalent amount of sequences (11) involved in pathogens (17%) followed by the (2) sequences were ribosomal RNA genes (2%), 1 sequence involved in food storage (2%). Moreover, and the sequences of unknown function was at 7%. They were shown in Table 3.7.

In order to confirm the differential display results, the semiquantative determination of some DD-PCR bands by RT-PCR. The total RNA was used as the template to construct first and second stranded cDNA. Then, the second stranded cDNA was used as a target cDNA for ATP syntase (N13), Thioesterase (N60) Phosphoglycerate (N107/sequence 1), Apolipophorin III (N139/sequence 1) and Transketolase (N141/sequence 1) quantitation.

Approximately, 50 ng of each total RNA of nurse and forager mandibular glands were used to amplify ATP syntase (N13), Thioesterase (N60) and the house keeping gene (Ribosomal protein: RpS8). The intensity of PCR product of the house keeping gene was found to be the same in both nurse and forager. In contrast, ATP syntase and Thioesterase were expressed at different levels in both nurse and forager bees. The expression level of ATP syntase in nurse bee was approximately 1.78 times higher than in forager bees. Likewise, the level of Thioesterase transcript in nurse bees was about 1.40 times greater than that in forager bees (Figure 3.5). Unfortunately, Phosphoglycerate, Apolipophorin III and Transketolase genes could be unsuccessfully amplified.

Table 3.6 Functional categories of the same sequence of DD-PCR bands with single nucleotide sequence in the cloning set

Gene ontology term	DD-PCR bands gave same sequence in cloning set	
	Number in category	% Blast analysis
Metabolism	9	35
Pathogens	3	12
Food storage (MRJPs families)	4	15
Ribosomal RNA genes	5	19
Regulatory and transporter	6	24

Table 3.7 Functional categories of the nucleotide sequences obtained from single DD-PCR band with showed more than one sequence

Gene ontology term	Sequence no. of DD-PCR bands in cloning set	
	Number in category	% Blast analysis
Metabolism	11	27
Pathogens	7	17
Food storage (MRJPs families)	1	2
Ribosomal RNA genes	2	5
Regulatory and transporter	11	27
Cell division and defense	2	5
Unknown function	3	7

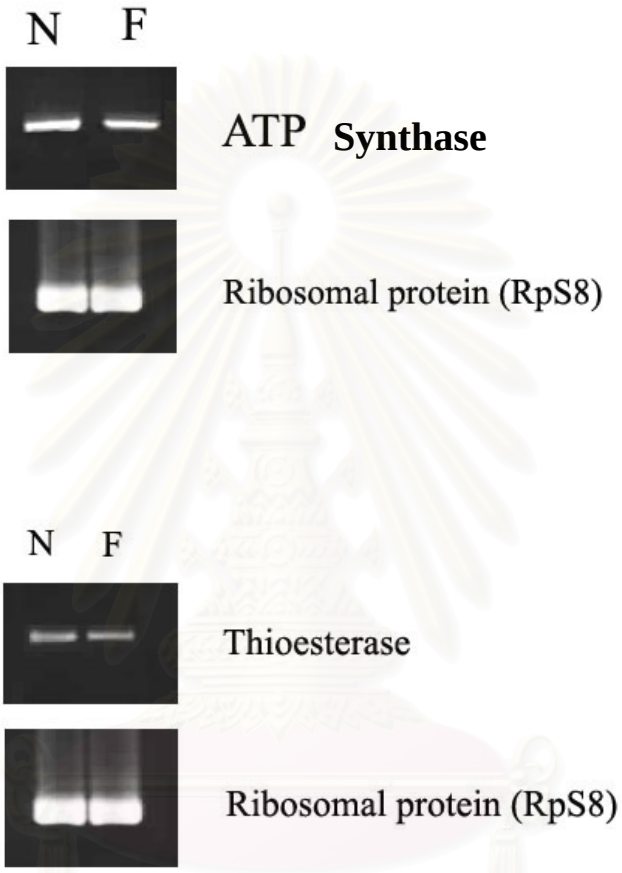

Figure 3.5 Agarose gel electrophoresis for quantification of ATP synthase (N13) and Thioesterase (N60) mRNA in mandibular gland total RNA of *Apis cerana*

Lane N nurse bee

Lane F forager bee

CHAPTER IV

DISCUSSION

In social insects such as bees, wasps, ants and termites, there are individual's variations in terms of their behavior and/or morphology. Caste differentiation is thought that some genes should be expressed in a caste-specific manner. In a given species, this phenomenon leads us to the phenotypes identification. In 1999, Miura *et al*. reported the caste-specific gene expression in mandibular glands of termite, *Hodotermopsis japonica*. This study suggested that the *SOL*1 gene encoding a secretary protein was specifically found in the mandibular glands of the mature soldiers. Honeybees are also the social insects, they may show the same caste differentiation as in termite. In previous studies, much attention has been paid to the hypopharyngeal glands (Srisuparbh, 2002, Imjongjirak, 2003, Cenphakdee, 2003). However, there are few reports about mandibular glands in bees. The mandibular gland was known as the pheromone secretory gland in queen. Most pheromones were the unusual cholesterols, triglycerides and carboxylic acids (Brown and Freure, 1959). Another function of mandibular gland related to the production of free fatty acids, the component in RJ of worker bees (Matsuyama *et al*., 1998). In 2002, Trongnipatt studied honeybee mandibular glands in order to understand the expressed genes involved in lipid metabolism. The cDNA library was generated from mRNA of dissected nurse bee mandibular glands using TimeSaver™ cDNA synthesis kit (Amersham Pharmacia Biotech, England). Unexpectedly, the cDNA library could not give be constructed. Then, the Delta™ Differential display kit (Clonetech, USA) was used to construct the cDNA clones. The constitutive genes such as rRNA genes, aminotransferase gene and the cell division control gene were found. Unfortunately,

genes involved in lipid metabolisms were not found (Trongnipat, 2002). Thus, in this study, the genes that express in mandibular glands of honeybee, *Apis cerana*, at nurse and forager stages were identified using differential display method.

Differential display is a technique which detects the differences in gene expression between tissues based on PCR amplification. This method was presented rapid identification of gene products and required small amounts of RNA. This method also exhibits the comparative display of all transcribed genes in any cell or tissue type and comparing differential gene expression of two or more cell populations and tissues. Moreover, it does not require the prior knowledge of sequence and availability of cDNA clones (Jurecic and Belmont, 2000). That can be said the differential display is one of the most suitable methods for tracking novel genes. The generation of false positive or nonspecific products remains one of its major drawbacks and the inability to confirm differential expression (Kozian and Kirschbaum, 1999). The methods available for differential analysis such as micro-array techniques, subtractive hybridization and classical differential display require extensive sequences information from the organism of interest or utilize large amounts of biological material (Sturtevent, 2000).

In this experiment, the nurse and forager bee samples were collected from Bee Research Center at Chumporn province. Nurse bee samples were collected when they were 5-15 days old. Newly emerged bees were marked on their thorax with color marker. Forager bee samples were captured near the hive entrance. In honeybee head, there are two mandibular glands joined with mandible. The mandibular gland is a small gland, thus to get enough total RNA for the experiment we had to collect 200 nurse bees from at least ten colonies. Usually, newly emerged bees are found in the

colony only when they want to expand their colony and/or prepare for swarming. Occurrence of the colony expansion and swarming depend on many factors such as number of bee in colony, healthy of the colony, weather, season and floral source. Therefore, collection of 400 honeybees sample (200 nurse bees and 200 forager bees) is time consuming step. Approximately, 200 of each nurse and forager bees' head were prepared for mandibular gland samples. This gland was cut from bee head under a binocular microscope at 4^{o}C. The mandibular bee glands contain high content of lipids therefore the tissues are very soft. It is vary difficult to completely take their out by forceps.

The total RNA was extracted from 400 mandibular glands of nurse and forager bees. Very low amount of extracted RNA of 24.38 and 20.33 µg per 200 bees (400 mandibular glands) were obtained from nurse and forager bee, respectively. However, the results from agarose gel exhibited that extracted RNA did not contained high molecular weight DNA. The spectrophotometrical measurement of OD_{260}/OD_{280} ratio was about 2 that indicated that acceptable quality of extracted RNA was obtained. The first stranded cDNAs were constructed in the reverse transcription reaction using oligo(dT) primer. The second stranded cDNAs were constructed using 36 combinations of arbitrary and oligo(dT) primers. The PCR reactions were used for long primers (25-30 mers) in combination with two steps of PCR procedure. In first cycle, primers were allowed to anneal under low stringency conditions followed by the higher annealing temperature increases stringency. In addition, hot start *taq* DNA polymerase (Clontech, USA) were used in amplification processes. DNA polymerase overall offers 3'- to 5'- exonuclease or proofreading activity and high accuracy in

amplification of target sequence. This can increase reproducibility, increase DD products size and reduce false positive results (Sturtevent, 2000).

The differential expression of the transcripts between nurse and forager mandibular glands were analyzed by denaturing 5% acrylamide/8M urea gels. A total of about 300 amplified transcripts can be visualized from DD-PCR using 36 primer combinations. Seventy differentially expressed bands of 19 primer combinations from nurse stage were selected for cloning and sequencing (Table 3.2). Seventeen primer combinations might not be annealed with first-stranded cDNA template. The limitation of primer in DD-PCR is that both of the primer matches and the abundance of each RNA dictate the display of a product (Sturtevant, 2000). In this study, most of selected DD-PCR bands for further study were cDNA of mRNA that could expressed in both stage but with different level of expression (50 bands (72%) from nurse, 1 band from forager). Only 20 DD-PCR bands (28%) were found to be nurse specific (expressed only in nurse stage) and 10 DD-PCR band (91%) were found to be forager specific. Most of forager specific DD-PCR bands (12%) were further identified to be Kakugo virus genomic RNA, whereas nurse specific DD-PCR bands were identified as mRNA from various genes as shown in Table 4.1.

Only fifty five (78%) of DD-PCR bands from nurse can be successfully reamplified. They were found in the range of 400 to 1500 bp in size (Table 3.2). Unsuccessful reamplification may be effected by the mismatch of primers and/or the mistake of DD-PCR band recovery by excision. Moreover, the amount of cDNA (template) used was another important factor that could affected the reamplification. Optimization of template concentration may get rid of this problem. The reamplified

Table 4.1 Nurse specific genes.

Sequence no. of DD-PCR bands	E-value	genes
N36	1e-78	(AF000632) *Apis mellifera* major royal jelly protein MRJP2 mRNA
N74/sequence 1	0.0	(XM_395455) *Apis mellifera* similar to eukaryotic translation initiation factor 4
N75/sequence 2	0.0	(XM_395158) *Apis mellifera* similar to CG33113-PA, neuroendocrine-specific protein
N76/sequence 2	1e-92	(XM_393632) *Apis mellifera* simila to Pgcp protein, peptidase
N78	0.0	(XM_394434) *Apis mellifera* similar to CG1782-PA, Ubiquitin activating protein
N81	e-138	(XM_392035) *Apis mellifera* similar to ENSANGP00000020019, Peptidase family M16
N127/sequence 1	4e-76	(XM_392236) *Apis mellifera* inositol 1,4,5-triphosphate receptor (ipr1)
N127/sequence 2	4e-93	(XM_394406) *Apis mellifera* similar to ENSANGP00000010230, Gamma-glutamyltransferase
N139/sequence 1	1e-85	(XM_392675) *Apis mellifera* similar to ENSANGP00000015140, Apolipophorin III
N139/sequence 2	e-127	(XM_392741) *Apis mellifera* similar to ENSANGP00000009256, lipid-binding protein
N141/sequence 2	e-105	(XM_392193) *Apis mellifera* similar to ENSANGP00000010075, Transketolase C ; pyridine binding domain

cDNA bands on agarose gel were recovered using QIAquick gel extraction kit (Qiagen, Germany) and ligated to pGEM-Teasy vector (Promega Co., USA.). The ligated plasmids were then transformed to bacteria *E. coli* JM 109 by electroporation. Blue/white colony screening was performed for each PCR products. Fifty two (94%) of reamplified PCR products of nurse can be successfully cloned.

The nucleotide sequences of 2-3 independent clones from each DD-PCR bands were analyzed (total 128 clones). Among 63 sets of clone from 63 DD-PCR bands (52 bands from nurse mandibular glands and 11 bands from forager mandibular glands), only 27 (43%) DD-PCR bands gave the same sequence of 2-3 clones in the cloning set. Thirty six (55%) sets of clones from 36 DD-PCR bands gave different sequence. The nucleotide sequences obtained were analyzed for the homology by searching from GenBank DNA and Protein database using Blastn and tBlastx programs through the National Center for Biotechnology Information (NCBI). Thirty six (55%) sets of clones from 36 DD-PCR bands gave different sequence (Table 3.4). The binding of more than one cDNA sequence from one DD-PCR bands might be the result from the excision of more than one band from the gel and the false positive. Usually, false positive was found in differential display method. The answer about the false positive rate was not clearly understood, because it depends on many factors, both intrinsic and extrinsic factors. It had been reported that the false positive would be much higher due simply to the fewer differences in gene expression in the samples (Liang, 1998). In this experiment, the false positive was quite high, this might due to not much difference in gene expression between two group of samples used. Since, nurse bee sample of 1-15 days old and forager bee sample of 15-21 days were used in

the experiment. Using nurse bee sample of 1-5 days instead of 1-15 days might show more different in gene expression and reduce the level of false positive.

Confirmation that recombinant clones differentially regulated genes (not artifact) was the necessary step. Many techniques can be used such as Northern blot analysis and reverse Northern blot assay (Sturtevent, 2000). This kind of test required large amount of RNA. The amount of extracted RNA obtained from mandibular gland of honeybee was vary low, so it was impossible to confirmed by this techniques.

Many DD-PCR bands selected from nurse bee were identified as cDNA of proteins/enzymes involved in lipid metabolism (Thioesterase, apolipophorin III, lipid binding protein, transketolase C and short chain alcohol dehydrogenase). All of these bands had higher intensity in nurse than those of forager, indicated that nurse had higher expression of these corresponding genes. This results corresponded to the report that lipid component of *Apis nigrocineta* nurse (1-4 days old) mandibular gland was higher than those in forager (Keeling *et al*., 2001).

The cDNA of MRJPs (AcMRJP1 and AmMRJP2) family was found in mandibular gland of nurse bee. This was believed to be the contamination during the mandibular dissecting process by hypopharyngeal gland. The MRJPs were reported to be expressed in hypopharyngeal gland (Srisuparbh, 2002; Ohashi *et al*., 1997). This two types of glands are closely located on head of honeybee.

The DD-PCR band containing cDNA of ribosomal RNA genes (18S ribosomal RNA and 28S ribosomal RNA genes) showed greater intensity than those of forager. Actually the ribosomal RNA genes have no poly (A) tail on 3’ end (Stryer *et al*., 2002). However, some part of sequence on ribosomal RNA genes may have poly (A)

that could be matched with Oligo (dT) primer. It can be reversed to first stranded cDNA, being template in DD-PCR condition.

Honeybees are attacked by the myriad of parasites and pathogens including viruses, bacteria, protozoa and parasitic mites. Hence, honeybees have been reported to be the host to multiple viruses infection. In this study many DD-PCR bands from forager were found to be sequences of viruses (Kakugo, Deformed wing and Varroa destructor virus). Kakugo and Deformed wing virus were found in forager stage (DD-PCR bands: F2, F6, F17, F39, F8/sequence 2 and F3/seqeuence 1,2, F7/sequence 8, respectively). This result was corresponding to the report by Fujiyuki *et al.*, (2004) that Kakugo virus was detected only in aggressive workers but not found in nurse. In addition, Deformed wing virus was reported highest exhibit in pupae and adult worker bees (Chen *et al.*, 2005). Varroa destructor virus 1 was found only in nurse stage (DD-PCR bands: N100/sequence1 and N147/sequence 2). Varroa destructor is one of mite which is parasite in honeybee. Varroa destructor virus 1 was closely related to Deformed wing virus. Both viruses replicated in the population of mite species and ultimately, high virus levels will be transmitted to the brood. The relationship between mite infection and virus infection is not clearly understood. Although the mite has been demonstrated to act as an activator of inapparent virus. (Devison *et al.*, 2003). Nurse and forager samples were collected from a many colonies, some of them may be infected by virus. Most honeybee viruses are single stranded RNA viruses also they could be amplified by RT-PCR (Chen *et al.*, 2004).

In this study semiquantitative PCR for estimating expression levels of DD-PCR bands of ATP synthase (N13) and Thioesterase (N60) were attempted to compare in mandibular gland from nurse and forager bee. House keeping gene

ribosomal protein (RpS8) was used as the control (Koywiwattrakul *et al*., personal communication). The same amount of frist stranded cDNA (approximately 50 ng) of each nurse and forager mandibular glands were used for amplified ATP synthase (N13) and Thioesterase (N60) with specific primers. The amplification of ATP synthase (N13) was followed to 30 cycles. Whereas the PCR condition of Thioesterse was performed 35 cycles. The result showed that the mRNA level of ATP synthase and Thioesterase of nurse mandibular gland were 1.78 and 1.40 times higher than those of forager. The PCR products of Phosphoglycerate, Apolipophorin III and Transketolase C were not sucessfully amplification. Uncessessful amplication may be the mismatch of primers and cDNA template. The optimal condition was unaviable. The amplification might be varying the factors for optimal condition.

Total of genes sequence (62 genes) can be divided in to three groups (Table 4.2). Twelve of them were unknown genes (*E*, $>10^{-6}$), which is approximately 20 % of total gene sequences. The number of database sequences observed in the search results was similar to the results previously reported in a recently published bee EST project. The results of bee EST project showed that 24 % were not matches to known DNA sequences of databases in GenBank (*E*, $>10^{-5}$) (Whitfield *et al*., 2002).

Table 4.2 Genes classificatios

Group	Number in category	Percentage group analysis
Known genes	39	63
Hypotilical protein	11	17
Unknown genes	12	20

CHAPTER V

CONCLUSIONS

1. A total of approximately 300 amplified transcripts that could be visualized from DD-PCR using 36 primer combinations.
2. Seventy differentially expressed bands of 19 primer combinations from nurse stage were selected for cloning and sequencing. Fifty (72%) out of seventy bands had higher intensity than forager and twenty bands (28%) were nurse specific genes.
3. Eleven differentially expressed bands of 7 primer combinations from forager were selected for cloning and sequencing. Only one bands (9%) showed higher intensity than that in nurse bee and ten bands (91%) were forager specific genes.
4. In forager stage, all DD-PCR bands could be successfully reamplified and cloned (100%).
5. In nurse stage, fifty five (78%) of DD-PCR bands could be reamplified and fifty two (94%) PCR products could be successfully cloned.
6. Blastn and Blastx analysis showed that 27 (42%) of two independent clones from DD-PCR bands were same sequence and thirty six (57%) of two or three clones were different sequence.
7. The GenBank search resulted of the sequence were sorted into eight different function categories including metabolism (22%), pathogens (10%), food

storage (7%), ribosome (5%), regulatory (11%), cell division and defense (2%), unknown function (8%) and unclassified (21%).

8. ATP synthase and Thioesterase showed higher expression level in nurse mandibular than those of forager.

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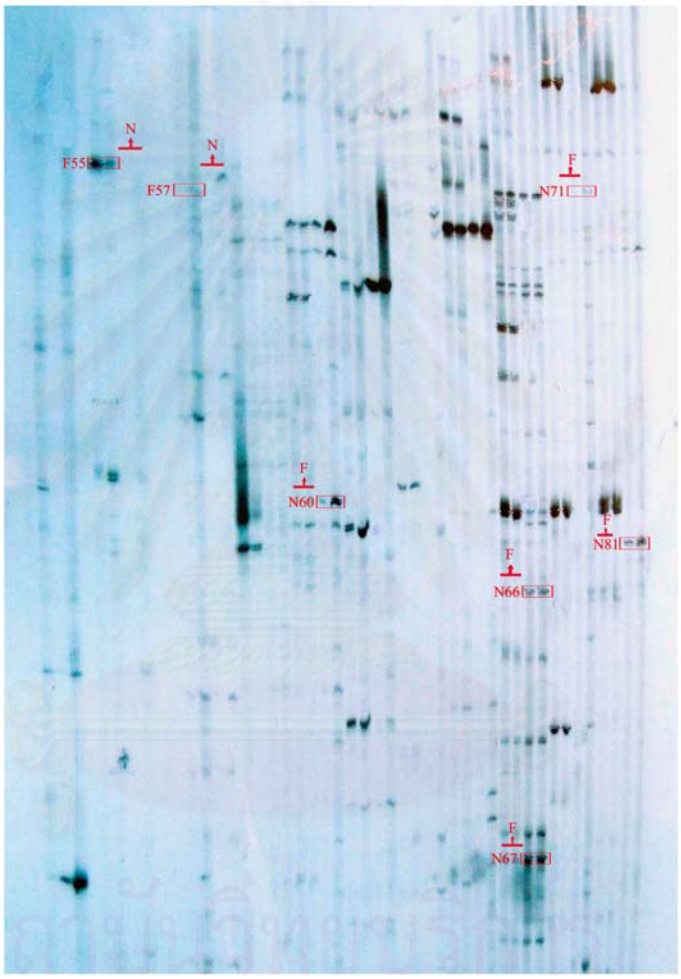
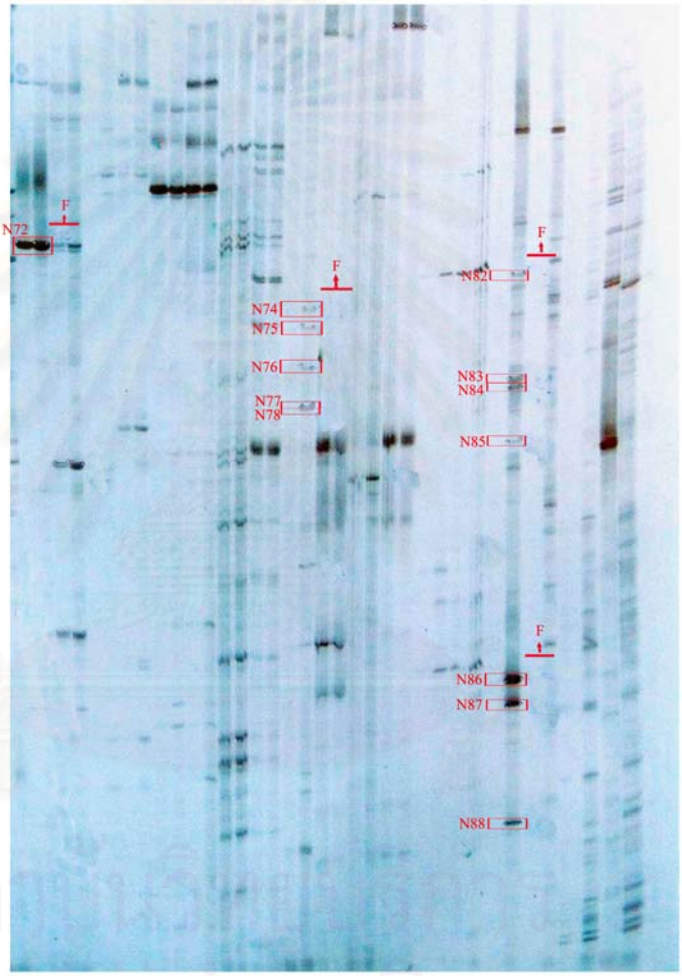
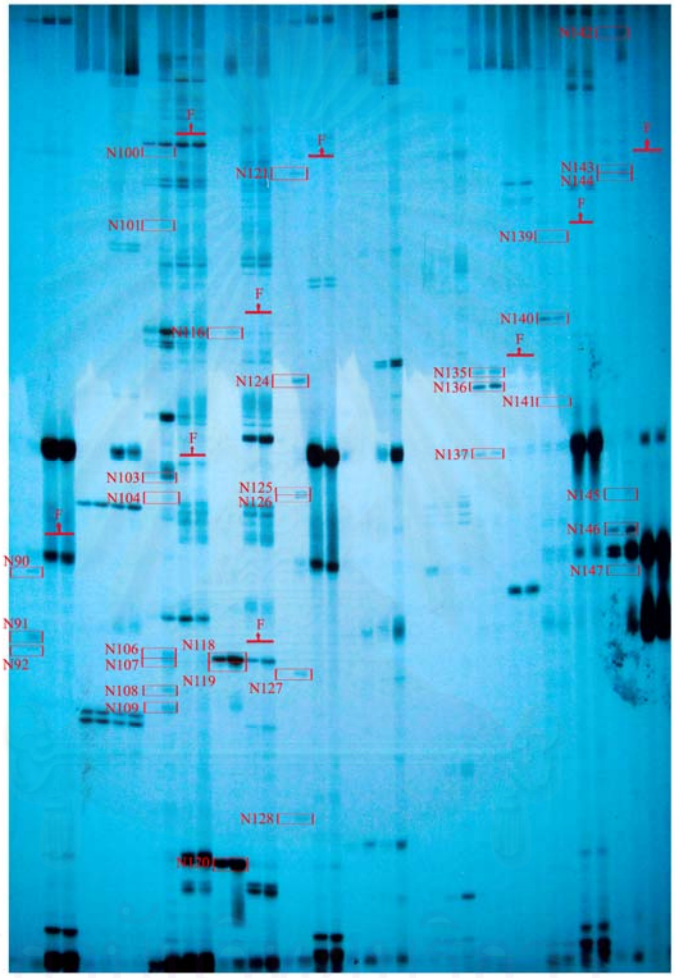
APPENDICES

APPENDIX A

Differential Display PCR profiles of gene in mandibular gland of nurse and forager

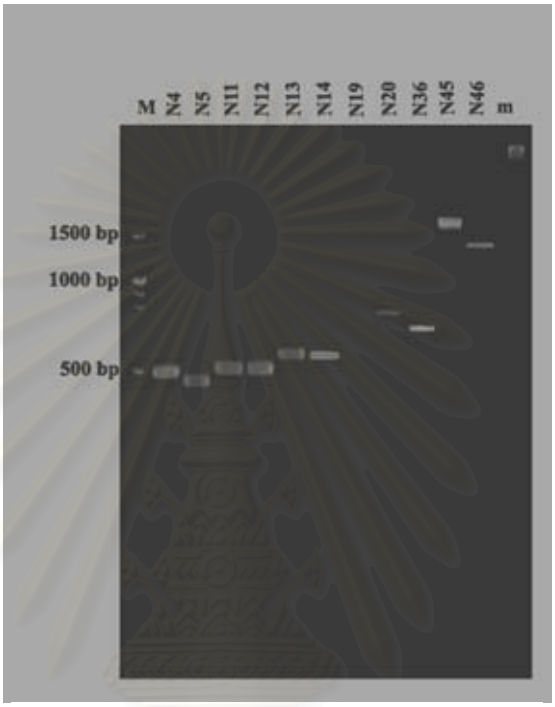


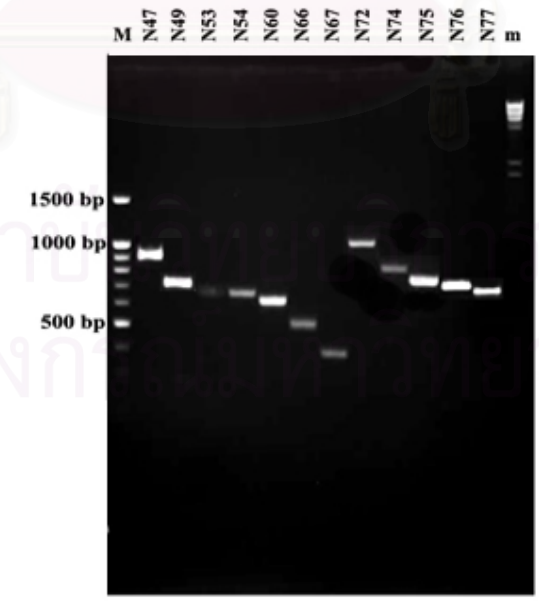


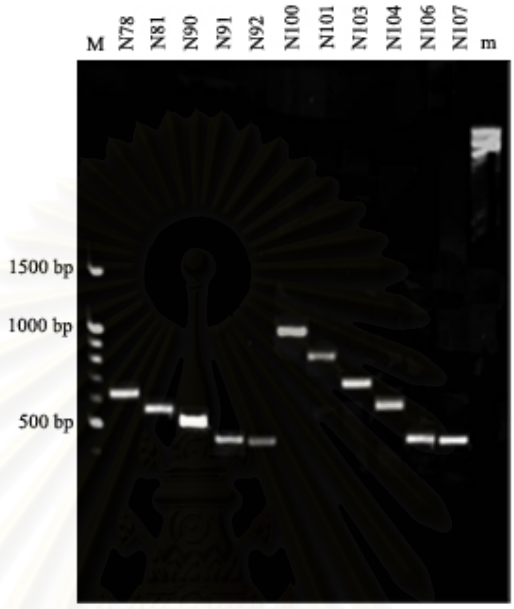


APPENDIX B

The reamplification profiles of DD-PCR fragments






M
N78
N81
N90
N91
N92
N100
N101
N103
N104
N106
N107
m
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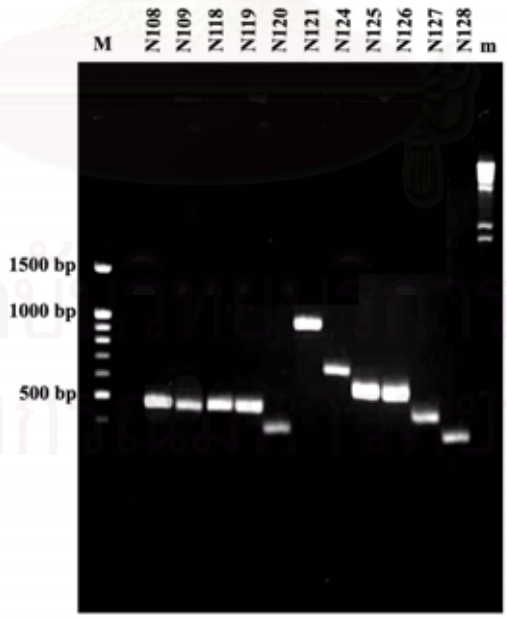
1000 bp
500 bp


M
N108
N109
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N119
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N121
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N125
N126
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N128
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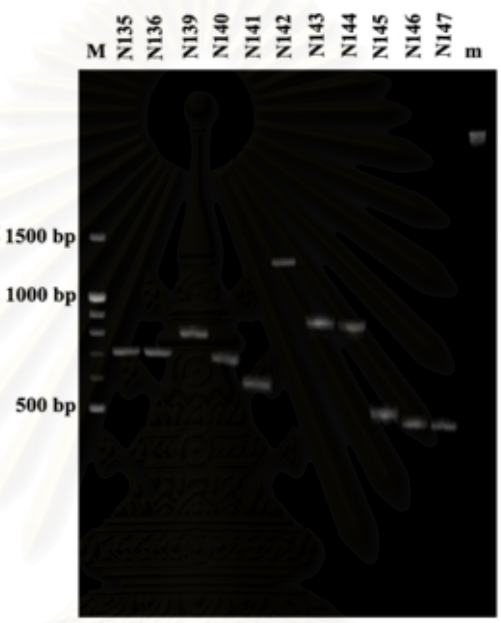
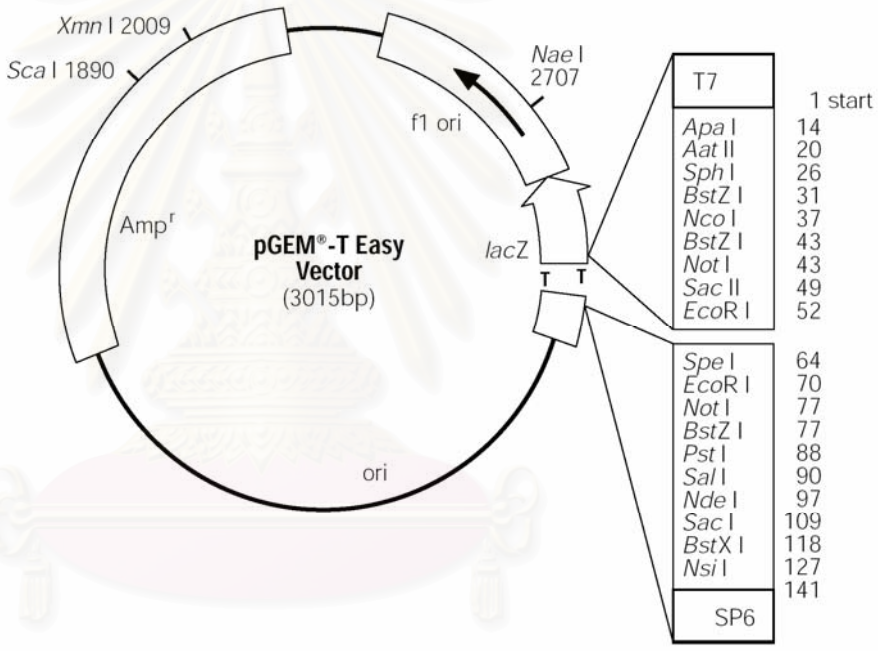
500 bp



Lane M and m 100 bp and λ/*Hin*dIII markers

APPENDIX C

Restriction map of pGem®-T Easy vector



APPENDIX D

cDNA clone sequences

>F1/sequence 1

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AGGTGACCGATGAGCCAGCACCGTCTGTCTCCCGGCTTGAGCACTGCTTGATGACTCCTTGATGGCCCT
GACTCTGGGCTAATGTCTAATGTCACCACGCTCCCGCGAGCTCATGCTTTTCTTTGTCTGTGTTATGTC
CGTGCAGGTGCAGGACAACTTCCAGAGTCACTCCTCAATCTCTGTCCACCCCCATTTGAAAGGCAGGGT
CTCTTGGGTGGCTTGGAACTCCTTAAGCAGGCTAGGCTAGCTGGCCGAAGGGCCCCAGGGNATCGGCCG
CCTCTGCCTCCCGAGTGCTGGGATTATAAGCACGTCCCACCACACTTGGGCTTTTATAGAGGTTCTGAG
GATTGAACTCAGGTCCTCATTGCCTGCAAAGCAAACACCTTACTGACTGAGCTATCCCACCAGCATTTA
GTGAGGGTTAATAATCACTAGT

>F1/sequence 2

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>F2/sequence 1

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TAACTCCATAGTAGAGTAGTTTTAATTACGATTAAAGTGGTATTCTAGATTAGGTATTACTCGCGTAAT
CCTGTGGTATAATAGGATNGCGTCCTAATTTTAGTATAGTTTTAATTATAATAGTGAAAAAAAAAGATA
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>F2/sequence 2

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>F3/sequence 1

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AAAACAGTGTAGATTAGACTACCATTTTTAGCTTATATATGGGAAAGGTTGAGTTGCCTCTAAAGACTT
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>F3/sequence 2

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TAACTCCATAGTAGAGTAGTTTTAATTACGATTAAAGTGGTATTCTAGATTAGGTATTACTCGCGTAAT
CCTGTGGTATAATAGGATTGCGTCCTAATTTTAGTATAGTTTTAATTATAATAGTGAAAAAAAAAGATA
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>F3/sequence 3

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>F6/sequence 1

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TGGGTTTTTAAAACATCCAACTAGACCTGTGTTTTTAGCTAATCTCGACAAAGTTTCAGTGGAAGGAAC
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ANAN

>F6/sequence 2

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>F7/sequence 1

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GCGAGTGTTTTACGATATGCATGTGTGGTGCATCTGGAATTGGAAAATCATATTTGACCGATTCCTTAT
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CTACAACTTTGGATAAGCAATTGAATATGTTATTCCAAGTACATTCGCCAATAGTTCTTTCTCCTCCTA
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>F7/sequence 2

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>F8/sequence 1

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CATCAAACTCACACATCATCAGAGTAGTCTAAGATCACCCAATGAGATAGAAAACTAAAGATTACATCA
TATCGAAGAGTGCACACATCTAGCAGATTGATGAGGGAATCTTCACTGATGCAGTCAGATCTTTAGTGA
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>F8/sequence 2

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>F9/sequence 1

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CNAACCNCTCANCGATCNGTTGNTGNCCGCCCTATNGCTCNTCCNNANNCNTNCNCTTCANGAANTTNT

CGTTNACNNAGNNTCTNCGTTTCNTCGGAATCCGGGTCTCCNCNCCNCGNNNCNATNCTNNGGANACNN
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>F9/sequence 2

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GGTCTTCATTTGTATCCCAGGTTTGCCTGCTTTGAAGATCCTCTCTCCTCAGACCCCTCAGTGCTGGAC
TGCTGGGTATGTCACCTCATCTCTCTCTCTGAGAAGCCTTGTCAGTCAGATCTTTAGTGAGGGTTAATA
ATCACTAGT

>F17/sequence 1

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TCCATACTATAGATAAACTTGTAGCCAAATAAGTCCATTCTAAACTATTCACGTCATATGCCAATACCA
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GGTAAATTGTACAAGGGCCTAATTACCATAAAATATTTCCTAAGTTTTTTTTCCCCCCGGGAAAAATTT
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>F17/sequence 2

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TAAAGAAATTTCTGGGGGACCCCAGGGTTTCAAGAATTTCCCTGAATGGTTTGCCACCCATTCCGGGCC
TCTT

>F39/sequence 1

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>F39/sequence 2

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>F39/sequence 3

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TAATAANTCACTAGT

>F55/sequence 1

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ATTGCTGTGATAAAAGACCAAGAACAAAGCAACTTGGGGAGGAAAGGGTTTGATTGACTTATACCTCCA
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>F55/sequence 2

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AGCCCNCGN

>F57/sequence 1

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>F57/sequence 2

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ATAATTGTAATTTGTATCATAATATTTTAATGAAAAATAGACAAATCGTATAACTTATTTTATTCTGCG
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>N4/sequence 1

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TTAAAAACCAAAT

>N4/sequence 2

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ATAATTATAATTAAAAAAAATCCCTTTAAAGNAATTTAAAAATTTATTAATTTAATTTAATTATATTAG
GGGGAAAACACCGAAAAAAAAAAAACCCCGAAAATGTCCTTGGTTCCTTAAAATTTTCCTTATTNGTGT
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>N5/sequence 1

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ACAAATAATTTATTTACATTAGTAATTTCCTACAGATTACAATAAACGTAAAAAAAACCTATTGTACTT
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>N5/sequence 2

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>N5/sequence 3

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>N11/sequence 1

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>N11/sequence 2

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>N11/sequence 3

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CATAATGAATCACTAGT

>N12/sequence 1

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>N12/sequence 2

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>N12/sequence 3

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>N13/sequence 1

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>N13/sequence 2

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>N13/sequence 3

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>N14/sequence 1

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>N14/sequence 2

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>N20/sequence 1

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>N20/sequence 2

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>N36/sequence 1

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>N36/sequence 2

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>N45/sequence 1

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>N46/sequence 1

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>N46/sequence 2

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>N46/sequence 3

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>N47/sequence 1

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>N49/sequence 1

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>N49/sequence 2

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>N53/sequence 1

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>N53/sequence 2

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>N53/sequence 3

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>N54/sequence 1

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>N54/sequence 2

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>N54/sequence 3

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>N60/sequence 1

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>N60/sequence 2

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>N66/sequence 1

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>N66/sequence 2

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>N67/sequence 1

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>N67/sequence 2

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>N72/sequence 1

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>N72/sequence 1

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>N74/sequence 1

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>N74/sequence 2

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>N75/sequence 1

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>N75/sequence 2

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>N76/sequence 1

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>N76/sequence 2

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>N77/sequence 1

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>N77/sequence 2

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>N78/sequence 1

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>N78/sequence 2

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>N81/sequence 1

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>N81/sequence 2

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>N90/sequence 1

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>N90/sequence 2

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>N91/sequence 1

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>N91/sequence 2

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>N92/sequence 1

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>N92/sequence 2

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>N100/sequence 1

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>N100/sequence 2

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>N104/sequence 1

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>N104/sequence 2

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>N106/sequence 1

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>N106/sequence 2

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>N107/sequence 1

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>N107/sequence 2

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>N108/sequence 1

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>N108/sequence 2

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>N109/sequence 1

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>N109/sequence 2

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>N118/sequence 1

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>N118/sequence 2

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>N119/sequence 1

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>N119/sequence 2

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>N120/sequence 1

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>N120/sequence 2

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>N124/sequence 1

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>N124/sequence 2

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>N125/sequence 1

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>N125/sequence 2

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>N126/sequence 1

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>N126/sequence 2

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>N127/sequence 1

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>N127/sequence 2

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>N128/sequence 1

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>N128/sequence 2

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>N135/sequence 1

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>N135/sequence 2

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>N136/sequence 1

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>N136/sequence 2

GATTATTAACCCTCACTAAATGCTGGTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCTCAG
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>N139/sequence 1

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TCAAGTTCTAATCTAGCTTTGTAATTTGGTTGTGATTCTATTTCATTTGCAATTNCTGCAGCTTTCTGT
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>N139/sequence 2

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CAAGAATTANTAGAAGCTGCAGAAGAATTTCATCATCCAAGATGTATTTTTCAAGATTCTATAATGCCN
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>N140/sequence 1

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GATGACCATCCACGCTGCCATAATAAATTGTATCCACAACCCCGAGCGGAACATATTTAACGCGCCAAT
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>N140/sequence 2

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CGCCATTTAAAGACAGCACTTTTTCCTTCAGGAATAGCATCAAGTTTTATTTCAATTTTTGCTAATGCA
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>N141/sequence 1

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CGGGTTTATCCAATCGGAATGTCGCGGATATTGAATCGTTGAATTAAAAGTCGTATACAAGTAGACTAA
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TNATATCGTAGTACATAATTAATGTATGATCGGTTTTANGATANGNGTTAGTATTTAANAAGGNANGAA
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>N141/sequence 2

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AAAGCTGCCATTCGAGATCCTGATCCAGTTGTTGTGTTAGAAAATGAAATATTATATGGTGTTCAATAC
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GCGGAGATTAGTGCTAGGATTTCGGAAAGTGAAGCTTTCTATCATCTTGATGCACCAGTAATGCGCGTT
ACAGGTGTTGATACACCTATGCCTTATGCCAGAACGTTAGAAAATGCATNCCTGNCNCATTTAGTGAGG
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>N142/sequence 1

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CTNAAGTATCATGNAATCCCNCCNAACCGGNTTGTNAAGGANTCGGGCCNTTANTGGANCAACCTCNCG
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CCCTTAANCNGANTTTT

>N142/sequence 2

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>N143/sequence 1

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>N143/sequence 2

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>N144/sequence 1

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>N144/sequence 2

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CACGTGTAAATAATATCAACGAGTTAAACGAGTATATCCCTTGTTTGATTTTGGATCGGTGTTGTTGAT
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CNTAATCGTATGGTTACTATCGGTACTGAACGTGCGNAGCNTTACTACGCTTCCTATTCCTTTTTCATA
CCNGGTGACNCNAATACGTTCGGCGNTNCCCGNANNAATGTGAGGCGNTNTNGTAACNNTATCGCCTNG
NCNTGANCNCTGNCNNGTNAGCNANGNACGNNNCNNANTCCNCNNNCNNAGANGGNNNNCNNNCCCCNN
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>N145/sequence 1

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TTCGTTTCTGCTTAAATAATTCACAATTCCTGCTCCGAGAGAATCACCTACCACATTCACTGTTGTTCT
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TAATAATCACTAGT

>N145/sequence 2

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CCATATGAAATCAAAGTCGATATTATTATCGGCAAGATTTGTAAATGCTAACAACAAACAAACGAAGAA
CGATGAGCGTTCGTTTTTTTTCTCCCCCCCGGTTAAATGTCGTACACTCCCACCGAACTTTAAATTTGA
AAGTCGAAGATGAAGTTCAGTACAAATATAATAATAAAATTCTTCGCAATCAACGATAATAGCAAATAA
TAGTGCACCTATTATATTTCATTCTATTTGTAGTAGTCAGTCTTCAAGAAGAGACTTTAATGATTCTTG
TACAATATTATTAGAAGCAATATTTTTTTGTATTTAACTACAAAAGCTGCTGCCACATTTAGTGAGGGT
TAAATCACTAGT

>N146/sequence 1

GATTATTAACCCTCACTAAAGCACCGTCCAAAATATCAAATGTACAACATGAGCCTTGACCTTTTTCAA
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ATTCACCCAATTCTAATGTTTCCAAATTCACTAAAGCTTGTAATATATCAATTCTTTTCTTTCCAAGTT
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TTAAGGAGTCACATTTTATTGACATTGCACTCAAGACTTCTAATTGAGGGCAACTACTAATAACTTCTT
CAACAACCATTACAGGACATTTACATAATTCTAATTTGACAAGGCTAGTTACGCGTGGTATTACTATTA
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>N146/sequence 2

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CAACACGAACACCAACATTAGCAAGATCTCGAGCTAATGGTAAAGTCATTGCAACAACTGCACCTTTAC
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CACGAAATCCATCTTTATCTGGTGTGTTGTTATACATAAGTGGTACACTTAATCTAATAACATTAAATG
TTCCAGTAACATTTACATTAAGCACTTTATTGAATTGATTTAAATCATGTGGCAAATTTGTATTAAAAT
TATATGTTTTGTGAGCAACAGCTACTCCTGCCACATTTAGTGAGGGTTAATAATCACTAGT

>N147/sequence 1

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CAACACCGTGGATATTTCACTTTTTTGTTCTTAAGTTTTTTCAAAGATATTCAAATTATTAATTAATCA
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GTATCTCTGTGTAAAAAGTGTAACTATAATGCTAATATAATAATAATTATAATAATAAGTTGCACAAGG
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CTTCCTGCCACATTTAGTGAGGGTTAATAATCACTAGT

>N147/sequence 2

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TATCCATACCATGGGGACGGTGCTTTAGTGAGGGTTAAATCACTAGT

APPENDIX E

Differentially expression of genes in mandibular gland from nurse and forager stages (Blastn)

Sequence no. of DD-PCR bands	*E*-value	DNA
F1/sequence 1	5e-28	(AC132102) *Mus musculas* BAC clone RP24-360K9 from chromosome 9
F1/sequence 2	1e-04	(XM_31290) Anopheles gambiae str. PEST ENSANGP00000012286
F3/ sequence1,2	1e-75	(AY292384) Deformed wing virus isolate PA
F3/sequence 3	0.0	(XM_392331) *Apis mellifera* similar to pDJA1 chapharone
F7/sequence 1	e-138	(AJ489744) Deformed wing virus genomic RNA
F7/sequence 2	2.1	(AE003692) *Drosophila melanogaster* chromosome 3R, section 30 of 118 the complete sequnce
F8/sequence 1	2e-09	(AC132226) *Mus musculas* chromosome 1 clone RP24-571 A14
F8/sequence 2	e-152	(AB070959) Kakugo virus genomic RNA
F17/sequence 1	7e-18	(AB070959) Kakugo virus genomic RNA
F17/sequence 2	2e-25	(AJ489744) Deformed wing virus genomic RNA
F55/sequence 1	0.0	(AC154814) *Mus musculas* chromosome 16 clone RP24-532L22
F55/sequence 2	2e-59	(XM_394418) *Apis mellifera* similar to ENSANGP00000004035, transmembrane receptor
F57/sequence 1	0.61	(AF100329) *Dendrobium* grex Madame Thong-IN ovg 15
F57/sequence 2	0.14	(AY691420) *Planococcus ficus* cytochrome b (cytb) psedudogene
N4/sequence 1	3e-55	(XM_392933) *Apis mellifera* similar to heat shock cognate 70 protein
N4/sequence 2	0.085	(BX530060) Zebrafish DNA sequence from clone DKEY-283F16
N11/sequence 1	0.0	(AC108399) *Mus musculus* chromosome 19 clone RP24-37507
N11/sequence 2	e-105	(XM_396268) *Apis mellifera* similar to ENSANGP00000016695, Thioesterase domain
N11/sequence 3	1.3	(AC073363) *Homo sapiens* 3 BAC RP11-293N1
N45/sequence 1	3e-05	(AF026945) *Homo sapiens* cig64 mRNA

Differentially expression of genes in mandibular gland from nurse and forager stages (Blastn) (continued)

Sequence no. of DD-PCR bands	***E*-value**	**DNA**
N46/sequence 1	3e-09	(U18676) *Bacteroides fragilis* catalase (kat B) gene
N46/sequence 2	3e-06	(AF026945) *Homo sapiens* cig64 mRNA
N46/sequence 3	0.12	(AF100329) *Dentrobium* grex Madame Thong-IN ovg 15
N47/sequence 1	0.53	(AC125170) *Mus musculus* BAC clone RP24-475C24 from chromosome 7
N49/sequence 1	0.008	(AC025116) *Mus musculus* chromosome 1 clone RP23-124O19
N49/sequence 2	1.7	(U40801) *Caenorhabditis elegans* cosmid F28E10
N67/sequence 1	7e-05	(AF100329) *Dendrobium* grex Madame Thong-IN ovg 15
N74/sequence 1	0.0	(XM_395455) *Apis mellifera* similar to eukaryotic translation initiation factor 4
N74/sequence 2	1e-04	(AF100329) *Dedtrobium* grex Madame Thong-IN ovg 15
N75/sequence 1	3e-05	(AL583651) Mouse DNA sequence from clone RP23-138F20 on chromosome 13
N75/sequence 2	0.0	(XM_395158) *Apis mellifera* similar to CG33113-PA, neuroendocrine-specific protein
N76/sequence 1	e-110	(AL713960) Mouse DNA sequence from clone RP23-44819 on chromosome 11
N76/sequence 2	e-155	(XM_393632) *Apis mellifera* simila to Pgcp protein, peptidase family
N90/sequence 1	1e-13	(U38230) *Pseudomonas aeruginosa* plasmid pSCH884
N90/sequence 2	4e-04	(AF095853)*Homo sapiens* asthmatic clone
N92/sequence 1	0.0	(AF525776) *Apis cerana* major royal jelly protein MRJP1 mRNA
N92/sequence 2	e-170	(XM_393220) *Apis mellifera* similar to EG:BACR25B3.1, Laminir-type epidermal growth factor-like domain
N100/sequence 1	1e-25	(AY251269) Varroa destructor virus 1
N100/sequence 2	0.0	(XM_394390) *Apis mellifera* similar to ENSANGP00000016661, Transcriptional repressor
N104/sequence 1	1e-04	(XM_319290) *Anopheles gambiae* str. PEST ENSANGP00000012286
N104/sequence 2	1e-60	(AY485644) *Triticum monococcum* phosphatidylserine decarboxylase

Differentially expression of genes in mandibular gland from nurse and forager stages (Blastn) (continued)

Sequence no. of DD-PCR bands	***E*-value**	**DNA**
N106/sequence 1	0.0	(AY703484) *Apis mellifera* 18S ribosomal RNA gene
N106/sequence 2	0.005	(AF450251) *Oryza sativa* blast-resistant mRNA
N107/sequence 1	0.0	(XM_393453) *Apis mellifera* similar to ENSANGP00000015691, phosphoglycerate mutase family
N107/sequence 2	0.0	(XM_392469) *Apis mellifera* similar to CG5735-PB, RNA-recognition motif
N108/sequence 1	0.0	(XM_395948) *Apis mellifera* similar to 00000011664, Glutamate-cysteine ligase
N108/sequence 2	0.0	(AY703484) *Apis mellifera* 18S ribosomal RNA gene
N118/sequence 1	5e-06	(AF026945) *Homo sapiens* Cig64 mRNA
N118/sequence 2	0.0	(XM_395162) *Apis mellifera* similar to potassium channel modulatory factor1
N126/sequence 1	0.0	(XM_396647) *Apis mellifera* similar to G protein-coupled receptor kinase type-2
N126/sequence 2	e-123	(XM_395871) *Apis mellifera* similar to protein expressed in T-cells and eosinophils
N127/sequence 1	e-173	(XM_392236) *Apis mellifera* inositol 1,4,5-triphosphate receptor (ipr1)
N127/sequence 2	0.0	(XM_394406) *Apis mellifera* similar to ENSANGP00000010230, Gamma-glutamyltranspeptidase
N128/sequence 1	0.0	(XM_397263) *Apis mellifera* similar to zinc finger protein 39
N128/sequence 2	0.004	(AF100330) *Dendrobium* grex Madame Thong-1N putative copper/zinc superoxide dismutase copper chaperone (ovg 23)
N139/sequence 1	e-174	(XM_392675) *Apis mellifera* similar to ENSANGP00000015140, Apolipophorin III
N139/sequence 2	0.0	(XM_392741) *Apis mellifera* similar to ENSANGP00000009256, lipid binding protein

Differentially expression of genes in mandibular gland from nurse and forager stages (Blastn) (continued)

Sequence no. of DD-PCR bands	***E*-value**	**DNA**
N140/sequence 1	0.0	(U00096) *Escherichia coli* K-12 MG 1665
N140/sequence 2	0.0	(XM_394657) *Apis mellifera* similar to Ubiquinol-cytochrome C reductase
N141/sequence 1	0.007	(AF100330) *Dendrobium* grex Madame Thong-1N putative copper/zinc superoxide dismutase copper chaperone (ovg 23)
N141/sequence 2	0.0	(XM_392193) *Apis mellifera* similar to ENSANGP00000010075, Transketolase C; pyridine binding domain
N142/sequence 1	3e-05	(AF026945) *Homo sapiens* Cig64 mRNA
N142/sequence 2	1e-44	(AL591983) *Listeria monocytogenes* strain EGD segment 11/12
N143/sequence 1	1.9	(AC123044) *Mus musculus* BAC clone RP24-548B7 from chromosome5
N143/sequence 2	0.007	(AE100330) *Dendrobium* grex Madame Thong-1N putative copper/zinc superoxide dismutase copper chaperone (ovg 23)
N144/sequence 1	5e-07	(BA000028) *Oceanbacillus iheyensis* HTE831 DNA
N144/sequence 2	4e-04	(AC020906) *Homo sapiens* chromosome 19 clone CTB-83J15
N146/sequence 1	0.0	(XM_396062) *Apis mellifera* similar to CG2247-PA
N146/sequence 2	0.0	(XM_395712) *Apis mellifera* similar to ENSANGP00000015136, short chain alcohol dehydrogenase
N147/sequence 1	2e-05	(AF026945) *Homo sapiens* Cig64 mRNA
N147/sequence 2	e-111	(AY251269) Varroa destructor virus 1

APPENDIX F

Differentially expression DD-PCR bands in mandibular gland from nurse and forager stages (tBlastx)

DD-PCR bands	E-value	genes
F2	4e-53	(AC125396) Kakugo virus genomic RNA
F6	2e-28	(AC125396) Kakugo virus genomic RNA
F9	4e-77	(AY508731) Cloning vector psilentGene Hygromycin
F39	3e-37	(AC125396) Kakugo virus genomic RNA
N5	0.18	(AY550116) *Megachile rotundata* 70 kDa heat shock cognate protein (HSC70) mRNA
N12	1e-45	XM_396268*Apis mellifera* similar to ENSANGP00000016695, Thioesterase domain
N13	3e-98	(XM_392639) *Apis mellifera* similar to ENSANGP00000009989, ATP synthase
N14	3e-89	XM_392639 *Apis mellifera* similar to ENSANGP00000009989, ATP synthase
N20	e-131	(XM_392962) *Apis mellifera* similar to putative activated protein kinase C receptor
N36	5e-59	(AY392758) *Apis cerana* major royal jelly protein MRJP2 mRNA
N53	7e-62	(XM_396268) *Apis mellifera* similar to ENSANGP00000016695, Thioesterase domain
N54	7e-62	(XM_396268) *Apis mellifera* similar to ENSANGP00000016695, Thioesterase domain
N60	3e-88	(XM_396268) *Apis mellifera* similar to ENSANGP00000016695, Thioesterase domain
N72	e-142	(AF525776) *Apis cerana* major royal jelly protein MRJP1 mRNA
N77	3e-89	(XM_394434) *Apis mellifera* similar to CG1782-PA, Ubiquitin activating protein
N78	1e-88	(XM_394434) *Apis mellifera* similar to CG1782-PA, Ubiquitin activating protein
N81	4e-60	(XM_392035) *Apis mellifera* similar to ENSANGP00000020019, Peptidase family M16

Differentially expression DD-PCR bands in mandibular gland from nurse and forager stages (tBlastx) (Continued)

DD-PCR bands	E-value	genes
N91	2e-46	(AB121788) *Myrmecia croslandi* gene for 28S rRNA
N109	3e-95	(AY703484) *Apis mellifera* 18S ribosomal RNA gene
N119	5e-81	(AY703484) *Apis mellifera* 18S ribosomal RNA gene
N120	4e-74	(AF525776) *Apis cerana* major royal jelly protein MRJP1 mRNA
N124	e-137	(AF525776) *Apis cerana* major royal jelly protein MRJP1 mRNA
N125	3e-67	(XM_397263) *Apis mellifera* similar to Zinc finger protein 39
N135	e-131	(AY703484) *Apis mellifera* 18S ribosomal RNA gene
N136	e-124	(AY703484) *Apis mellifera* 18S ribosomal RNA gene
N145	2e-76	(XM_393410) *Apis mellifera* similar to high-affinity Na^{+}-dependent glutamate transporter

BIOGRAPHY

Mr. Puttarat Saechana was born on March 14, 1979. He graduated with Bachelor degree of Science in Biochemistry from KhonKaen University in 2000. He has studies his Master's degree at the department of Biochemistry, Faculty of science, Chulalongkorn University.