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Original Research Article

Identification, molecular characterization and phylogenetic analysis of cytochrome c oxidase gene from bacterial-infected *Culex pipiens* (Diptera: Culicidae)

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Abstract

The present work aims to identify, molecular characterization and phylogenetic analysis of the induced antibacterial gene, cytochrome c oxidase, from the whole body of bacterial-fed fourth instar *Cx. pipiens* larvae using differential display technique. For achievement this research, the fourth instar *Cx. pipiens* larvae were fed on gram (+) bacteria, *S. aureus*, gram (-) bacteria, *K. pneumoniae* and mix. Whole body infected larvae were collected at 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66 and 72 h postfeeding. The differential display technique was employed to screen the genetic variation (at RNA level) between bacterial-fed and control fourth instar *Cx. pipiens* larvae. Nine reproducible bands were eluted and sequenced to characterize the full length cDNA of the induced genes. The results indicated the presence of differentially displayed bands in the bacterial- fed larvae and not observed in controls. The resulting sequences were blasted to cytochrome c oxidase.

Keywords: antibacterial gene, *Cx. pipiens,* cytochrome c oxidase, DD-PCR, immune response

INTRODUCTION

The growing problem of resistance of microorganisms to current antibiotics has fostered the search for novel antimicrobial therapies (Breithaupt, 1999). Insects represent one of the most successful groups of evolution within the animal kingdom (about 75% of all animal species), accounting for nearly one million species. The amazing diversity and evolutionary success argue for an effective system of defense against infections. During evolution, insects developed a complex and effective innate immune system, which apparently differs from the adaptive immune system of vertebrates. However, there is no evidence for clonal selection mechanisms in insects and their immune system that shows no memory, their defense mechanisms are rapid, lasting up to a few days, and offering a particularly powerful resistance to microbial infections (Royet, 2004; Ratcliffe *et al.,* 2011 and Vilcinskas, 2013).

Mosquitoes significantly contribute to insect biodiversity and biomass, representing around 3500 described species (Fang, 2010). Mosquitoes are the most important arthropod vectors of disease (Youdeowei and Service, 1983). Like other insects, mosquitoes have highly effective immune systems that protect them from pathogens such as bacteria or fungi (Marquardt and Beaty, 1996).

The insect immunity is a complex of several distinct systems, both cellular and humoral in nature, that cooperate together in a more or less coordinated way to provide protection of the body cavity from invading microorganisms (Dunn, 1986 and Boman and Hultmark,

1987). The cellular arm involves haemolymph coagulation, melanization, phagocytosis and encapsulation. The humoural arm includes constitutive and inducible antimicrobial peptides (AMPs). These responses are based on the recognition of the pathogen as non-self, the induction of suitable genes and biochemical pathways (Bulet *et al.,* 2003; Bulet and Stocklin, 2005; Ratcliffe *et al.,* 2011; Seufi, 2011; Seufi *et al.,* 2011; Seufi, 2012; Seufi *et al.,* 2012 and Seufi *et al*., 2017). Antibacterial peptides constitute the key defense elements in response to bacterial challenges or trauma (Hoffmann and Hetru, 1992 and Cociancich *et al.,* 1994). AMPs defined as critical defense molecules that can protect the host from the invasion of bacteria, viruses or fungi. AMPs are conserved evolutionally in their innate immune response, which have served as natural first-line of defense system for the majority of living organisms (Gallo and Nizet, 2003; Beutler, 2004 and Kang *et al.,* 2012). There are about 559 antimicrobial peptides identified and isolated from plants, vertebrates and invertebrates (Wang and Wang, 2004).

The mitochondrial protein cytochrome oxidase c is a highly conserved electron transport protein coded by multiple genes (Lunt *et al.,* 1996). *AeCOI* expression levels following pathogen infections seem warranted (Bossy-Wetzel *et al.,* 1998). Also *COI* expression levels increase in the late stages of infection of *Bombyx mori* cells infected with nucleo-polyhedovirus (Okano *et al.,* 2001). In invertebrate host-pathogen systems, cytochrome oxidases have been shown to be up-regulated in response to immune stimulation as shrimp: (James *et al.,* 2010) clams: (Gestal *et al.,* 2007). Rensburg and Coyne (2009) found that two electron transport system genes, cytochrome b and cytochrome c oxidase III upregulated in a cDNA microarray experiment performed on haemocytes from immune-stimulated abalone *Haliotis midae*. Freitak *et al.,* (2009) showed that two different cytochrome c related genes were up regulated in 2 and 7 days old *Trichoplusia ni* larvae fed on plant and bacterial diet. Abumourad (2011) identified The complete sequence of the cytochrome c oxidase subunit 1 (*ONCOX1*) in Tilapia (*Oreochromis niloticus*) immunized by formalin-killed *Flavobacterium columnarae* and suggested that this member of *COX* genes is probably involved in the general immune response against the pathogenic bacteria.

MATERIALS AND METHODS

Insects and bacterial species

Mosquito samples were obtained from breeding habitat in Giza Governorate, Egypt. Mosquito larvae reared in sectary to obtain adults for morphological identification using taxonomic keys (Harbach, 1985) and colonized in the in sectary of the Department of zoology, Faculty of Science, South Valley University. Stock colony of the adult mosquitoes was maintained under laboratory conditions (27 $\pm$ 2 ℃ and 60-70% RH) for supplying clean adults of known ages. According to the method described by Adham *et al.,* (2003).

Gram (+) bacteria, *Staphylococcus aureus* and gram (-) bacterial, *Klebsiella pneumoniae* were obtained from the Unit of Genetic Engineering and Agricultural Biotechnology, Faculty of Agriculture, Ain Shams University and used for insect immunization. Bacteria were grown in a peptone medium (1%), supplemented with 1% meat extract and 0.5% NaCl, at 37 ℃ in a rotary shaker. Bacterial challenge was performed by feeding newly moulted fourth instar larvae with diluted bacterial solution (106 cells/ml). Bacterial species were used for immunization separately and in combinations.

Bacterial feeding and whole body collection

Cx. pipiens fourth instars larvae were kept without food for 6 hrs then they classified into four groups . the first group was kept without any treatment (C), the second group (T_k) was treated by feeding on diluted culture of *Klebsiella pneumoniae* (-) for 24 hrs, the third group (T_s) was treated by feeding on diluted culture of *Staphylococcus aureus* (+) for 24 hrs, the fourth group (T_m) was treated by feeding on diluted mixture of *Staphylococcus aureus* (+) and *Klebsiella pneumoniae* (-) for 24 hrs. Both control and bacterial-challenged fourth instars larvae were collected after 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66 and 72 hrs postfeeding (h.p.i.) at 4℃. A bout 1 µl Phenyl Methyl Sulfonyl Fluoride (PMSF) were added to the collected sample to prevent protein degradation and stored at -80℃ until processing.

Differential display technique (DD-PCR)

DD-PCR is used in the present study to record the genetic differences between control and bacterial-fed fourth instar larvae of *Cx. pipiens* at 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66 and 72 h.p.i.

Total RNA of the insect was extracted using biozol reagent (Bioflux) according to the manufacturer's instructions. RNA was dissolved in DEPC-treated water, quantified using a BioPhotometer 6131 (Eppendorf) and analyzed on 2 % denatured agarose gel to ensure its integrity. The 260/280 and 260/230 ratios were examined for protein and solvent contamination.

A total of 2 µg of DNA-free total RNA was converted into cDNA using RevertAid First Strand cDNA Synthesis kit according to the manufacturer's instructions. Synthesis of the first cDNA strand was performed in a thermal cycler (PeQlab, USA) programmed at 42℃ for 1 h, 72℃ for 10 min and a soak at 4℃. The cDNA was ali-quoted and stored at -80 until processed (within a weak).

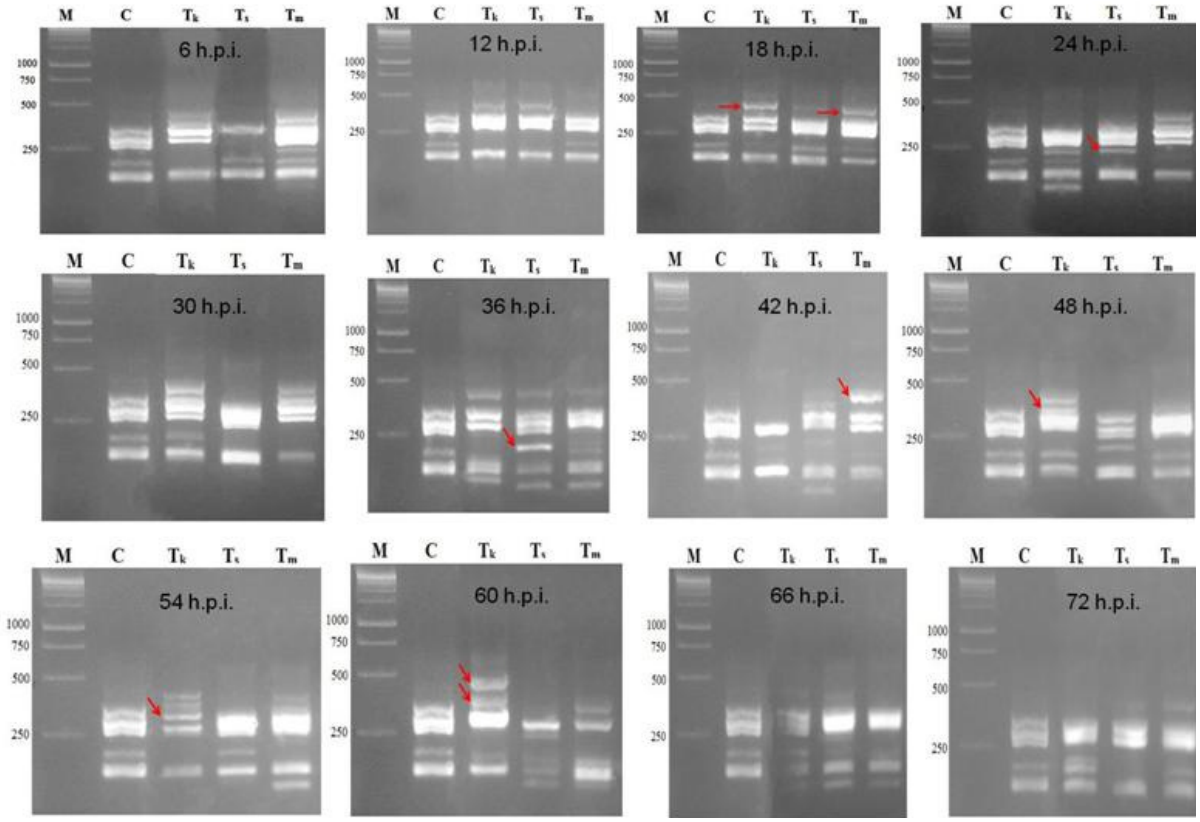

Figure 1. 2% agarose gel of DD-PCR patterns of different studied groups for different times using **RAPD8** primer. Lane **M**: DNA Ladder 1 kbp, lane **C**: normal fed larvae, lane $\mathbf{T_k}$: gram – bacteria, *K. pneumonia* - fed larvae with, lane $\mathbf{T_s}$: gram + bacteria, *S. aureus* fed larvae with and lane $\mathbf{T_m}$: both gram – bacteria, *K. pneumoniae* and gram + bacteria, *S. aureus* fed larvae. The arrows pointed to sequenced bands.

PCR was performed in a DNA thermal cycler (PeQlab, USA). Total PCR volume was 25 µl containing 12.5 µl PCR master mix (promega, USA), 7.00 µl primer (10 pmol, Sigma) RAPD8–12P primer 5´ ACC TGA ACG G3`, 1.00 µl template DNA, 4.5 µl H_2O. For DNA contamination assessment, a no–reverse transcription control reaction was performed. The PCRs were programmed for one cycle at 95 °C for 5 min followed by 45 cycles of 1 min at 95 °C, 1 min at 36 °C, and 1 min at 72 °C. The reaction was finally incubated at 72 °C for 10 min for final extension. PCR product was visualized on 2 % agarose gel and photographed using gel documentation system.

The excised bands were purified using Wizard® SV Gel and PCR Clean-Up System kit (Promega, USA) according to the manufacturer's instructions. The eluted DNA was stored at 4°C or –20°C until sequenced.

DNA Sequencing, Sequence Analysis (Alignment) and phylogenetic construction

DNA sequencing for the 9 purified reproducible bacterial-induced bands were performed by Sigma Aldrich Company, Munich (Germany).

Analyses of nucleotide and deduced amino acid sequences were carried out using ExPasy database (http://expasy.org/tools/dna.html). Blast search for alignment of the obtained sequence with the published ones was done using database of NCBI GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic tree was constructed using MEGA4.0 program and the sequence alignment was compared with the other defense genes that were available in the Genbank database using ClustalW (2.1) program (http://www.ebi.ac.uk/clustalw2).

RESULTS

Differential display technique (DD-PCR)

As the identification of the induced antibacterial genes was the main objective of this study, differential display technique was used to characterize the genetic variation (at RNA level) between bacterial- fed and control *Cx. pipiens* fourth instar larvae. Figure (1) represents DD-PCR patterns generated from control and bacterial- fed whole body samples for different times using RAPD8 primer. Whole body samples (fed on *K. pneumoniae*, *S.*

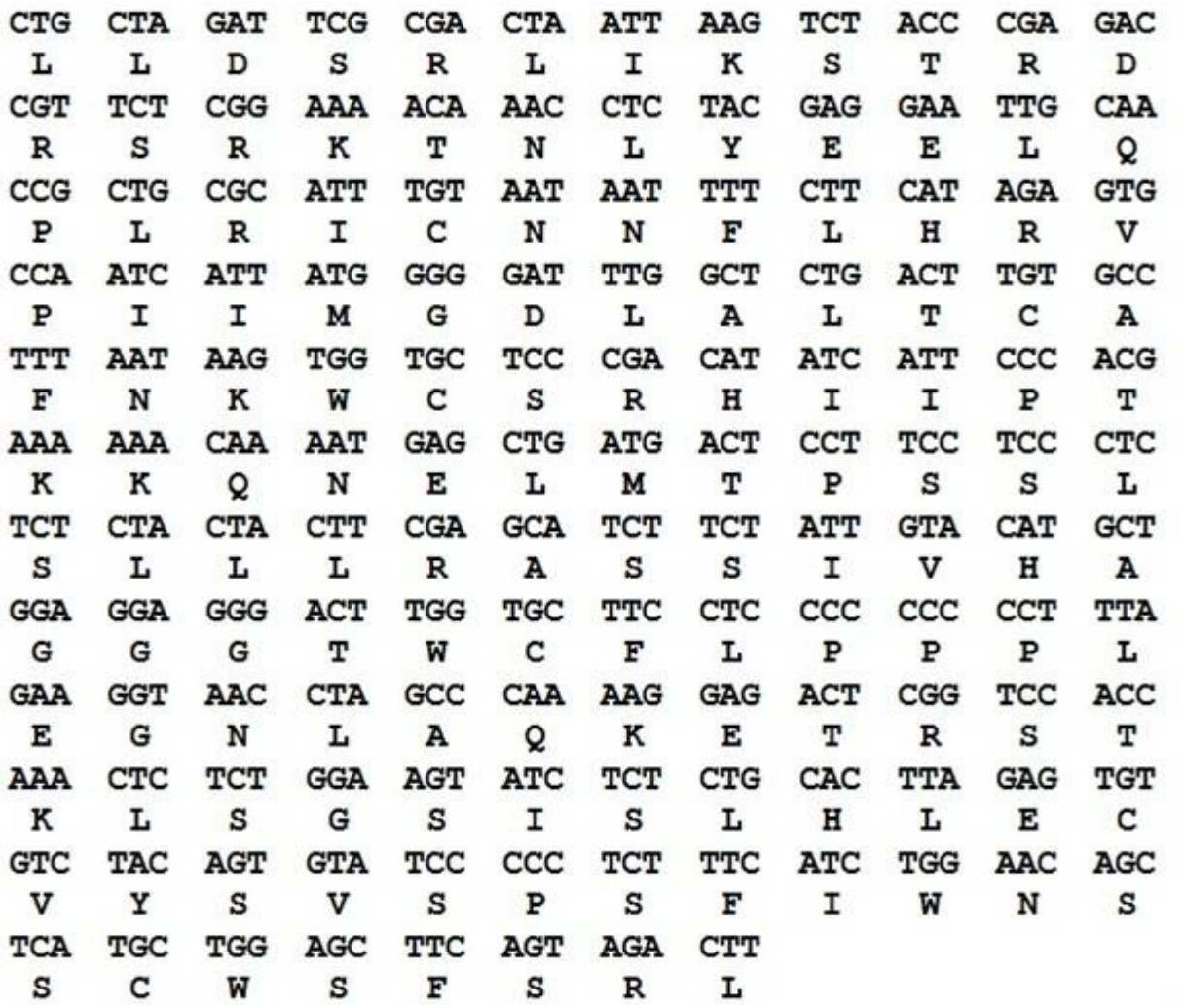

Figure 2. Nucleotide and corresponding deduced amino acid sequence of *Cx. pipiens* whole body cytochrome c oxidase gene (*CxpCOI*$_{WB}$).

aureus and mix of both bacterial species) were differentially displayed at 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66 and 72 h.p.i. The total number of amplified bands resolved in 2% agarose gel for both control and bacterial-fed larvae was 13 (molecular size ranged from > 450 to ~130 bp). 12 polymorphic bands (92.31%) were differentially displayed in the case of *K. pneumonia,* while the total number of amplified bands was 12 (molecular size ranged from > 450 to ~130 bp). 11 polymorphic bands (91.67%) were differentially displayed in the case of *S. aureus,* also in the case of mix the total number of amplified bands was 12 (molecular size ranged from > 450 to ~130 bp). 11 polymorphic bands (91.67%). Nine reproducible, treatment induced bands were sequenced.

Elution and sequencing results

The reproducible bands indicated by arrows in Figures (1) were eluted and sequenced using the previous primer.

These sequences were subjected to BLAST (Basic local alignment search tool), translated into their corresponding amino acids (deduced amino acids), and their phylogenetic analysis at the nucleotide and amino acid level were determined.

Cytochrome c oxidase nucleotide sequence and sequence analyses

Nucleotide sequence of *cytochrome c oxidase* and its deduced amino acid sequence are shown in Figure (2). The nucleotide sequence of *CxpCOI*$_{WB}$ was blasted to all cytochrome-related sequences in GenBank database. Blast search of putative *CxpCOI*$_{WB}$ peptide created no identity with other insect cytochromes -published peptide sequences however created significant identity with

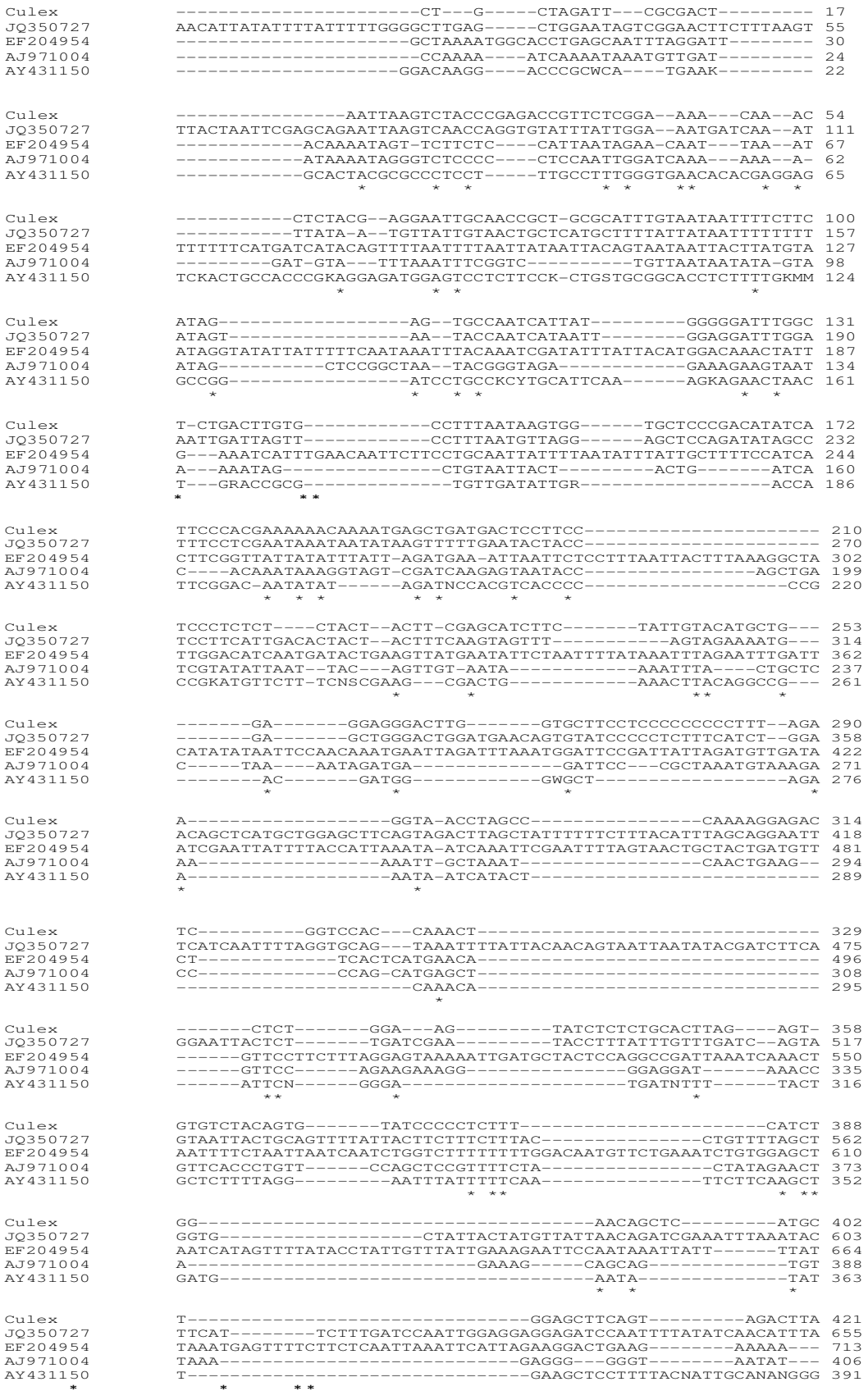

Figure 3. *CxpCOI*$_{WB}$ nucleotide sequence multiple alignment with other cytochrome oxidases isolated from other *Culex* species.
* refers to identical bases

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AFI80759        TLYFIFGAWAGMVGTSLSLLIRAELSQPGVFIGNDQIYNVIVTAHAFIMIFFMVMPIMIG 60
CDK31363        TLYFIFGAWAGMIGTSLSLLIRAELSQPGVFIGNDQIYNVIVTAHAFIMIFFMVMPIMIG 60
AAX09948        ------------------------------------------------------------
Culex           -----------LLDSRLIKSTRDRSRKTNLYEELQPLR--------ICNNFLHRVPIIMG 41
AAZ22855        ----------------------------------------------VNLTFFPQH--FLG 12


AFI80759        GFGNWLVPLMLGAPDMAFPRMNNMSFWMLPPSLTLLLSSSLVENGAGTGWTVYPPLSSGT 120
CDK31363        GFGNWLVPLMLGAPDMAFPRMNNMSFWMLPPSLTLLLSSSLVENGAGTGWTVYPPLSSGT 120
AAX09948        DFGNWLVPLMLGAPDMAFPRMNNMSFWMLPPSLTLLLSSSLVENGAGTGWTVYPPLSSGT 60
Culex           DLALTCAFNKWCSRHIIPTKKQNE--LMTPSSLSLLLRASSIVHAGGGTWCFLPPPLEGN 99
AAZ22855        LAGMPRRYSDFPDSYLAWNIVSSLGSTISLFGIVFFL---FII------WESMISQRTPS 63
                  .           :     ..    :   .: ::*    :       *    .    .

AFI80759        AHAGASVDLAIFSLHLAGISSILGAVNFITTVINMRSSGITLDRMPLFVWSVVITAVLLL 180
CDK31363        AHAGASVDLAIFSLHLAGISSILGAVNFITTVINMRSSGITLDRMPLFVWSVVITAVLLL 180
AAX09948        AHAGASVDLAIFSLHLAGISSILGAVNFITTVINMRSSGITLDRMPLFVWSVVITAVLLL 120
Culex           LAQKETRSTKLS----GSISLHLECVYSVSPSFIWNSSCWSFSRL--------------- 140
AAZ22855        FPMQLSSSIEWY--------HTLPPAEHTYAELPLLSSNF-------------------- 95
                     : .             *  .    . :   **

AFI80759        LSLPVLAGAITMLLTDRNLNTSFFDPIGGGDPILYQHLF-------------- 219
CDK31363        LSLPVLAGAITMLLTDRNLNTSFFDPIGGGDPILYQHLF-------------- 219
AAX09948        LSLPVLAGAITMLLTDRNLNTSFFDPIGGGDPILYQHLFWFFGHPEVYILIFT 173
Culex           ------------------------------------------------------
AAZ22855        ------------------------------------------------------
```

Figure 4. *CxpCOI$_{WB}$* deduced amino acid sequence multiple alignment with other cytochrome oxidases isolated from mosquitoes.
* identical amino acid
: different but highly conserved (very similar) amino acids
. different amino acids that are somewhat similar

mammalian cytochromes (Ac# AGX29586, AEK98509, ABV02962), amphibian cytochromes (Acc# AGE11471) and avian cytochromes (Acc# ACH55528). Meanwhile, the *CxpCOI$_{WB}$* nucleotide sequence created 100% identity with 99 *Culex sp*. cytochrome oxidase (Acc# KM233149, KM233148, KM233147, KM233146, KM233145, LM000940, HE997157, HE997156, HE997154, HE997153, HE997152, HE997151, HE997150, HE997149, HE997146, HE997145, HE997144, HE997143, HE997141, HE997135, HE997133, HE997132, HE997131, HE997130, HE997120, HE997117, HE997113, HE997112, HE997111, HE997110, HE997109, HE997108, HE997107, HE997106, HE997105, HE997104, HE997097, HE997095, HE997092, HE997090, HE997088, HE997086, HE997085, HE997075, KM258185, KM258182, KM258178, KM258170, KM258167, KM258166, KM258161, KM258160, KM258159, KM258157, KJ858518, KJ858517, HG793622, HG793602, HG793590, HG793568, HG793558, HG793556, HG793541, HG793539, HG793517, HG793516, HG793491, HG793489, HG793473, HG793472, HG793467, HG793445, HG793443, HG793442, HG793437, HG793409, HG793406, KJ680549, KF407800, KF407797, KF407767, KF407738, KF407725, KF407712, KF407709, KF407687, KF407675, KF407655, KF407632, KF407630, KF407620, KF407610, KF919190, KF919189, KF919188, HG793452, HE997147, KM258168, HG793395).

On comparing the present cytochrome oxidase nucleotide sequence *CxpCOI$_{WB}$* (Figure 3) with other cytochromes isolated from other *Culex* sp. (Acc# JQ350727, EF204954, AJ971004 and AY431150), and deduced amino acid sequence with other cytochromes isolated from other *Culex* sp. (Acc# AFI80759, CDK31363, AAX09948 and AAZ22855), conserved regions were observed throughout the five sequences.

Phylogenetic analyses of the *CxpCOI$_{WB}$* sequence

Phylogenetic analysis has been performed on the *CxpCOI$_{WB}$* nucleotide seuquence and its deduced polypeptide and the results of this analysis are shown in Figures (5 and 6). In the case of nucleotide seuquence, a phylogenetic tree was generated from 96 cytochrome-related sequences including 57 *Culex* species, 17 *Culiseta* species, 7 *Mansonia* species, 3 *Aedes* species, 3 *Psorophora* species, 2 *Lutzia* species and 3 *Uranotaenia* species by neighbor-joining distance analysis with maximum sequence difference 0.9. (Figure 5). The topology shows two distinct lineages including 96 cytochrome-related sequences from family Culicidae. The maximum nucleotide sequence divergence was exhibited in the second lineage. The *CxpCOI$_{WB}$* was clustered with, *C. vishnui* cytochrome (Acc# AB690844), and *Uranotaenia_sapphirina* (Acc# GU908125), in a monophyletic sister clade (Figure 5) with the other cytochrome sequences. Meanwhile, *Culex* cytochrome (Acc# DQ181433, DQ181434, DQ181429, DQ181430, DQ181435 and DQ181438) were diverged in different phylogenetic clades (Figure 5). In the case of *CxpCOI$_{WB}$* deduced amino acid seuquence, a phylogenetic tree was

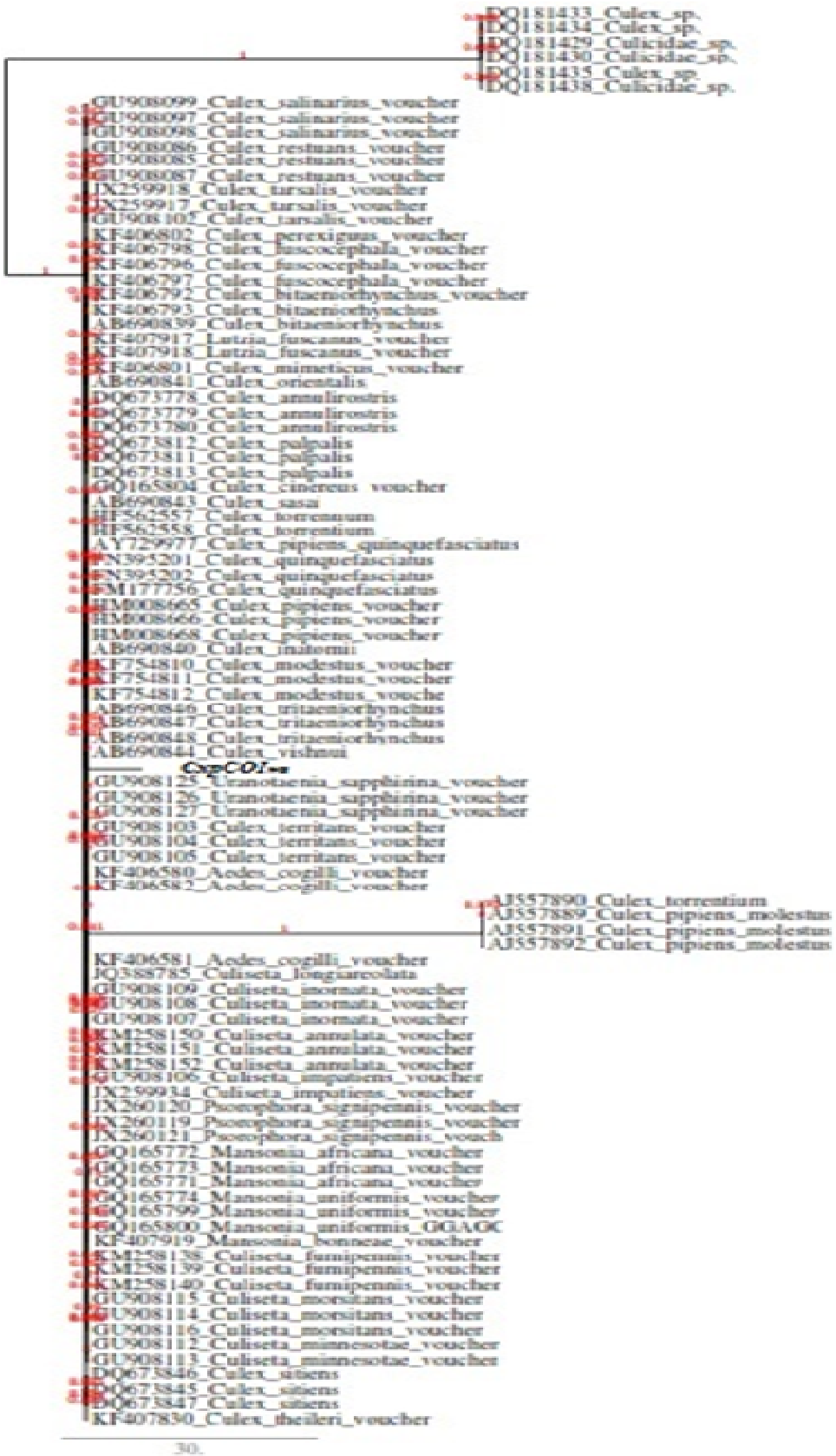

Figure 5. Phylogenetic analysis of *CxpChi*$_{WB}$ nucleotide sequence compared to sequences registered in NCBI.

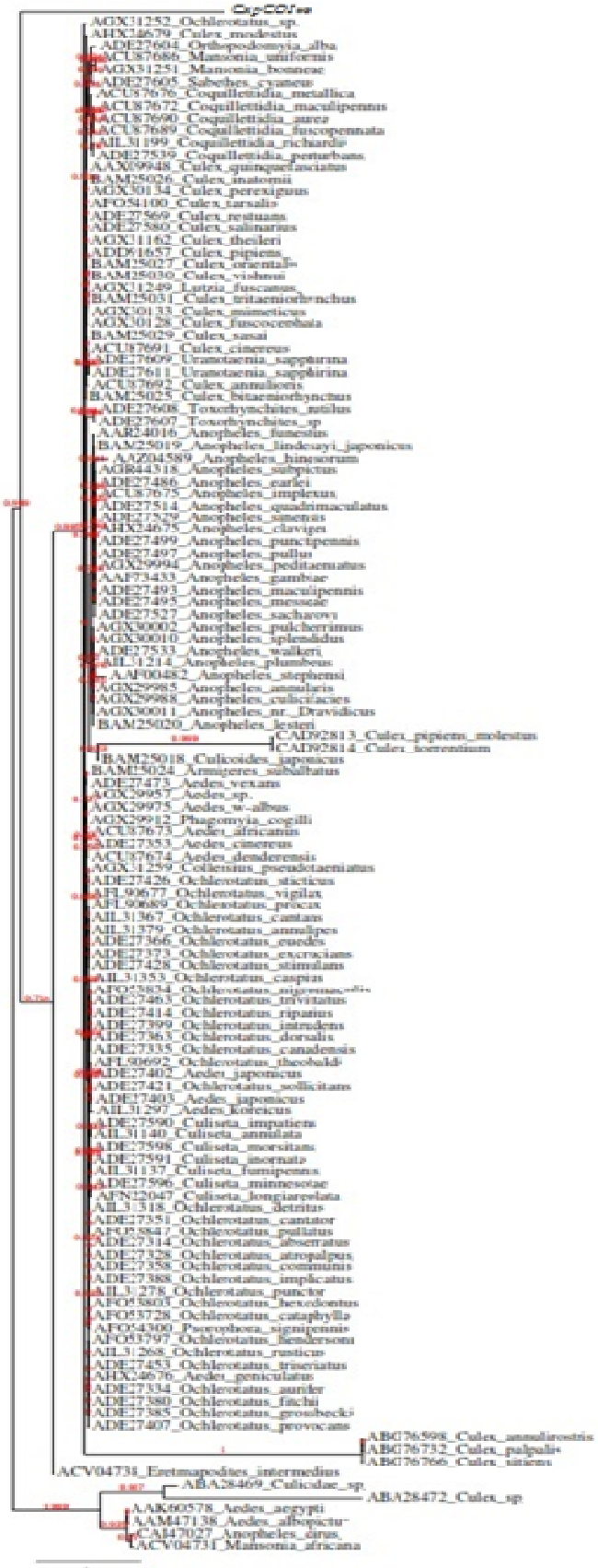

Figure 6. Phylogenetic analysis of *CxpCOI*$_{WB}$ deduced amino acid sequences compared to sequences registered in NCBI.

generated from sequence data of 128 sequences including 12 *Aedes* species, 26 *Anopheles* species, 25 *Culex* species, 3 *Mansonia* species, 1 *Eretmapodites* species, 34 *Ochlerotatus* species, 7 *Culiseta* species, 1 *Phagomyia* species, 1 *Armigeres* species, 1 *Culicoides* species, 2 *Uranotaenia* species, 6 *Coquillettidia* species, *1 Sebthes* species and 1 *Orthopodomyia* species by neighbor-joining distance analysis with maximum sequence difference 1 (Figure 6). The topology shows two distinct lineages of cytochrome peptides. The maximum divergence of amino acid sequences was exhibited in lineage II and the *CxpCOI*$_{WB}$ putative peptide was clustered in a separate monophyletic cluster clade in the other lineage. Meanwhile, other cytochromes are grouped in a separate cluster clade. Generally, clustering cytochrome from dipterous insects in monophyletic sister clades is a very strong clue that insect cytochrome may share a common ancestor (Figure 6).

DISCUSSION

The main objective of the current work is to study and characterize the immune response specially the induction of the antibacterial peptides from the mosquitoes, *Cx. pipiens* after infection with bacteria. Where, the antibacterial peptides have a great importance in the medical applications as they considered anew panel of natural antibiotics which destruct bacteria or even other microorganisms. To accomplish this objective, fourth instar larvae were fed with gram (+) bacteria (*S. aureus*), gram (-) bacteria (*K. pneumoniae*) and combination of the two types (mix) to trigger the innate immunity of larvae to respond and fight against infection. RNA extraction for the control and infected larvae were carried out. Then, the cDNAs of the control and infected larvae were differentially displayed. A group of the induced fragments were sequenced and analyzed using NCBI programs.

DD-PCR technique is considered a powerful genetic screening tool for complicated dynamic tissue processes, particularly when multiple, limited-sized samples are involved, because it allows for simultaneous amplification of multiple arbitrary transcripts (Soo *et al*., 2002). This technique has been developed as a tool to detect and compare altered gene expression in eukaryotic cells (Liang *et al*., 1993), to screen mRNAs, and to characterize differentially expressed mRNAs (Dimopoulos *et al*., 1996; Ramalho-Ortigão *et al*., 2001; Mong *et al*., 2002 and Santana *et al*., 2006). Here, as we used the DD-PCR technique to differentiate between normal and bacterial-infected *Culex pipiens* larvae, Seufi (2011 and 2012), Seufi *et al.* (2011 and 2012) also used this technique to compare between cDNAs of uninfected and bacterial-infected larvae of cotton leaf worm, *Spodoptera littoralis*. Asling *et al.* (1995) used DD-PCR to compare between uninfected and bacterial-infected *Drosophila*. Recently, Seufi *et al.* (2017) used DD-PCR technique to differentiate between normal and bacterial-infected *Musca domestica*.

In the present study, DD-PCR revealed that some common bands were observed in both control and infected samples which known as housekeeping genes. On the other hand, some bands were recorded in the normal larvae but disappeared in bacterial-fed (T_k, T_s or T_m) ones. These bands indicate that these genes were down-regulated or turned off in the case of infection. Otherwise, some bands were induced as a result of bacterial feeding at different h.p.i. using RAPD8 primer.

The resulted induced bands at different h.p.i. of *Cx. pipiens* may lead to either the expression of antibacterial peptides or the expression of peptides which responsible for signaling and communication between immune cells that consequently stimulated the production of AMPs (cascading action).

The first probability agree with the previous studies of Kang *et al.* (1996); Dimopoulos *et al.* (1997); Lowenberger (2001); Vizioli *et al.* (2001a); Bartholomay *et al.* (2003); kim *et al.* (2004); Marquardt and Kondratieff (2005); Waterhouse *et al.* (2007) and Coggins *et al.* (2012) who described the enhancement of insect immune system and induction of AMPs due to stress and/or bacterial challenge in different species of mosquitoes. Also, the same probability agrees with Lopez *et al.* (2003); Wang *et al.* (2010); Seufi (2011 and 2012), Seufi *et al.* (2011 and 2012) and Seufi *et al.* (2017) who described it in other insects.

The results of the induced bands showed that there were characteristic bands which appeared only in either *K. pneumoniae*-fed larvae (T_k), *S. aureus*-fed larvae (T_s) or mix-fed larvae (T_m). *S. aureus* (T_s)-fed group showed a band of 340 bp. The same band was shown in *K. pneumoniae* (T_k)-fed group after 6 and 30 h.p.i. using RAPD 8 primer (figure 1). These bands were different from each other and this proved that the defense system in *Cx. pipiens* can discriminate between various classes of microorganisms. In our case it differentiates between gram + and gram – classes. In addition, this differential level of induction of antibacterial genes by gram + and gram – suggests a degree of selectivity in response as confirmed by Nasr and Fallon (2003) and Vierstraete *et al.* (2004).

On the other hand, the results of new induced bands after feeding with *K. pneumoniae* or *S. aureus* as well as mix group showed appearance of some bands at 410, 365, 300, 230 and 130 bp using RAPD 8 primer. This may be related to the appearance of new protein in these groups without differentiation between them and it disagrees with results of Nasr and Fallon (2003) and Vierstraete *et al.* (2004).

Many publications described the enhancement of the insect immune system and induction of AMPs due to stress and/or bacterial challenge (Lamberty *et al*., 1999; Lopez et *al*., 2003; Volkoff *et al*., 2003; Lee *et al*., 2004; Freitak *et al*., 2007; and Wang *et al*., 2010).

In the present study, sequencing of the eluted bands was performed and the blast search generated sequence similarity to cytochrome c oxidase gene. The sequencing of target genes is one of the most promising tools for detection and identification of antibacterial genes. In invertebrate host-pathogen systems, cytochrome oxidases have been shown to be up-regulated in response to immune stimulation as claims: (Gestal *et al.,* 2007) and shrimp: (James *et al.,* 2010). Abumourad (2011) suggested that cytochrome c oxidase subunit 1 (*CO1*) in *Tilapia* (*Oreochromis niloticus*) involved in the general immune response against the pathogenic bacteria.

CONCLUSION

Herein we have isolated and characterized cytochrome c oxidase gene from bacterial-fed larvae of *Cx. pipiens.* The present work is the first step that claims a role of cytochrome c oxidase in the immune response of *Cx. pipiens.* Further studies are required to explore the exact role of this gene in the immune response of *Cx. pipiens* due bacterial infection. Studies on the expression profile of this gene and antibacterial activity of its corresponding purified protein are recommended, too.

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