# Identification of differentially regulated genes in blood-fed *Anopheles sinensis*

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# Identification of differentially regulated genes in blood-fed *Anopheles sinensis*

Directed by Professor Bang-Bu Youn

The Doctoral Dissertation submitted to the Department of Medicine, the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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December 2007

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The Graduate School
Yonsei University

December 2007

**ACKNOWLEDGEMENTS**

I appreciate much help from all of you for me during this time from the bottom of my heart. First of all, thank professor Bang-Bo Youn very much. You have thought me how to think and investigate as a scientist. I appreciate your teaching from the depth of my heart. And thank professor Tai-Soon Yong for your great concerns and encouragements though you are far away.

I appreciate great deal of encouragements and pieces of advice for the better investigation from professor Kyung-il Im, professor Won-Young Lee and professor Duk-Chul Lee. Thank Mr. Jongweon Lee for many pieces of advice and much help. I appreciate to Sir Yong Ki Lee who is my master and superior.

The last, I appreciate to my parent, my father-in-law, my mother-in-low, my wife and my lovely two daughters who encouraged me. I appreciate to God who blesses me.

**TABLE OF CONTENTS**

## LIST OF FIGURES



## LIST OF TABLES

ABSTRACT

# Identification of differentially regulated genes in blood-fed *Anopheles sinensis*

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(Directed by Professor Bang-Bu Youn)

Blood feeding, or hematophagy, is a behavior exhibited by female mosquitoes required both for reproduction and for transmission of pathogens. Identification of the genes that are differentially expressed between blood-fed mosquitoes and sugar-fed mosquitoes is very important for understanding the molecular basis of malaria vector mosquitoes. The patterns of differentially expressed gene (DEG) analyzed by annealing control primer (ACP)-based PCR and cDNA microarray in adult female *Anopheles sinensis* at a day following completion of the blood-fed and compared their expression to transcript levels in sugar-fed mosquitoes. Using the technique of ACP-based PCR, we isolated 68 differentially expressed genes (DEGs) that are expressed in blood-fed mosquitoes at least three-fold above as compared to sugar-fed mosquitoes. Thirty-four from 68 DEGs were cloned and sequenced. Twenty-six DEGs showed homology to genes of *An. gambiae* and *Aedes aegypti*. Six DEGs (1, 2, 2-3, 4, 5 and 110-3) encoded an odorant-binding protein. Interestingly, DEG 3 and DEG 110-2 were found to encode a chitin-binding peritrophin A and peroxidase, respectively. These proteins were believed to play a role in innate immune response of the mosquito vector. Other DEGs match with various proteins, including $Cu^{+2}$/$Zn^{+2}$

superoxide dismutase, cathepsin b, DnaJ, serine/threonine protein kinases, serine hydroxymethyltransferase, aryldialkylphosphatase, ADP/ATP carrier protein 2, DNA polymerase III gamma/tau subunits and vasa-like protein. Then, we determined the expression patterns of 8,467 gene transcripts by cDNA microarrays prepared using the *Anopheles gambiae* genome data (2002) in blood-fed adult female *An. sinensis* and compared their expression to transcript levels in sugar-fed mosquitoes. In blood-fed mosquitoes, 139 cDNAs were expressed at least seven-fold above or below their levels in the sugar-fed mosquitoes. The 139 cDNAs were found to represent 131 unique mosquito transcripts. More unique transcripts are up-regulated than down-regulated in response to blood feeding: 95 are up-regulated at least sevenfold and 36 are down-regulated at least sevenfold. Differentially expressed gene products up-regulated in the blood-fed mosquitoes were involved in the digestion of blood (trypsin, chymotrypsin, serine protease, aminopeptidase), formation of peritrophic matrix (chitin-binding peritrophin A), chemosensory system (odorant-binding protein), embryogenesis and vitellogenesis (vitellogenin). Importantly, increased understanding of *An. sinensis* biology at the molecular level may open new avenues for intervention against malaria transmission.

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Key words : ACP-based PCR, *Anopheles sinensis*, blood feeding, differentially expressed gene, microarray, odorant-binding protein, peritrophin A

# Identification of differentially regulated genes in blood-fed *Anopheles sinensis*

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## I. Introduction

Malaria remains a common and devastating human disease, with over 300 million cases reported annually. The disease produces extensive morbidity and over one million deaths annually, resulting in enormous economic impact in endemic regions. Malaria is caused by several *Plasmodium* species that are transmitted to humans exclusively by mosquitoes of the *Anopheles* genus. The emergence of treatment-resistant strains of *Plasmodium* has refocused efforts on malaria prevention to control the disease. Therefore, understanding the molecular basis for human host recognition by *Anopheles* mosquitoes should drive us to devise new rational approaches to interfere with this process and thereby prevent malaria.

In the republic of Korea, vivax malaria had been eradicated in 1979. However, malaria re-emerged in 1993 and an outbreak occurred in northern parts of Kyonggi-do and Kangwon-do. *Anopheles sinensis* was confirmed as the main vector species in Korea[1,2,3]. In order to understand the epidemiological features of vivax malaria in the Korea, taxonomical studies on the *An. sinensis* complex

(*An. hyrcanus* group) are required, as several vector species are included in the complex. The Hyrcanus group of the genus *Anopheles* (Diptera: Culicidae) consists of 18 related species[4,5], of which five species, *An. sinensis, An. lesteri, An. pullus, An. yatsushiroensis* and *An. sineroides* are found in Korea[6,7]. Species identification of malaria vectors is a prerequisite step with a vital importance for determination of the differentially expressed genes in vector mosquitoes.

Blood feeding is a behavior exhibited by female mosquitoes required both for reproduction and for transmission of pathogens[8]. Blood feeding initiates a complex series of physiological events. These events may be required for parasite development; they certainly can be modulated by the presence of parasites[9,10] and may provide points of intervention for mosquito control. By comparing the level of transcription of a gene over time between two states, *e.g.* blood-fed *vs*. sugar-fed, an expression signature for each gene can be defined in response to blood feeding. Feeding behavior was compared between infected and uninfected field-collected groups of *Anopheles gambiae* and *An. funestus* from western Kenya[11]. A significantly greater percentage (81%) of *Plasmodium falciparum*-infected *An. gambiae* females probed on experimental hosts (hamsters) than did uninfected females (38%). This finding provides evidence that natural malaria infection modifies the feeding behavior of *Anopheles* females. Also, there was published evidence that documented changes in feeding behaviour associated with malaria infection also contribute to higher mortality of infected mosquitoes relative to uninfected individuals. In a natural situation, infection by the sporozoites of the malaria parasite *P. falciparum* significantly reduced survival of blood-feeding *An. gambiae*, the major vector of malaria in sub-Saharan Africa. The increase in mortality was probably due to decreased efficiency in obtaining blood and by increased feeding activity of the sporozoite-infected mosquitoes that elicited a greater degree of defensive behaviour of hosts under attack[12]. However, no data as above was available using *P. vivax* so far, since *P. vivax* is not possible to culture in the laboratory at

all not like *P. falciparum.*

The study of gene expressions among the mosquitoes that blood-fed and sugar-fed can be very important because it will provide a broadened basis for understanding vector-parasite interactions. Therefore, differentially expressed genes were screened in female mosquitoes at 24 hours after a blood-fed using ACP-based PCR and microarray. This study certainly provides insights into the physiology of the malaria vector *An. sinensis.* Understanding of *An. sinensis* biology at the molecular level may open new avenues for intervention against malaria transmission regulation eventually.

## II. MATERIALS AND METHODS

### 1. Trapping and rearing of Mosquitoes

Female mosquitoes of *Anopheles* complex were caught with light traps (Fig. 1) in Paju, Gyunggi-Do (37° 71° of north and 126° 93° of east) in 2004. *An. sinensis* among four *Anopheles* species was determined using PCR method[13]. These field-collected mosquitoes were used for blood-fed mosquitoes in the following experiment. Each of the blood-fed *An. sinensis* was kept in separate cup, in which water was provided for laying eggs. Each batch of the eggs from a female was reared in a separate pan in insectarium (temperature of 26$^{o}$C and 14 hours of light and 10 hours of dark condition). $F_1$ progenies of adults (Fig. 2) were obtained and used for molecular analysis.

Fig. 1. *Anopheles* mosquitoes were caught with a light trap in Paju, Gyunggi-do.

Fig. 2. An $F_1$ progeny of *An. sinensis* obtained in an insectarium.

**2. Preparation of total RNA**

Total RNA was purifeid from field collected blood-fed and laboratory reared sugar-fed *An. sinensis* using TRIZOL reagent (Gibco/BRL, Gaithersburg, MD) according to the manufacturer's instructions. The concentration and quality of each RNA sample was measured for the synthesis of high quality cDNA determined by spectrophotometry of a 260/280 ratio.

**3. First-strand cDNA Synthesis**

Total RNAs extracted from *An. sinensis* were used for the synthesis of first-strand cDNAs by reverse transcriptase. Reverse transcription was performed for 1.5 h at 42ºC in a final reaction volume of 20 $\mu\ell$ containing 3 $\mu g$ of the

purified total RNA, 4 ㎕ of 5X reaction buffer (Promega, Madison, WI, USA), 5 ㎕ of dNTPs (each 2 mM), 2 ㎕ of 10 μM dT-ACP1 (5'-CTGTGAATGCTGCGACTACGATIIIIIT(18)-3'), 0.5 ㎕ of RNasin® RNase Inhibitor (40 U/ ㎕; Promega), and 1 ㎕ of Moloney murine leukemia virus reverse transcriptase (200 U/ ㎕; Promega). First-strand cDNAs were diluted by addition of 80 ㎕ of ultra-purified water for the GeneFishing™ PCR, and stored at -20°C until use.

**4. ACP-based PCR**

Differentially expressed genes were screened by ACP-based PCR method[15] using the GeneFishing™ DEG kits (Seegene, Seoul, Korea). Briefly, second-strand cDNA synthesis was conducted at 50ºC during one cycle of first-stage PCR in a final reaction volume of 20 ㎕ containing 3-5 ㎕ (about 50 ng) of diluted first-strand cDNA, 1 ㎕ of dT-ACP2 (10 μM), 1 ㎕ of 10 μM arbitrary ACP, and 10 ㎕ of 2X Master Mix (Seegene). The PCR protocol for second-strand synthesis was one cycle at 94°C for 1 min, followed by 50°C for 3 min, and 72°C for 1 min. After second-strand DNA synthesis was completed, the second-stage PCR amplification protocol was 40 cycles of 94°C for 40 s, followed by 65°C for 40 s, 72°C for 40 s, followed by a final extension at 72°C for 5 min. The amplified PCR products were separated on 2% agarose gel stained with ethidium bromide.

**5. Cloning and Sequencing**

The differentially expressed genes were extracted from the gel by using the GENCLEAN® II Kit (Q-BIO gene, Carlsbad, CA, USA), and directly cloned into a TOPO TA cloning vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The cloned plasmids were sequenced with ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA)

using M13 forward primer (5'-CGCCAGGGTTTTCCCAGTCACGA-3') or M13 reverse primer (5'-AGCGGATAACAATTTCACACA GGA-3'). For data analysis, the nucleotide sequences of the selected cDNA clones were compared with EMBL and GenBank databases, and all six reading frames were also searched for homologous mosquito proteins and proteins of other organisms using BlastX.

**6. Northern blot analysis**

cDNA probes were labeled with $^{32}P$ using a Random labeling kit (Takara, Japan). For Northern hybridization, samples of total RNA (10 μg) were fractionated in 1% formaldehyde agarose gels, blotted on Hybond-N membranes by capillary transfer in 20X SSPE (3 M NaCl, 0.2 M $NaH_2PO_4$, 0.02 M EDTA) and immobilized on the membranes using an UV-crosslinker. Blots were prehybridized in 5X SSPE, 50% formamide, 5X Denhardt's solution, 0.5% SDS and 100 μg/ml salmon sperm DNA for 2 h at 42°C, and hybridization at 42°C was continued overnight in the presence of a probe, which had been labeled with $^{32}P$. Membranes were washed twice in 2X SSPE/0.1% SDS at room temperature for 15 min, and then twice at 60°C for 30 min in 0.2X SSPE/0.5% SDS. Blots were hybridized with a labeled β-actin DNA fragment as an internal RNA control.

**7. Real-time PCR**

Total RNAs isolated from blood-fed and sugar-fed mosquitoes were processed by reverse-transcription. Real-time PCR was performed in ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) by using SYBR Green PCR Master Mix (Applied Biosystems) with three-stage program parameters provided by the manufacturer, as follows: 2 min at 50 °C to require optimal AmpErase uracil-N-glycosylase activity, 10 min at 95 °C to

activate AmpliTaq Gold DNA polymerase, and then each cycle 15 s at 95 °C, 1 min at 60 °C for 50 cycles, 2 min at 50 °C, 10 min at 95 °C, and then 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Table 1 lists primers specific for the genes examined in the present study. Each sample was tested in triplicate and data obtained from three independent experiments were expressed as a subtraction of the quantity of specific transcripts to the quantity of the control gene (β-actin) in mean arbitrary units.

Table 1. Primer sequences of genes applied in real-time RT-PCR

| DEGs | Sense | Anti-sense | Size |
|---|---|---|---|
| **β-actin** | 5′-CCATCATGAAGTGTGACGTTG | 5′-CAATGATCTTGATCTTCATGGTG | 150bp |
| **DEG 1** | 5′-CTTCGACCTGTTCGACATGCT | 5′-CTGCAACAAGAGCAAGTGCGA | 141bp |
| **DEG 2** | 5′-AGCTCGCCGGACGCTGCTT | 5′-TGAAGAACCTGTACACCCAG | 121bp |
| **DEG 3** | 5′-GTGGATAATCCGTGCAAAGCT | 5′-CACTCTGGCGAGAGTCAGTGTT | 148bp |
| **DEG 4** | 5′-CTGCAGCAGTCGTACGACCTGT | 5′-ACTTCGTGACCCGCTCCAAG | 125bp |
| **DEG 5** | 5′-GATACTCGTTCGATCTGTGCA | 5′-GAATGGACTGATGCCCAT | 114bp |
| **DEG 6** | 5′-GTTCGAGGAATGTATTCA | 5′-GTTCGAGGAATGTATTCA | 139bp |

## 8. Microarray chip fabrication

cDNA microarray chip of 8,467 gene transcripts prepared using the *Anopheles gambiae* genome data (2002). The cDNAs were constructed using whole body of adult female *An. sinensis* which had been sugar-fed and blood-fed. Clones were subjected to PCR-based insert amplification using λTriplEx2 vector specific primers (3' LD Amplimer Primer 5'-ATACGACTCACTATAGGGCGAATTGGC-3'; 5' LD Amplimer Primer: 5'-

CTCGGGAAGCGCGCCATTGTGTTGG-3'). Amplification reactions contained 1.0 µL eluted phage, 0.03 pmol of each primer, 1X Taq polymerase buffer (Invitrogen), 3 mM $MgCl_2$, 1 mM of each dNTP, and 0.2 U Taq polymerase (Invitrogen), in a total volume of 100 µL. Reactions were conducted in 96-well plates on a Perkin-Elmer 9700 Thermocycler using the following cycling conditions: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing/elongation at 70°C for 2 min, and a final elongation step at 68°C for 3 min. PCR products were purified on a Beckman Biomek FX using Montage PCR 96 Cleanup kits (Millipore), eluted in 100 µL of water, evaporated overnight and the pellets resuspended in 30 µL of 3 × SSC microarray spotting buffer. A total of 8,467 resuspended cDNA inserts and 108 controls were spotted in triplicate on CMT-Gaps II slides (Corning, NY, USA) using the Affymetrix Arrayer 417 at 19 – 20°C with a relative humidity between 50 – 60%. Slides were post-processed by baking at 80°C for three hours, incubation in 1% SDS for 2 min, in 95°C purified water for a further 2 min, and then plunged 20 times into 100% ethanol kept at -20°C and air-dried via centrifugation at 500 RPM for 5 min.

**9. Microarray target preparation and hybridization**

First strand cDNA synthesis and labeling with Cyanine 3 (Cy3) or Cyanine 5 (Cy5), were performed on 15 µg of total RNA from each sample using the Genisphere 3DNA Array 50 kit according to the manufacturer's protocol. Hybridizations were conducted following the two step protocol recommended by the manufacturer: 1) cDNA hybridization to the amplified cDNA probes spotted on the slides, 2) hybridization of 3-DNA fluorescent dendrimers (Genisphere) to cDNAs via the capture sequences incorporated into them during first strand synthesis. All cDNA and fluorescent dye hybridizations were performed in a volume of 50 µL using the formamide based hybridization buffer provided by the manufacturer. The cDNA hybridizations were performed

at 45°C overnight. The slides were then washed according to the 3DNA Array 50 kit protocol and air dried by centrifugation for 3 min at 800 RPM. The 3-DNA hybridizations were performed at 53°C for 2 hours as described above, except that 0.5 mM DTT was added to the first two wash solutions to protect the fluorochromes from oxidation. These included two dye-swap experiments performed to eliminate dye fluorescence bias.

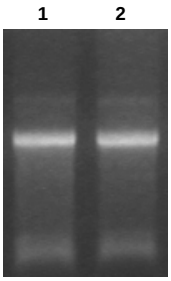
**10. Microarray data acquisition and statistical analysis**

Following hybridization and washing, microarray slides were scanned successively at 532 and 635 nm using the Affymetrix 428 Array Scanner. Raw signal intensities were acquired using the adaptive circle algorithm and spot intensities quantified using the Jaguar 2.0 segmentation and data analysis software (Affymetrix, CA, USA).

## III. RESULTS

### 1. Preparation of total RNA

The concentration and quality of each RNA sample was measured for the synthesis of high quality cDNA determined by spectrophotometry of a 260/280 ratio (Fig. 3). The concentration of each RNA sample was 0.49 μg/μl and 0.52 μg/μl from one blood-fed and one sugar-fed mosquito, respectively. Total RNA was treated with DNase I at 37°C for 15 min, at 65°C for 10 min, to get rid of genomic DNA contamination.




- OD determination
- Electrophoresis of 1.5 ug total RNA

| Sample | A260 | A260/A280 | Conc. (ug/ul) |
|---|---|---|---|
| 1. Blood-fed | 12.310 | 1.99 | 0.492 |
| 2. Sugar-fed | 12.962 | 1.99 | 0.518 |

Fig. 3. RNA quality check by electrophoresis and optical density (OD) read at 260/280nm.

### 2. ACP-based PCR

To determine the gene expression patterns, differentially expressed gene (DEG) were screened by ACP-based PCR using the GeneFishing™ kits (Seegene) in adult female *An. sinensis* after a day following blood meal by comparison of their expression with transcript levels in sugar-fed mosquitoes (Fig.4). Total 68 DEGs were identified at least three-fold above their levels in the sugar-fed

mosquitoes.

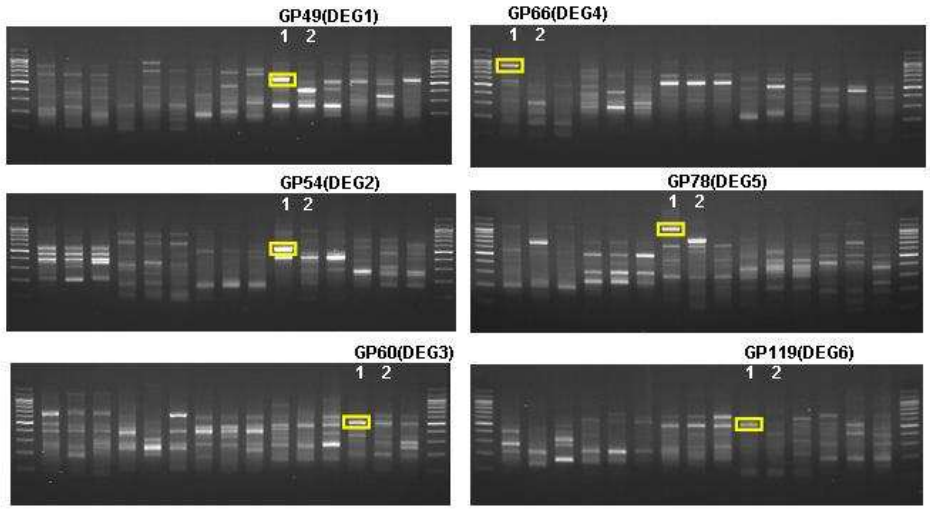



Fig. 4. To determine the gene expression patterns, differentially expressed genes (DEGs) were screened by ACP-based PCR using the GeneFishing$^{TM}$ kits in adult female *Anopheles sinensis* after one day following blood-fed (Lane 1), and compared their expression to transcript levels in sugar-fed mosquitoes (Lane 2). GP: GeneFishing Primer. The boxes indicate DEGs.

### 3. Cloning and Sequencing

The DEGs were cloned into TOPO TA cloning vectors. Thirty-four DEGs were cloned and sequenced. The nucleotide sequences of the selected DEGs were compared with EMBL and GenBank databases. Results of the sequence analysis are summarized in Table 2. The most frequently identified DEGs were genes for odorant-binding proteins. Six DEGs (1, 2, 2-3, 4, 5 and 110-3) were found to encode an odorant-binding protein, which is a mosquito chemosensory system

protein and is potential targets for novel inhibitors that could be applied to the skin to disguise human hosts. Interestingly, DEG 3 was found to encode a chitin-binding peritrophin A earlier identified to play a role in innate immune response of the mosquito vector in *An. gambiae* upon infection by the malaria parasite[14]. Also, DEG 110-2 showed homology to an *An. gambiae* peroxidase, which is also known to be a protein that may be upregulated to protect the tsetse fly against reactive oxygen species which are generated during the tsetse immune response against trypanosomes.

Three of the cloned DEGs, 2-2, 2-4 and 20-1, were found to encode DnaJ. DnaJ domains (J-domains) are associated with hsp70. DEG 6-1 and 19-4 showed homology to a mitochondrial metabolic enzyme, cytochrome c oxidase subunit IV. DEG 99-2 and 113-4 was found to be homologous to the $Cu^{+2}/Zn^{+2}$ superoxide dismutase and cathepsin b, respectively. DEGs 1-1, 1-2 and 3-2, 3-4 matched ribosomal protein S4E; it is known to be a stress proteins and ribosome biogenesis protein. One DEG 120-1 showed homology to a gene encoding 45 kDa immunophilin FKBP45 of *Bombyx mori*, which is known as an immunosuppressive agent. Remaining six DEGs were a group of genes involved in metabolism, including serine/threonine protein kinases, serine hydroxymethyltransferase, aryldialkylphosphatase, ADP/ATP carrier protein 2, DNA polymerase III gamma/tau subunits and vasa-like protein. BlastX searches for 5 DEGs (9-4, 56-1, 56-2, 80-3 and 110-1) encoded a hypothetical conserved protein in *An. gambiae, Ae. aegypti* and *C. elegans*. The Blast searches with the sequence information of three DEGs, 6, 8-2 and 8-3, did not produce any meaningful results.

Table 2. DEGs in blood-fed *An. sinensis* identified by ACP-based PCR

| DEG | Homologue | Organism | E-value |
|---|---|---|---|
| 3-2<br>3-4 | ribosome biogenesis protein tsr1 (EAT44108) | *Aedes aegypti* | 3e-32 |
| 20-1<br>2-2<br>2-4 | DnaJ (XP 311513) | *Anopheles gambiae* | 1e-11 |
| 1<br>2<br>4<br>5<br>2-3<br>110-3 | odorant-binding protein (AAO12071) | *Anopheles gambiae* | 7e-05 |
| 99-2 | Cu+2/Zn+2 superoxide dismutase (ABF18094) | *Aedes aegypti* | 3e-37 |
| 113-1 | vasa-like protein (AAY41942) | *Anopheles gambiae* | 1e-34 |
| 113-3 | Cathepsin b (EAT40702) | *Aedes aegypti* | 2e-12 |
| 1-1<br>1-2 | Ribosomal protein S4E (XP 308886) | *Anopheles gambiae* | 2e-29 |
| 2-1 | DNA polymerase III, gamma/tau subunits (NP 299094) | *Xylella fastidiosa* | 1.3 |
| 3-1 | ADP,ATP carrier protein 2 (ADP/ATP translocase 2) (Q7PQV7) | *Anopheles gambiae* | 6e-51 |
| 6-1<br>19-4 | Cytochrome c oxidase subunit IV (XP 314839) | *Anopheles gambiae* | 2e-41 |
| 6-3 | Aryldialkylphosphatase (YP 001068518) | *Mycobacterium* sp. | 3.1 |
| 89-1 | Serine hydroxymethyltransferase (EAA13500) | *Aedes aegypti* | 5e-65 |
| 8-1 | Serine/Threonine protein kinases (EAU88898) | *Coprinopsis cinerea* | 6.4 |
| 110-2 | peroxidase (XP563016) | *Anopheles gambiae* | 1e-45 |
| 120-1 | 45kDa immunophilin FKBP45 (AAY80706) | *Bombyx mori* | 7e-39 |
| 3 | Chitin binding peritrophin A (AAR02439) | *Anopheles gambiae* | 2e-25 |
| 110-1 | Hypothetical protein K02A2.6. (Q09575) | *Caenorhabditis elegans* | 6.7 |
| 9-4 | Uncharacterized protein (XP307850) | *Anopheles gambiae* | 0.42 |
| 56-1 | hypothetical protein (EAA05981) | *Aedes aegypti* | 1e-40 |
| 56-2 | hypothetical protein (XP564794) | *Anopheles gambiae* | 7e-15 |
| 80-3 | hypothetical protein (EAT48189) | *Aedes aegypti* | 9e-12 |
| 6<br>8-2<br>8-3 | No significant similarity found | - | - |

**4. Northern blot analysis**

Northern blot analysis also showed increased expressions of DEGs 1~6 in the blood-fed mosquitoes, ranging from 3 to 62 times (Fig. 5).

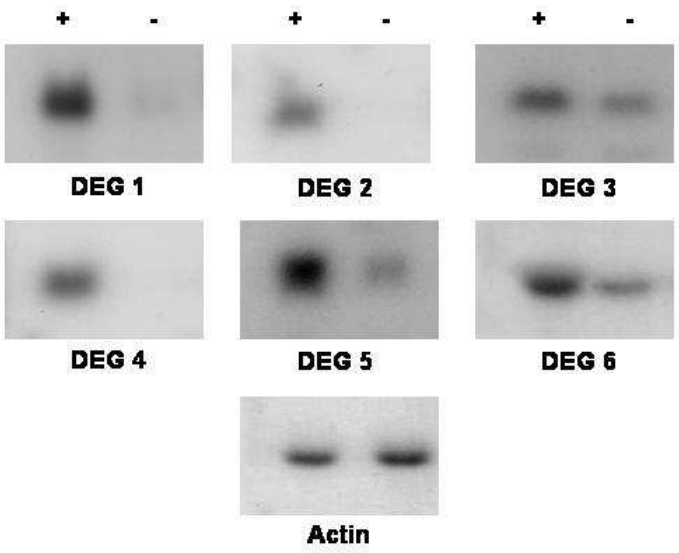



Fig. 5. Northern blot analysis of *An. sinensis* total RNA obtained from blood-fed (+) and sugar-fed (-). The same membrane was stripped and re-hybridized with a non-regulated probe encoding β-actin as an RNA loading control.

**5. Real-time PCR**

To verify DEGs observed in ACP-based PCR, we performed real-time PCR analysis for selective DEGs (Table 1) in blood-fed mosquitoes. Expression profiles of six selected genes and the β-actin control gene were re-confirmed using a quantitative real-time PCR strategy (Fig. 6). Transcript levels for each

of the six genes were quantified using SYBR Green technology and differences in their expression between sugar-fed and blood-fed mosquitoes at 24 hours. Although the magnitudes of the changes in transcript abundances of all the genes whose expression levels were quantified differed between the techniques, the changes in direction of expression, whether positive or negative, remained consistent for the majority of them.

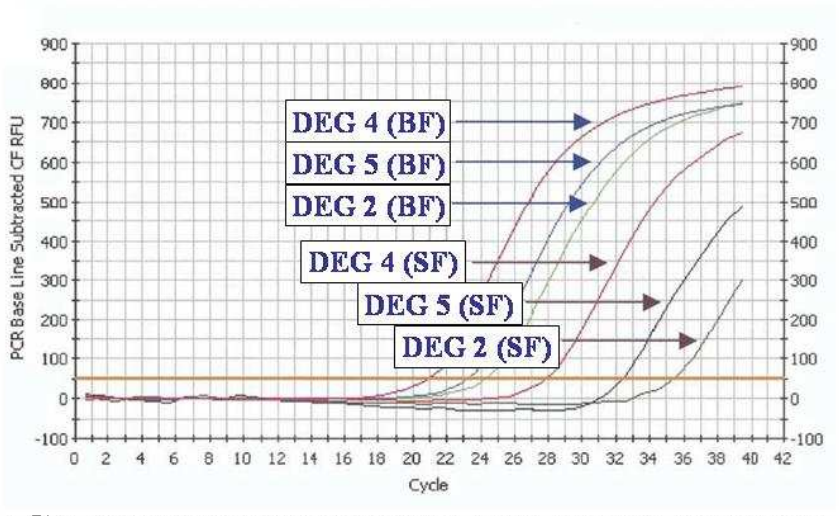



Fig. 6. Quantitative real-time PCR analysis showing upregulated mRNA levels of selective DEGs (DEG 2, 4 and 5) which were identified on the ACP-PCR in blood-fed mosquitoes. BF: Blood-fed, SF: Sugar-fed.

**6. Microarray**

DEGs of greater than seven-fold up-regulation or down-regulation were identified by comparing transcript levels beqween blood-fed *An. sinensis* adult female mosquitoes and sugar-fed ones. One hundred thirty-nine cDNAs and the positive control were expressed more than seven-fold above or below the

control sugar-fed levels, which represented 131 unique transcripts. More transcripts were up-regulated than down-regulated in response to blood feeding: 95 were up-regulated at least sevenfold, and 36 were down-regulated at least sevenfold. Bioinformatic analyses of these 131 unique transcripts showed that most of the sequences shared sequence homology with *An. gambiae* genome, 90% of which shared sequence similarity with an entry in Nr of dbEST (Table 3 and 4). The prominent DEGs up-regulated in the blood-fed mosquitoes were involved in the digestion of blood (trypsin, chymotrypsin, serine protease, aminopeptidase), formation of peritrophic matrix (chitin-binding peritrophin A), chemosensory system (odorant-binding protein), embryogenesis and vitellogenesis (vitellogenin). Digestion of the two different food sources, blood and nectar sugars, requires changes in the types of enzymes present within the digestive tract of the mosquito. Considering that blood contains large quantities of protein, the mosquito requires a variety of proteolytic enzymes to digest the recently acquired meal. In the present study, 6 genes were identified whose products are most likely required for protein digestion. These include previously characterized digestive enzyme genes, two trypsins, two chymotrypsins, a serine protease and an aminopeptidase. The peritrophin gene would be transcribed in response to blood meal acquisition and their products would be used immediately in the formation of the peritrophic matrix. The microarray also identified the odorant-binding protein gene expression was increased in blood-fed mosquitoes as in ACP-based PCR. The majority of *An. sinensis* genes upregulated at least sevenfold following a blood meal appear to function in egg production.

Table 3. List of up-regulated genes in female *An. sinensis* at 24 hours post-blood meal by microarray gene expression studies.

| Spot ID | Microarray | Gene Product |
|---|---|---|
| Asi.30996 | Up | Actin 4 |
| Asi.23845 | Up | Adenylosuccinate lyase-like protein |
| Asi.1852 | Up | ADP/ATP translocase |
| Asi.1760 | Up | ATP synthase beta chain |
| Asi.39252 | Up | ATP synthase gamma chain |
| Asi.97, Asi.34323 | Up | beta-arrestin |
| Asi.28295 | Up | beta-galactosidase |
| Asi.23911 | Up | Cathepsin B precursor |
| Asi.69, Asi.18643 | Up | Chymotrypsin |
| Asi.2260 | Up | Cyclin B |
| Asi.1583 | Up | Cytosolic aminopeptidase |
| Asi.47641 | Up | Elongation factor 1-alpha |
| Asi.2229 | Up | Elongation factor 2 |
| Asi.18046 | Up | Enhancer of Delta KP135 |
| Asi.24528 | Up | Enolase |
| Asi.2726 | Up | Eukaryotic initiation factor 4A |
| Asi.23825 | Up | Extracellular matrix protein 1 (ECM1) |
| Asi.1597 | Up | Ferritin 1 heavy chain |
| Asi.1755 | Up | Ferritin 2 light chain |
| Asi.2176 | Up | Fructose-bisphosphate aldolase |
| Asi.1751 | Up | Gelsolin |
| Asi.24242 | Up | Glyceraldehyde-3-phosphate dehydrogenase |
| Asi.2193 | Up | Guanine nucleotide-binding protein |
| Asi.72 | Up | Histone H2B |
| Asi.7390 | Up | Hypothetical protein 13 |
| Asi.255 | Up | Insect allergen related repeat protein |

| Spot ID | Microarray | Gene Product |
|---|---|---|
| Asi.102 | Up | Iron-responsive element-binding like protein |
| Asi.866 | Up | Laminin-receptor |
| Asi.3665 | Up | Lysophospholipase_L1 |
| Asi.47627 | Up | Mucin |
| Asi.764 | Up | Nucleoside Diphosphate Kinase |
| Asi. 3854,Asi. 6132, Asi. 6182 | Up | Odorant-binding protein |
| Asi.39176 | Up | Opsin Rh6 |
| Asi.1337 | Up | Ornithine decarboxylase antizyme |
| Asi.3257 | Up | Peritrophin A |
| Asi.34356 | Up | Peroxidase |
| Asi.20698 | Up | Peroxiredoxin |
| Asi.17154 | Up | Plasminogen |
| Asi.1141 | Up | Protein translation factor SUI1 |
| Asi.2115 | Up | Salivary glue protein SGS-3 precursor |
| Asi.2321 | Up | Serine protease 3 precursor |
| Asi.33374 | Up | Structure-specific recognition protein 1 |
| Asi.1732 | Up | Sugar transporter 4 |
| Asi.35326 | Up | TBP-associated factor |
| Asi.24240 | Up | TCTP (Translationally controlled tumor protein) |
| Asi.16548 | Up | Trypsin 1 |
| Asi.7037 | Up | Trypsin 2 |
| Asi.1940 | Up | Trypsin-like serine protease |
| Asi.1626 | Up | Tubulin alpha |
| Asi.39045 | Up | Tyrosine kinase |
| Asi.16939 | Up | ubiquinol-cytochrome C reductase |
| Asi.2336, Asi.34016, Asi.3156 | Up | Uncharacterized Protein |

| Spot ID | Microarray | Gene Product |
|---|---|---|
| Asi.22143, Asi.31977, Asi.34315, Asi.34346 | Up | Vitellogenin precursor |
| Asi.34335, Asi.27771, Asi.34331 | Up | Vitellogenin_N |
| Asi.2243 | Up | Voltage-dependent anion-selective channel protein |
| Asi.47733 | Up | zinc-finger protein |
| Etc | Up | Ribosomal protein L1, 2, 3, 5, 6, 7, 10, 11, 13, 18, 21, 23, 27, 44<br>Ribosomal protein S2, 3, 4, 5, 6, 7, 8, 9, 13, 14, 15, 20, 26, |

Table 4. List of down-regulated genes in female *An. sinensis* at 24 hours post-blood meal by microarray gene expression studies.

| Spot ID | Microarray | Gene Product |
|---|---|---|
| Asi.20145 | Down | 3-deoxyglucosone reductase |
| Asi.29127 | Down | Actin |
| Asi.29128 | Down | Actin 11 |
| Asi.24196 | Down | Actin 2 |
| Asi.40641 | Down | Actin 4 |
| Asi.24241 | Down | Adenosine nucleotide translocator |
| Asi.24165 | Down | ATP synthase |
| Asi.34122 | Down | ATP synthase alpha subunits |
| Asi.34241 | Down | Ca2+-transporting ATPase 2 |
| Asi.15731 | Down | Calcium-binding protein 2 |
| Asi.2725 | Down | Calcium-transporting ATPase 3 |
| Asi.35562 | Down | Calmodulin 1 |
| Asi.24258, Asi.30989, Asi.20266 | Down | Chitin_bind_4 |
| Asi.19165 | Down | Collagen |
| Asi.1264 | Down | Flightin |
| Asi.19888 | Down | Glutathione S-transferase |
| Asi.34433 | Down | Glycerol-3-phosphate dehydrogenase |
| Asi.1757 | Down | Mucin 2 precursor |
| Asi.16587 | Down | Myosin heavy chain 2 |
| Asi.34314 | Down | Myosin heavy chain 4 |
| Asi.34557 | Down | Myosin heavy chain B |
| Asi.34834 | Down | Myosin heavy chain C |
| Asi.24200 | Down | Myosin, essential light chain |
| Asi.25947, Asi.1393 | Down | Opsin Rh6 |
| Asi.21165 | Down | PDZ_signaling doman |

| Spot ID | Microarray | Gene Product |
|---|---|---|
| Asi.6705 | Down | Proline-rich protein |
| Asi.1576, Asi.28665 | Down | Protein kinase |
| Asi.30883 | Down | Retinin_C |
| Asi.16612 | Down | Tropomyosin 1 |
| Asi.37821 | Down | Tropomyosin 2 |
| Asi.1045 | Down | Troponin I |
| Asi.21306 | Down | Troponin T-like protein |
| Asi.24334 | Down | Trypsin 29F |
| Asi.16969 | Down | Ubiquitin |
| Asi.24412 | Down | Uncharacterized Protein |

## IV. DISCUSSION

The patterns of differentially expressed gene (DEG) analyzed by annealing control primer (ACP)-based PCR and cDNA microarray in adult female *An. sinensis* at the 24 hours following completion of the blood-fed and compared their expression to transcript levels in sugar-fed mosquitoes. We have identified 34 DEGs and 139 cDNAs as being induced following blood-feeding using the ACP-based PCR and microarray analysis, respectively. Limitations on this approach include the numbers of DEGs obtained from each technique, the limited number of time points or physiological states that can be examined, and the requirement that their mRNAs be of intermediate or high abundance in at least one of the conditions studied. Thus some, indeed, many blood-feeding and immune-responsive genes may not have been identified, simply because they are expressed at low levels despite the importance of their roles in these processes. In addition, transcript abundances do not always correlate highly with protein levels. Some mRNAs have high turnover rates while others may be stabilized yet not translated except under specific conditions.

Infection of the adult female anopheline mosquito with malaria parasites elicits both local and systemic responses from a range of vector organs and tissues. *Plasmodium* infection is also coincident with the ingestion of a blood meal which sets in motion a complex set of events including digestion and egg production[13]. The majority of *An. sinensis* genes upregulated at least sevenfold following a blood meal appear to function in egg production. Paramount to the development of the embryo is the massive accumulation of vitellogenin by the oocyte. Microarray analysis identified seven cDNAs, greater than sevenfold induced, as vitellogenin gene homologs. Following synthesis in the fat body, vitellogenins are released into the hemolymph. The increased number of gene products involved in receptor-mediated endocytosis before and after the height of vitellogenin gene transcription may reflect a preparation for the increase in receptor-mediated endocytosis when the oocytes are accumulating vitellogenins

and other yolk constituents during the trophic phase of the ovarian cycle. Digestion of the blood meal is required for oocyte development and vitellogenesis, and consequently these are coordinated processes. During the first several hours following a blood meal, the mosquito undergoes physiological changes in addition to hormonal ones. Acquisition of a blood meal stimulates midgut proteolytic activity such that approximately 80% of the protein content is digested within one day[14]. Serine proteases including trypsins and chymotrypsins are responsible for the majority of endoproteolytic activity[15]. The role of trypsins in blood digestion has been well documented in *Aedes aegypti*, and more recently it has been investigated in *An. gambiae*. Several types of trypsins are also found in *An. gambiae*. Trypsins 1 and 2 are both induced by a blood meal and exhibit similar expression profiles. In contrast to Trypsins 1 and 2, Trypsins 3, 4, and 7 are constitutively expressed in unfed females[16]. In addition to the trypsins, chymotrypsin genes have been isolated and characterized in *An. gambiae*. Chymotrypsins, AnChym 1 and 2, are expressed in the midgut by 12 hours post blood meal and their transcripts are abundant until 48 hours, as determined by PCR, unlike the levels of trypsins 1 and 2 that have decreased dramatically by this time[17]. In contrast, the other characterized chymotrypsin, AgChyL, exhibits transcript level changes more similar to those of Trypsins 3–7[18]. Multiple aminopeptidases have been isolated from hematophagous insects, and it has been suggested that they may play different roles in digestion[19-24]. *An. gambiae* aminopeptidase N (AgAPN1) as the predominant jacalin (a lectin) target on the mosquito midgut luminal surface and there is an evidence for its role in ookinete invasion. Anti-AgAPN1 IgG strongly inhibited both *P. berghei* and *P. falciparum* development in different mosquito species, implying that AgAPN1 has a conserved role in ookinete invasion of the midgut[25]. In addition to dramatic changes in physiology, blood feeding also induces changes in mosquito morphology. Following gut distension by blood ingestion, midgut epithelial cells secrete a peritrophic matrix (PM) that is continuous along the length of the midgut[17,26]. In *An.*

*gambiae*, the PM visualized by electron microscopy as early as 12 hours post-blood meal is fully formed by 48 hours post-blood meal[27]. The exact functions of the PM remain unknown, but it has been suggested that this semi-permeable porous structure may function as a restrictive layer protecting the midgut epithelium from proteolytic digestive enzymes, from haematin crystals that form following hemoglobin breakdown and as a barrier to blood-borne pathogens including bacteria and malaria parasites[28]. The PM is a biochemically complex structure containing not only chitin and other proteoglycans, but as many as 20–40 different proteins[29]. However, only one gene encoding a peritrophic matrix protein (chitin binding peritrophin A) has been cloned in *An. gambiae*[30] so far. Interestingly, DEG 3 (Table 2) and Asi.3257 (Table 3) were found to encode a chitin binding peritrophin A, earlier identified to play a role in innate immune response of the mosquito vector, *An. gambiae*, upon infection by the malaria parasite[31]. Transsmission of malaria parasites occurs by relatively few species of mosquitoes. One proposed mechanism of refractoriness is an inability of certain *Plasmodium* spp. to cross the peritrophic matrix in midgut of an incompatible mosquito. Peritrophin A containing chitin-binding domain which may has an adhesive function and play a role in immunity and may play a defensive barrier role in the midgut[32]. One of the prominent genes was found to encode an odorant-binding protein (DEG 1, 2, 2-3, 4, 5, 110-3 and Asi. 3854, 6132, 6182). These proteins are the most abundantly expressed in olfactory tissues[33], and they have been postulated to either act as odorant carriers and/or mediate the catalytic removal of odorants from the lymph. Olfaction principally mediates host preference that in turn significantly influences the ability of anopheline and other mosquitoes to act as vectors for pathogens responsible for malaria or other serious human diseases such as dengue fever and West Nile encephalitis[34]. A total of 79 candidate odorant binding proteins (AgORs) have been identified[35] so far. Comparative genomics showed that the AgOR family of mosquitoes is rapidly expanding compared with the odorant receptors of *Drosophila*. Subsequent studies utilizing the genome data currently being

generated for several vector and non-vector insects are helping to elucidate the role of the AgORs in shaping species-specific chemosensory processes that are likely to have evolved in the extremely anthropophilic *An. gambiae* mosquitoes. The gene families implicated in olfactory processes are regarded as promising novel targets for the design of novel mosquito attractants and/or repellents, and for the development of other pharmacological applications for mosquito control[36]. DEG 110-2 and Asi.34356 showed homology to an *An. gambiae* peroxidase. In tsetse flies, comparing midguts from tsetse which had infected or self-cleared trypanosome infections to equivalent non-trypanosome challenged midguts, two peroxidase homologs are upregulated in both self-cleared and infected flies. It is known that trypanosomes are particularly susceptible to ROS[37] and it is an interesting speculation that these genes may be upregulated to protect the fly against ROS which are generated during the tsetse immune response against trypanosomes.

The identification of 34 DEGs and 139 cDNAs were the first step towards determining their biological roles in this medically important *An. sinensis*.

## V. CONCLUSION

In this study, we identified 34 DEGs and 131 genes as being up or down regulated following blood feeding of *An. sinensis* relative to their expression levels in sugar fed females using ACP-based PCR and a microarray, respectively. Differentially expressed gene (DEG) were screened by ACP-based PCR using the GeneFishing$^{TM}$ kits (Seegene) in adult female *An. sinensis* after a day following blood meal by comparison of their expression with transcript levels in sugar-fed mosquitoes. The most frequently identified DEGs were genes for odorant-binding proteins, which is a mosquito chemosensory system protein and is potential targets for novel inhibitors that could be applied to the skin to disguise human hosts. Interestingly, DEG 3 was found to encode a chitin-binding peritrophin A earlier identified to play a role in innate immune response of the mosquito vector in *An. gambiae* upon infection by the malaria parasite. Results of microarray gene expression studies, the prominent DEGs up-regulated in the blood-fed mosquitoes were involved in the digestion of blood (trypsin, chymotrypsin, serine protease, aminopeptidase), formation of peritrophic matrix (chitin-binding peritrophin A), chemosensory system (odorant-binding protein), embryogenesis and vitellogenesis (vitellogenin). The microarray also identified the odorant-binding protein gene expression was increased in blood-fed mosquitoes as in ACP-based PCR. Importantly, increased understanding of *An. sinensis* biology at the molecular level may open new avenues for intervention against malaria transmission.

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## 국문요약


흡혈 중국얼룩날개모기에서 발현 조절되는 유전자의 동정

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모기의 흡혈은 생식 및 말라리아와 같은 병원체의 전달을 위해 꼭 필요한 행위이며, 흡혈 시 모기체내에서 복잡한 분자적 변화가 시작된다. 이런 흡혈에 따른 많은 분자적 변화들은 모기나 이를 통해 전파되는 말라리아를 포함하는 모기매개 기생충들에게 중요한 과정이며, 이런 분자적 변화의 양상을 연구하는 것은 매우 중요한 일이다. 따라서 한국의 매개모기인 중국얼룩날개모기를 재료로 모기의 흡혈 여부로 달리 발현되는 유전자를 규명하고자 하였다. 흡혈 후 24시간 지난 흡혈모기와 당섭취 모기의 total RNA를 재료로 ACP (Annealing Control Primer)-based PCR과 Microarray 분석을 수행하였다. ACP-based PCR 결과, 흡혈 모기에서만 유전자 발현이 3배 이상 증가되는 DEG (Differentially Expressed Gene)를 총 68개 얻을 수 있었으며, 이 중 34의 DEG를 TOPO TA 클로닝 벡터에 클로닝하고 염기 서열 분석을 하였다. 분석한 염기 서열은 EMBL과 GenBank database에서 상동성 검색을 수행한 결과, 6개의 DEG (1, 2, 2-3, 4, 5 and 110-3)는 열대열 말라리아의 주 매개모기인 *An. gambiae*의 odorant-binding protein를 encoding하고 있었다. 이 단백질은 잠재적 모기 억제제로서 표적 단백질로 중요성이 있는 것으로 알려진바 있다. 또한 DEG 3과 DEG 110-2는 innate immune response에 중요한 물질로 알려진 peritrophin A와 peroxidase와 각각 상동성을 가졌다. 그 외에 hsp70 heat-shock system과 관련이 있는 DnaJ, 그리고 cytochrome c oxidase subunit IV, $Cu^{+2}/Zn^{+2}$ superoxide dismutase,

cathepsin b, ribosomal protein S4E, 45kDa immunophilin FKBP45, serine/threonine protein kinases, hydroxymethyltransferase, aryldialkylphosphatase, ADP/ATP carrier protein 2, DNA polymerase III gamma/tau subunits, vasa-like protein과 상동성을 가지는 단백질로 밝혀졌다. 흡혈시 발현의 차이를 보이는 유전자 정보를 더욱 많이 밝히기 위해, 2002년에 완성된 *Anopheles gambiae* genome 자료를 이용하여 cDNA microarray를 제작하고, 8,467 gene transcripts의 발현 양상을 분석하였다. 그 결과, 흡혈 모기에서 7배 이상의 발현이 증가 또는 감소되는 139 cDNA의 정보를 알 수 있었다. 95개의 cDNA는 흡혈시 발현이 up-regulation되었으며, 36개의 cDNA는 down-regulation되었다. 흡혈시 발현이 up-regulation되는 유전자는 주로 혈액을 소화하거나 (trypsin, chymotrypsin, serine protease, aminopeptidase), peritrophic matrix를 형성하거나 (chitin-binding peritrophin A), 화학주성 system (odorant-binding protein) 또는 embryogenesis와 vitellogenesis에 관련된 유전자들이었다. 이런 결과들은 한국의 말라리아 매개 모기인 중국얼룩날개모기의 분자적 연구에 새로운 이해를 다지는 초석이 될 것이다.

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핵심되는 말 : ACP-based PCR, 중국얼룩날개모기, 흡혈, differentially expressed gene, microarray, odorant-binding protein, peritrophin A