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Identification of genes involved in pyrethroid-, propoxur-, and dichlorvos- insecticides resistance in the mosquitoes, *Culex pipiens* complex (Diptera: Culicidae)

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ABSTRACT

Culex pipiens pallens and *Cx. p. quinquefasciatus* are important vectors of many diseases, such as West Nile fever and lymphatic filariasis. The widespread use of insecticides to control these disease vectors and other insect pests has led to insecticide resistance becoming common in these species. In this study, high throughout Illumina sequencing was used to identify hundreds of *Cx. p. pallens* and *Cx. p. quinquefasciatus* genes that were differentially expressed in response to insecticide exposure. The identification of these genes is a vital first step for more detailed investigation of the molecular mechanisms involved in insecticide resistance in *Culex* mosquitoes.

1. Introduction

The mosquitoes *Culex pipiens pallens* Coquillett and *Cx. pipiens quinquefasciatus* Say are the primary vectors of West Nile virus, St. Louis encephalitis virus, Eastern Equine Encephalitis virus, Japanese Encephalitis virus, Chikungunja virus, *Wucheria bancroftii* and the pathogens that cause lymphatic filariasis (Miller and Nasci, 1996; Turell, 2012). These mosquitoes are widespread globally and especially common in tropical and temperate regions (Cupp et al., 2011; Fonseca et al., 2004). They are often resistant to insecticides, a feature that may be related to their habit of breeding in habitats that are frequently exposed to insecticide runoff, such as sewers and drainage ditches (Cui et al., 2006). Pyrethroid and organophosphate insecticides have been widely used for decades in China and resistance to these chemicals is common in many Chinese mosquito species (Meng et al., 2011). Highly resistant populations of both species have been reported in several Chinese provinces (Liu et al., 2013).

Insecticide resistance is typically characterized by a variety of genetic modifications, such as transcriptional changes, changes in gene amplification and point mutations in coding regions, which allow increased rates of insecticide detoxification (metabolic detoxification), or reduce the sensitivity of target proteins (target site insensitivity) (Raymond et al., 1998; Hemingway et al., 2002; Scott, 1999). Analysis of mRNA has provided significant insights into the molecular basis of insecticide resistance (Hemingway et al., 2004; Ffrench-Constant et al., 2004). The advent of suppression subtractive hybridization (SSH) and microarray platforms has allowed research on the genetic basis of resistance in the *Cx. pipiens* species complex to progress from the analysis of a small number of candidate genes to high-throughput genetic profiling. Some previous research used SSH and specific microarray platforms to identify the genes associated with deltamethrin resistance in this group of mosquitoes (Tian et al., 2001; Wu et al., 2004; Liu et al., 2007). The genes identified were homologous with cytochrome P450, ribosomal RNA, ribosome proteins, trypsin and chymotrypsin-like proteins, all of which are known to play vital roles in cellular and molecular metabolism, signal transduction, vesicular and molecular transport, protein biosynthesis, ubiquitination and neuronal survival. Subsequent studies have found that other genes, such as opsin and ribosomal protein L22, are also involved in insecticide resistance (Gong et al., 2005; Hu et al., 2007; Tan et al., 2007,2012; Yang et al., 2008a,b; Xu et al., 2008; He et al., 2009; Sun et al., 2011,2012; Zhang et al., 2011; Zhou et al., 2012; Hong et al., 2013).

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Before recent developments in mosquito genetics, high throughput screening using the above methods was thought to have achieved very good results. However the limited understanding of the mosquito genome at that time meant that only a few hundred of the many thousand genes in the mosquito genome could be screened for anti-insecticidal activity. Consequently, knowledge of the genetic basis of insecticide resistance was limited to this small proportion of the total genome.

Insecticide resistance in mosquitoes is a very complex phenomenon. In addition to insecticide metabolizing genes, a large number of gene amplification products, such as ribosomal protein, myosin and actin, are involved in drug resistance. In addition, many other proteins with unknown functions have been identified in other insects. The development of resistance involves not only the regulation of several known families of genes, but a variety of complex, multi-level metabolic changes that allow individual insects to withstand the toxic effects of insecticides and insect populations to respond to the selective pressure imposed by widespread insecticide use.

The sequencing of the *Culex quinquefasciatus* genome in 2010 (Arensburger et al., 2010) made it possible to screen all 18.9 thousand genes in the mosquito genome for anti-insecticidal activity. We hypothesize that many gene expression patterns have become altered in *Culex* mosquitoes in response to selection for insecticide resistance. To test this hypothesis, and to isolate the genes or factors correlated with insecticide resistance, we used high throughput Illumina sequencing to monitor the differential expression of genes within the entire genome of two species of *Culex* mosquitoes in response to insecticide exposure. Using this approach we identified hundreds of genes that are up-regulated in insecticide resistant mosquitoes. The identification of these genes play a very important role for more detailed investigation of the molecular mechanisms involved in insecticide resistance in mosquitoes.

2. Materials and methods

2.1. Mosquito strains

Larvae of *Cx. pipiens pallens* and *Cx. pipiens quinquefasciatus* were collected from twelve sites in China in 2010 (Fig. 1). Samples were collected from open water such as sewages, ditches, sewers and puddles in rural and urban environments. No specific permissions were required to collect larvae these locations and their collection did not involve, or affect, endangered or protected species.

Collected larvae were transported to the laboratory and reared to adulthood. Bioassays for insecticide resistance were conducted on the larvae of this F_1 generation and the results compared to those of a susceptible strain of *Cx. pipiens pallens* that had been maintained in a laboratory for more than 30 years without exposure to insecticides. Larvae were flash-frozen at −70 °C after insecticide exposure trials.

2.2. Mosquito larval bioassays

To assess the susceptibility of *Cx. pipiens pallens* and *Cx. pipiens quinquefasciatus* larvae to insecticides, late 3rd and early 4th instar larvae were exposed to a total volume of 199 ml water treated with different concentrations of insecticides using the methods proposed by the WHO (1996). Five insecticides were tested in the study: deltamethrin (≥98%, Sigma, USA), beta-cypermethrin (≥98%, Sigma, USA), cyhalothrin (>99%, Sigma, USA), propoxur (>99%, Sigma, USA) and dichlorvos (>99%, Sigma, USA). All analytical grade of insecticides were diluted to five–seven concentrations with acetone. Each concentration of insecticide diluted in 1 ml acetone was applied onto the water surface with an automatic pipette. Each concentration of insecticide was tested on 30 larvae. All experiments were repeated three times and larval mortality recorded 24 h after treatment. Larval mortality was determined by dividing the number of dead larvae by the total tested. No food was provided to the larvae and a 14L:10D photoperiod, 75% relative humidity and temperature of 26 ± 1 °C were maintained in the laboratory during all bioassays.

LC_{50} values were calculated using the probit analysis program of Schoofs and Willhite (1984). As a measure of resistance we calculated the resistance ratio (RR) (Orshan et al., 2005), which was the ratio of the estimated LC_{50} of larvae of the wild-caught F_1 generation to that of larvae of the susceptible strain. In order to examine whether the resistance to insecticide was correlated among the sampling sites, the relationships between the estimated RR values of various insecticides were analyzed by Pearson correlation analysis. *P* value (significant at $P < 0.05$) was performed to determine if the correlation was significant.

2.3. Preparation of cDNA libraries for RNA-Seq

Total RNA of 30 mosquito larvae of each population (seven *Cx. pipiens pallens* population and five *Cx. pipiens quinquefasciatus* population) was extracted using the TRIzol reagent according to the manufacturer's protocol (Invitrogen, Burlington, ON, Canada). These samples came from the same generation that was used for the resistance bioassay and RNA-extracted in the same age (late 3rd and early 4th instar). The yield and purity of the extacted RNA were assessed by determining its absorbance (Abs) at 260 and 280 nm. RNA was only used if its Abs260 nm/Abs280 nm ratio was >1.8. RNA integrity was checked using a 1% agarose gel and the RNA 6000 Nano Assay Kit and Agilent 2100 Bioanalyzer. Extracted total RNA was stored at −70 °C for later use.

Total RNA samples were pooled and 10 mg from each pool was used to isolate poly(A) mRNA and to prepare non-directional Illumina RNA-Seq libraries with an mRNA-Seq 8 Sample Prep Kit (Illumina). There were two biological replicates in each mosquito population. Library quality control and quantification were performed using a Bioanalyzer Chip DNA 1000 series II (Agilent). Each library had an insert size of 200 bp; 42- to 50-bp sequences were generated via Illumina HiSeq™ 2000.

2.4. Mapping reads to the reference genome and annotated genes

The *Cx. pipiens quinquefasciatus* genome and gene information were downloaded from the *quinquefasciatus* Genome Annotation Project (Megy et al., 2009) (https://www.vectorbase.org/organisms/culex-quinquefasciatus, GenBank accession number: AAWU00000000.1). Sequencing-received raw image data were transformed by base culling into sequence data. Prior to mapping reads to the reference database, we filtered all sequences to remove adaptor sequences and low-quality sequences (the percentage of low quality bases with a quality value ≤5 was >50% in a read). The remaining reads were aligned to the mosquito genome using SOAPaligner/soap2, allowing up to two base mismatches. Reads that failed to be mapped were progressively trimmed off, one base at a time, from the 3′ end and mapped to the genome again until a match was found (unless the read had been trimmed by <27 bases). The insert between paired reads was set as 1 base-5 kilobases, allowing them to span introns of various sizes in the genome. The same strategy was used to align single-end reads to non-redundant genes, except that the insert was changed to 1 base-1 kilobase.