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Developmental Expression of Mitochondrial Gene Cytochrome *b* in *Culex pipiens* (L.) (Diptera: Culicidae)

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ABSTRACT

Cytochrome *b*, coded by mitochondrial DNA, is one of the cytochromes involved in electron transport in the respiratory chain of mitochondria. A 257 bp fragment of Cytochrome *b* gene (*Cytb*) was generated by reverse transcription-polymerase chain reaction (RT-PCR) products and sequenced directly. Sequence analysis of PCR product of *Cytb* shared a similarity in sequence compared to *Culex Cytb* found in GenBank. To reveal whether cytochrome *b* of the mosquito, *Culex pipiens* (L.) (Diptera: Culicidae) (*CxpCytb*) was developmentally regulated; the real-time quantitative polymerase chain reaction (qPCR), was used to examine *Cytb* gene expression levels in different developmental stages of *Cx. pipiens*. The qPCR showed that *CxpCytb* was expressed in each developmental stage, with high expression at eggs, the RNA relative expression level of *CxpCytb* decreased dramatically in 1st and 2nd instar larvae and increased again in 4th instar larvae and pupae of mosquitoes, than the lowest point in adult males. Expression of *CxpCytb* in teneral adults (2-days-old female) of *Cx. pipiens* was higher than that found in male adults of the same age. These results suggest that *CxpCytb* gene plays an important role in the development of *Cx. pipiens* and may provide critical information needed for designing novel control strategies for medically important disease vectors and identifying new pathways to target for the development of new molecular pesticides.

Key words: Cytochrome *b*, *Culex pipiens*, development, RT-PCR, quantitative polymerase chain reaction (qPCR), molecular pesticides.

INTRODUCTION

Insect mitochondrial DNA (mtDNA) consists of thirty-seven genes including two ribosomal RNA (rRNA) genes, twenty two transfer RNA (tRNA) genes and thirteen protein coding genes (Clay and Wolstenholme, 1985). The cytochrome *b* coded by mitochondrial DNA, is one of the cytochromes involved in electron transport in the mitochondrial respiratory chain (Esposti *et al.*, 1993). Cytochrome *b* is the largest polypeptide in the cytochrome *bc*1 complex, which catalyzes the redox transfer of electrons from ubiquinone to cytochrome *c* (Crofts *et al.*, 1999). Cytochrome *b* also contains various inhibitors and quinone antagonists that bind and inhibit the activity of oxidoreductase (Esposti *et al.*, 1993). In all eukaryotic and some prokaryotic respiratory chains, energy is obtained from the transfer of electrons through multisubunit complexes (membrane-bound) to cytochrome *c* oxidase (CcO) (Zhen *et al.*, 1999). To provide electrons rapidly, cytochrome *c* must interact with several proteins at a high rate of speed and specificity in the mitochondrial intermembrane space (Zhen *et al.*, 1999).

The role of cytochrome *b* (*CxpCytb*) during the development in the mosquito, *Culex pipiens* (L.) (Diptera: Culicidae), the common and widely distributed mosquitoes across Egypt and was incriminated as the main vector of bancroftian filariasis (Southgate, 1979) and the Rift Valley fever (RVF) (Meegan *et al.*, 1980), has not been explored.

By using real time quantitative polymerase chain reaction (qPCR), the present study aimed to examine the role of the *CxpCytb* gene in developmental expression in *Cx. pipiens* and determine the expression profile of *CxpCytb* in different life stages of the mosquito, *Culex pipiens* in order to facilitate the elucidation of the role of this gene.

MATERIALS AND METHODS

Mosquito Strains

Culex pipiens was reared in the insectary of the Research Institute of Medical Entomology, Ministry of Health and Population, Giza, Egypt. Different developmental stages 1-2 day old (eggs, larvae, pupae and adults) were used for the experiments. Adult females used in these experiments were not fed blood but were given sucrose (10%) as a carbohydrate source for routine maintenance (Pridgeon *et al.*, 2007).

RNA Extraction

Total RNA was extracted from five egg rafts and 25 specimens from each stage (1st, 2nd and 4th instar larvae, pupae and male and female adult life stages), using Qia Amp RNA blood Mini Kit (Qiagen) according to the manufacturer's instructions. RNA samples were quantified by spectrophotometer. Experiments were repeated three times and mRNA was reverse transcribed into cDNA.

Design of Gene-Specific Primers for RT-PCR

To design gene-specific primers, a detailed analysis of the nucleotide sequence of the

mitochondrial gene *CxpCytb* (NCBI accession no. DQ440235.1) was performed, using PRIMER3-Design Primer Pairs and Probes program from Biology Workbench (http://workbench.sdsc.edu). The primers for *Cx. pipiens* GAPDH also were used for an internal control and comparison.

RT-PCR Amplification and Sequencing

One µg of total RNA was reversely transcribed by cDNA Archive cDNA kit (Applied Biosystem) according to manufacturer instructions. PCR amplification was performed in 50 µl total volume with the following forward

5′ CTCCTGTCCATATTCAACCAGA 3′ and reverses
5′ TAAGGATCTTCAACAGGGCG 3′ primers.

PCR conditions were as follows: an initial denaturation step at 95 °C for two min, 30 cycles of 95 °C for 1min, 55 °C for 30 sec, and 72 °C for 1min, and a final extension step at 72 °C for 10 min, using Perkin Elmer Gene Amp 9600 (www.perkinelmer.com). PCR products were checked by electrophoresis using 1% agarose gel in 1×TAE buffer. The products were then purified using QIAQuick Gel Extraction Kit #28706 (QIAGEN, www.quiagen.com) following manufacturer instructions and sequenced by automated DNA sequencing reactions, which were performed using a sequencing ready reaction kit (Life Technologies, www.invitrogen.com) in conjunction with ABI PRISM and ABI-PRISM big dye terminator cycler. Primers used for the sequencing of the PCR products were the same as for the amplification.

Real-Time PCR Amplification

The qPCR assay for cytochrome *b* gene expression in *Cx. pipiens* (*CxpCyb*) used Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen) in a volume of 15 µl on a 7500 Fast Real-Time PCR system (Applied Biosystems). The PCR mixture consisted of 1 µl of diluted cDNA, 7.5 pmole primers, and 1x master mix. In every RT-PCR run, *GAPDH* was used as an internal control to normalize variation in the amount of cDNA template. The PCR primers used were

CYTB-F (5′-CTCCTGTCCATATTCAACCAGA-3′) and
CYTB-R (5′-TAAGGATCTTCAACAGGGCG-3′).

The PCR primers for GAPDH were
F (5′-CAAGGTCATCCATGACAACTTTG-3′) and
GAPDH-R (5′-GTCCACCACCCTGTTGCTGTAG-3′).

The PCR thermal cycling parameters were 50°C for 2 min, 95°C for 10 min., followed by 40 cycles of 95°C for 15 s and 60°C for 1min. This was followed by the dissociation stage at 95° C for 15 s, 60°C for 30 s, and 95°C for 15 s. Data analysis was based on three replicates of RT-PCR. Relative expression levels were calculated as follows: First, CxpCytb transcript levels relative to a standard (GAPDH) by using the formula:

$$\Delta Ct = Ct\ (CxpCytb) - Ct\ (GAPDH).$$

Second, an average ΔCT value for each sample was calculated. Third, relative expression levels were calculated using the modified equation

$1 \times 2^{-\text{average}\ \Delta Ct}$ (Portereiko *et al.*, 2006).

Statistical Analysis

Data were subjected to analysis of variance (ANOVA) with at least three repeats.

RESULTS AND DISCUSSION

Amplification of the *cytochrome b* coding sequence

To amplify the *cytochrome b* coding sequence, the mRNA corresponding to the *cytochrome b* gene was reversely transcribed to corresponding cDNA. PCR primers were tested by cDNA, extracted from different developmental stages. A 257bp fragment corresponding to *CxpCytb* was amplified and visualized on 1% agarose gel and stained with Ethidium Bromide. This result showed that a single specific PCR product was obtained with a molecular size of approximately 257 bp from egg rafts, 1st, 2nd and 4th instar mosquito larvae as well as pupae, adult males and females but with difference in band intensity (Fig. 1). Semiquantification of *Cytb* levels in different developmental stages showed that expression level in eggs was higher than the rest of stages under investigation meanwhile, the lowest expression was observed in males. The band intensity was detected by determination of its integrated display value (IDV), using gel documentation system (Bio-Rad). The band intensity of *Cytb* specific PCR products was 3868, 477.09, 1498.8, 2367.8, 2235.8, 371.7 and 826.3 in eggs, 1st, 2nd, 4th, pupae, males and females, respectively. The expected 257bp fragment of *CxpCytb* was recovered and sequenced (Fig. 2).

***Culex pipiens cytochrome b* sequence homologies**

DNA sequencing of *Cytb* was done to confirm the isolation of *Cytb* and to compare the sequence of *Cytb* with the sequence of homologues *Cytb* from other organisms. The sequence was provided by MACROGEN Company, Korea. Obtained sequence was analyzed for similarities to other known sequences found in the GenBank database using non redundant GenBank + EMBL + DDBJ + PDB sequences database. The DNA of four insects from those resulted from BLASTN search were chosen for multiple alignment with *Cx. pipiens cytochrome b* using PC/GENE program and revealed a 82% - 85% similarity with the another insects cytochrome b on GenBank (Table 1 and Fig. 2).

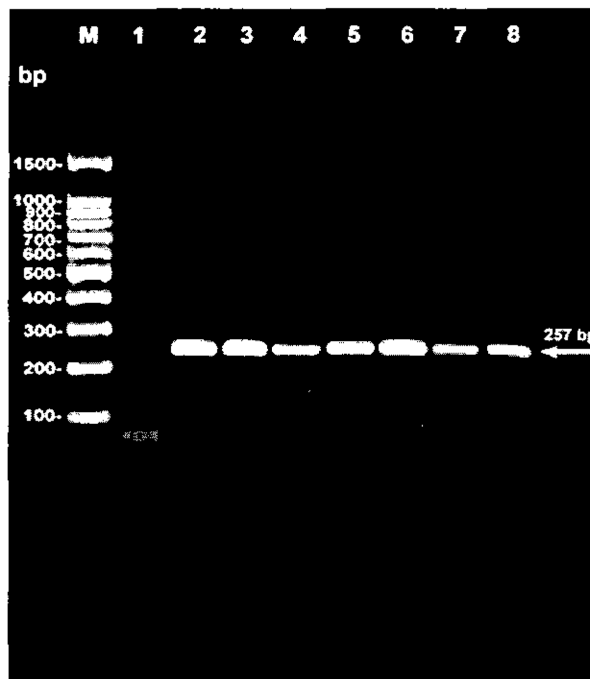

Fig. (1): Ethidium bromide-stained agarose gel of PCR amplified fragments using specific primers for cytochrome *b* gene, M: Molecular size marker (GeneDireX # DM001-R500), Lane1, negative control (nuclease free water); Lane 2-6, eggs stage, pupae, 1st, 2nd, 4th instar larvae; lanes 7and 8 adult males and females 2 days-old, respectively. The arrow shows the *Cx. pipiens* PCR amplified fragment (257bp).

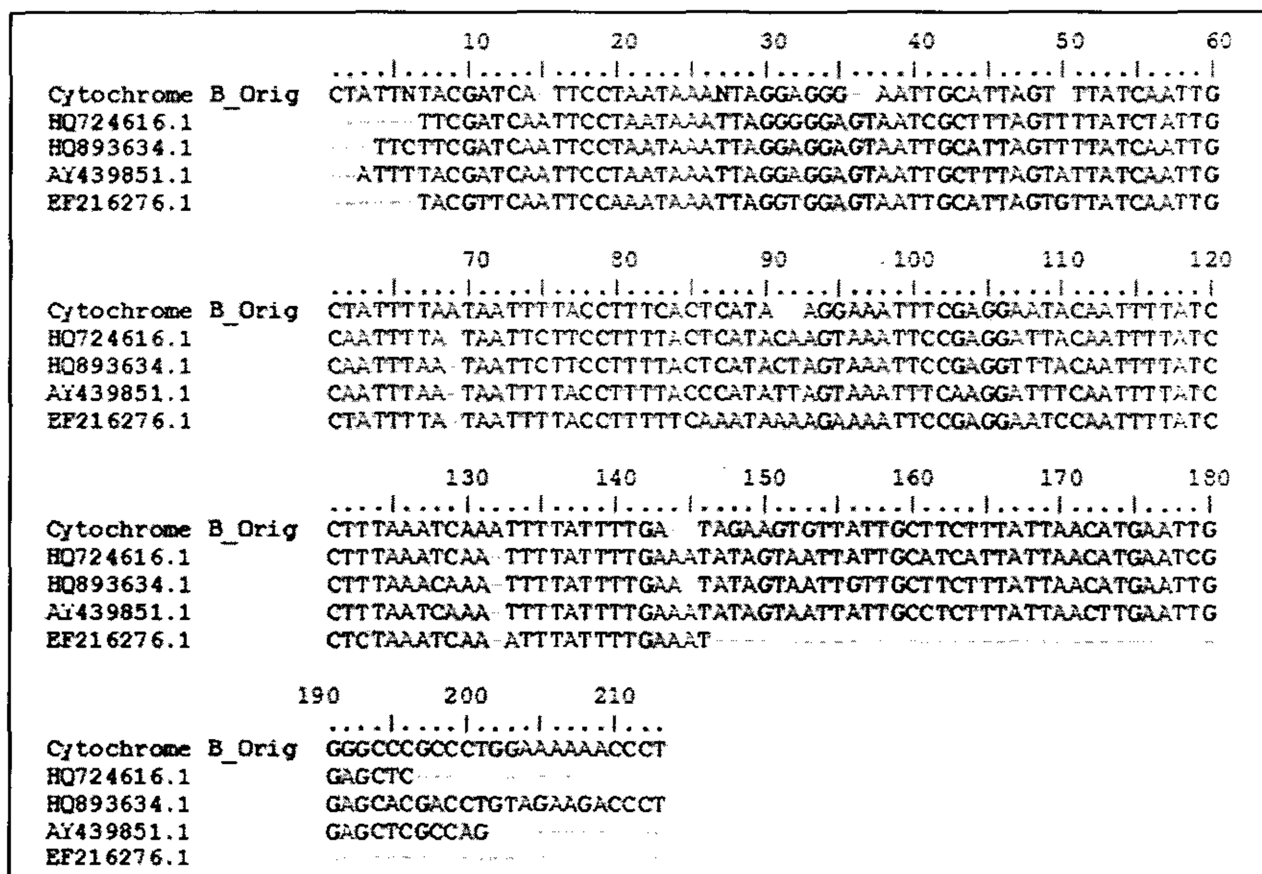

Fig. (2): Alignment of sequence of PCR amplified fragment with some of the sequences resulted from homology search of BLASTN; HQ724616.1(*Culex pipiens pipiens* from Turkey), HQ893634.1(*Culex theileri*), AY439851.1 (*Armigeres subalbatus*)and EF216276.1(*Drosophila miranda*). Identical nucleotide sites are shown by dots.

Table (1): Cytochrome *b* of four insects resulted from BLASTN search

Accession No.	Description	Max. ident.
HQ724616.1.1	*Culex pipiens pipiens* from Turkey mitochondrion, complete genome	82%
HQ893634.1	*Culex theileri* isolate H4-2 cytochrome b gene, partial cds; tRNA-Ser gene, complete sequence; and NADH dehydrogenase subunit 1 gene, partial cds; mitochondrial	84%
AY439851.1	*Armigeres subalbatus* ASAP ID: 43877 cytochrome b mRNA sequence	84%
EF216276.1	*Drosophila miranda* cytochrome b (Cytb) gene, partial cds; mitochondrial	85%

Table (2): Expression of *CxpCytb* in different developmental stages of *Cx. pipiens*

Sample stage	Cycle threshold (Ct) ±SD		Relative *CxpCytb expression level*
	CxpCyb	GAPDH	$2^{-\Delta Ct}$±SD
Eggs	27.00±0.40	28.1±0.013	53.3±1.587451
1st instar larvae	38.60±0.42	27.7±0.014	5.6±1.700157
2nd instar larvae	35.05±0.37	27.44±0.021	18.8±1.463193
4th instar larvae	32.35±0.16	27.95±0.012	31.7±0.643739
pupae	32.8±0.10	28.1±0.034	30.5±0.4
Adult female	39.48±0.38	27.44±0.016	1.5±0.801332
Adult male	40.43±0.10	28.1±0.16	Standard (low expression)

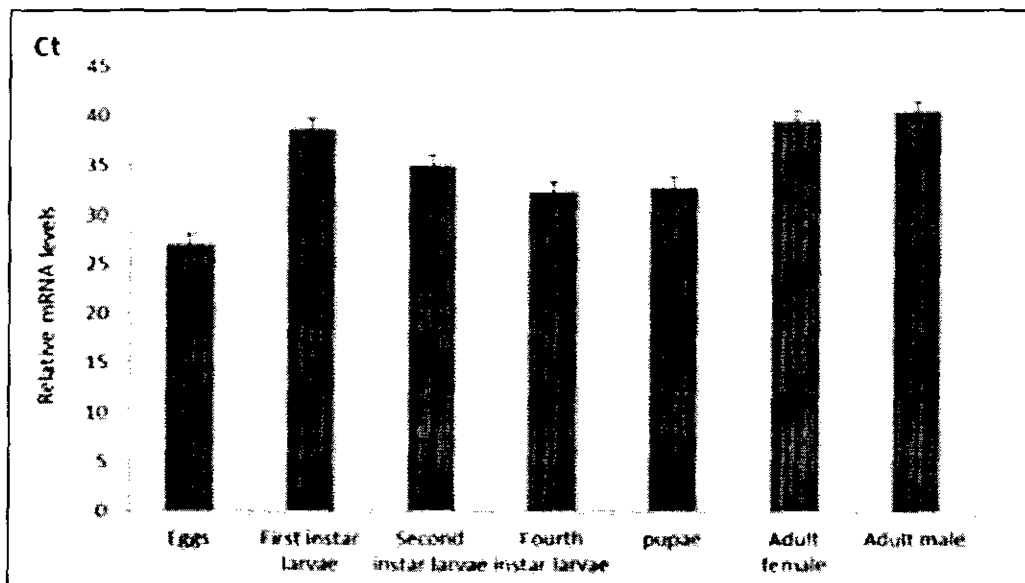

Fig. (3): *CxpCyb* gene expression levels in developmental stages of *Cx. pipiens* quantified by qPCR, with SD for three replicates. Ct: Cycle threshold.

Sequence confirmation of RT-PCR specificity

Sequence alignment of the amplified fragment of *Cytb* with published sequences at GenBank resulted in sequence similarity between 82-85%. The high species identification capability of *Cytb* was confirmed by the study of the homology of this gene in various species of Diptera recorded in the GenBank. Thus, the genetic differences among species may be expected to increase as the evolutionary distance between them becomes greater. *Cytb* seemed to have more variable positions inside genera than the common insects' identification locus, COI (Gilarriortua *et al.*, 2013).

Mitochondrial Gene Cytochrome *b* Expression during *Cx. pipiens* Development

Changes in the mitochondrial gene expression of cytochrome *b* were analyzed in *Cx. pipiens* eggs, larvae, pupae, and adults. The mitochondria gene was expressed 100-fold higher than nuclear genes, such as *Aedes aegypti CytC,* during all developmental stages examined (Zhao *et al.*, 2008). The *CxpCytb* gene was expressed at relatively high levels in eggs, the late larval and pupal stages and was expressed in varying quantities during early larval instars. In adults, *CxpCytb* expression differed in males and females of the same age. This in

agreement with the results obtained by Zhao *et al.* (2009), who reported that in females, *Ae. aegypti* *Cytb* gene expression was higher than in males for all ages examined. There were significant differences in the expression of *Cytb* genes between teneral and 14-day-old *Ae. aegypti* females. In addition, *Cytb* gene expression was significantly different between teneral males and females. However, the relative RNA levels of *Cytb* expression was undetectable in the older male mosquitoes (7-day-old), which may suggest that *Cytb* played an important and different role depending on adult *Ae. aegypti* sex and age (Zhao *et al.*, 2009).

Numerous physiological changes occurred during the development of male and female mosquitoes as they grew older and *CxpCytb* gene expression in mature mosquitoes was critical for mitochondrial functions and might be related to mosquito aging. The age-related elevation in adenine nucleotide translocase carbonyl content indicated that proteins in mitochondrial membranes were selectively modified during aging (Yan and Sohal, 1998). Integral membrane protein component of the cytochrome *bc*1 complex, which catalyzed the redox transfer of electrons from ubiquinone to cytochrome *c*, and the *CxpCytb* gene expression, might correlate to the cytochrome *c* genes expression in the adult mosquitoes (Zhao *et al.*, 2008). As reported previously, human mitochondrial DNA cytochrome *b* gene expression could be detected from mosquitoes after blood meal ingestion (Oshaghi *et al.*, 2006). For this reason, females that were not blood fed but had sucrose available during the course of the experiments were used. Mosquito development can be regulated by multigenes (Severson *et al.*, 2004, Fontenille *et al.*, 2005; Raibaud *et al.*, 2006; Strode *et al.* 2006 and van den Hurk *et al.*, 2007), and cytochrome *b* might be one of these genes.

In conclusion, *CxpCytb* gene expression in the life cycle of *Cx. pipiens* was highly regulated during developmental stages. Gene expressed at higher levels in eggs, fluctuated in the larval instars and pupae meanwhile, *Cytb* gene expression was higher in adult females than in males. Cytochrome *b* activity had been examined, for the first time, in detail for all developmental stages of a mosquito. High levels of *CxpCytb* expression in different developmental stages might direct the design of double-stranded RNA (dsRNA) from *CxpCytb* to knockdown the *CxpCytb* gene, as dsRNA knockdowns was shown to kill mosquitoes in the study of Pridgeon *et al.* (2008).

The present study suggests that the mitochondrial gene *CxpCytb* plays an important functional role in the development of *Cx. pipiens* and may provide critical information needed for designing novel control strategies for medically important disease vectors and identifying new pathways to target for the development of new molecular pesticides.

CxpCytb Gene Regulation in Different Developmental Stages of *Cx. pipiens*

To understand how mitochondrial genes were regulated during the development of *Cx. pipiens*, cytochrome *b Cx. pipiens* (*CxpCytb*) relative expression levels were examined in eggs, larvae, pupae and adults by using quantitative real time PCR (qPCR) (Table. 2). During the development of *Cx. pipiens*, the real-time PCR data showed that the lowest *CxpCytb* RNA expression was in the adult male stage. This point was set as standard, and the *CxpCytb* RNA expression in other stages was compared with this point. *CxpCytb* expression was relatively high in the eggs (53.3±1.58 folds), while it was 5.6±1.7 folds in first instar larvae and 18.8±1.46folds in second instar larvae, 4th instar larvae showed 31.7±0.64 folds as well as 30.50±0.4 folds in pupae. However, the RNA relative expression level of *CxpCytb* decreased dramatically in 1st and 2nd instar larvae and increased again in 4th instar larvae and pupae of mosquitoes, than the lowest point in adult males. Expression of *CxpCytb* in teneral adults (2-day-old female) of *Cx. pipiens* was 1.5±0.80 folds than that found in male adults of the same age. *CxpCytb* expression was relatively high in eggs and the 4th instar larvae as well as pupae than the 1st and 2nd instar larvae, and adult males and females. *Cytb* gene expression was significantly different $P < 0.0001$ between *Cx. pipiens* developmental stages (Fig. 3).

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