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

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Palavras-chave

Culex pipiens pallens, Culex pipiens quinquefasciatus, Gene, Insecticide resistance

Resumo (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.

Corpo do Texto

Analysis of correlation between mosquito immunity-related genes expression levels and insecticide resistance level

Among mosquito immune signaling molecules, there was a significant positive correlation between serpin and cyhalothrin and propoxur, dorsal and cyhalothrin resistance.

A significant positive correlation was found between seven immune-related genes expression levels and resistance to different insecticides.

The degree of correlation between dozens of mosquito immunity-related genes and the level of insecticide resistance was analyzed (Table 7).

cecropin was also correlated with resistance to all three pyrethroids.

C-type lectin is associated with resistance to five insecticides, whereas, excluding propoxur, fibrinogen is associated with resistance to four.

Among mosquito immune recognition molecules, a significant positive correlation was found between the peptidoglycan recognition protein (PGRP) and beta-cypermethrin resistance.

Among mosquito immune effector molecules, there was a significant positive correlation between prophenoloxidase and cyhalothrin resistance.

Analysis of differential gene expression in twelve mosquito populations

GO functional enrichment analysis was carried out using Blast2GO.

The R package DEGseq was applied to identify DEGs with a random sampling model based on the read count for each gene in each mosquito population.

We used “FDR $\leq$0.001 and the absolute value of log2 Ratio $\geq$1” as the threshold for judging the significance of each gene expression difference (Wang et al., 2010).

More stringent criteria with a smaller FDR and bigger change value can be used to identify DEGs.

Analysis of gene expression

Although the majority of gene is expressed at low levels, a small proportion is highly expressed.

Redundancy is a term typically used to describe situations where a given biochemical function is redundantly encoded by two or more genes.

Although 11,964 genes are expressed in all twelve mosquito populations (shown in Supplementary Table 1), many of which are differentially expressed.

Genes with little variation are thought to fulfill housekeeping functions.

Heterogeneity and redundancy are two significant characteristics of mRNA expression.

The distribution of distinct tags over different tag abundance categories showed similar patterns for all the RNA-Seq libraries.

ERANGE (version 4.0; http://woldlab.caltech.edu/gitweb/) measures gene expression in reads per kilobase per million mapped sequence reads (RPKM), a normalized measure of read density that allows transcription levels to be compared both within and between samples.

Heterogeneity is a phenomenon in which a single phenotype or genetic disorder may be caused by any one of a multiple number of alleles or non-allele mutations.

One of the primary goals of RNA sequencing is to compare gene expression levels between populations.

As ERANGE distributes multi-reads at similar loci in proportion to the number of unique reads recorded, we included in the analysis both unique reads and reads that occurred up to ten times to avoid undercounting genes with closely related paralogs.

The similarity distribution had a comparable pattern with more than 20% of the sequences having a similarity >80%, while approximately 80% of the hits had a similar range.A total of 14,807 (Xiuying), 15,083 (Longhua), 15,264 (Qiongshan), 14,489 (Meilan), 14,338 (Guangzhou), 14,322 (Nanjing), 15,167 (Dalian), 15,062 (Chaoyang), 14,920 (Haidian), 14,994 (Shunyi), 15,351 (Shijingshan) and 15,467 (Tongzhou) genes, ranging from 75–≧2,000 bp, were detected in the samples.

Therefore, the distribution of tag expression was used to evaluate the normality of our RNA-Seq data.

Data deposition

Detailed transcription data for the 17,757 genes detected in the present study are presented in Additional file 1 (Supplementary Table 1, GEO number is GSE57307).

All gene accession numbers mentioned in the present manuscript are compatible with VectorBase http://cquinquefasciatus.vectorbase.org genome databases.

Discussion

Previous research has demonstrated that increasing the expression levels of immune genes can affect mosquitoes' vector capability.

As a protein related to olfaction, we speculate that OBPjj7a may increase the ability of mosquitoes to detect the odor of beta-cypermethrin and deltamethrin.

In this study, we found a positive correlation between expression of several immune genes and resistance, irrespective of whether these were immune recognition molecules, signaling molecules or effector molecules, and there was no evidence of negative correlation between the expression of immune genes and resistance.

The analysis indicates that not as many metabolic enzyme genes are associated with resistance as previously thought.

This result could lead to the development of a novel biosensor based on this protein to detect insecticide residues in the environment.

pipiens quinquefasciatus.

p. quinquefasciatus (Reid et al., 2012).

The further understanding of genes in resistance will provide us the important information for monitoring resistance development and delaying resistance development.

We speculate that this is because, either the mosquitoes used in this study had not yet developed high levels of resistance to propoxur and dichlorvos, or we were unable to identify the genes related to propoxur and dichlorvos resistance.

It is an interesting research direction to understand the function of proteases in insecticide resistance and deserves further investigation.

Cytochrome P450 has been considered the main metabolic enzyme involved in metabolizing pyrethroids in insects.

The GO functional analysis indicated that the percentage of genes related to pyrethroid resistance was much higher than the percentage related to propoxur and dichlorvos resistance.

Chitinase plays an important role in insect postembryonic development, especially larval molt and emergence (Eijsink et al., 2010; Arakane and Muthukrishnan, 2010).

All these results show that resistance involves diverse aspects of mosquito physiology, including some that have yet to be discovered.

By correlating gene expression with different levels of insecticide resistance, hundreds or even thousands of genes are found either positively or negatively associated with different levels of insecticide resistance.

With the development of new molecular techniques, such as suppression subtractive hybridization (SSH) and gene chips, high-throughput sequencing has been widely applied to identify the genes involved in resistance.

This changes cellular redox capacity, which could affect the survival of viral pathogens (Hemingway and Ranson, 2000).

There has been a long history of research on the relationship between mosquito resistance and pathogen transmission levels.

Moreover, The new information presented here will provide fundamental new insights into designing novel strategies to control the mosquito vectors, reducing the prevalence of mosquito-borne diseases.

These results are consistent with a gradual increase in insecticide resistance in response to increased insecticide use.

Therefore, we only analyzed genes that were expressed in more than 6 populations.

p. quinquefasciatus have been found to be up-regulated in the permethrin-selected strain, suggested the importance of the up-regulation of proteases in insecticide resistance.

In recent years, RP has been identified as the main protein involved in protein synthesis.

In conclusion, our study strongly supported the hypothesis that biological and physiological changes and genetic signatures are significantly altered in response to insecticide selection.

There was a significant positive correlation between this gene and resistance to beta-cypermethrin and deltamethrin, but not cyhalothrin.

Among the genes with a negative correlation coefficient >0.9, we found genes that influence the alternative splicing of mRNA, such as splicing factor 3B subunit 5, and also genes that affect reproductive and embryonic development, such as protein slowmo and protein pangolin.

Moreover, a miscellaneous RNA gene was significantly negatively correlated with cyhalothrin resistance.

The results indicate a correlation coefficient >0.9 between chitinase expression and resistance to beta-cypermethrin and deltamethrin.

Gene overlap analysis indicates that a total of 11 kinds of metabolic enzymes were significantly associated with resistance to the three insecticides, including eight CYP genes.

If future studies confirm this conjecture, this will be the first evidence that an olfactory gene enhances insects' ability to detect insecticides.

However, some of these could only be detected in less than 5 populations.

However, one of the limitations of gene chip technology is that it can only be used for known genes.

Among genes with a positive correlation coefficient >0.9, the odorant-binding protein jj7a (OBPjj7a) is unique.

It is also involved in regulating gene transcription and translation, and thereby cell proliferation and apoptosis (Warner and McIntosh, 2009; Lindström and Zhang, 2008; Matragkou et al., 2008).

In the case of cytochrome P450, only about 10% of genes are associated with resistance, some of which have been confirmed in studies of other resistance related enzymes, such as CYP6AA1.

Studies of susceptible and resistant strains of Cx.

We speculate that one mechanism of beta-cypermethrin and deltamethrin resistance is increasing the generation of chitin thereby reducing the penetration of these insecticides via the cuticle.

This suggests that insecticide resistance has evolved through multiple physiological pathways.

We found that expression of many ribosomal protein genes was negatively correlated with cyhalothrin resistance.

The metabolic resistance of mosquitoes to chemical insecticides has been widely reported worldwide and several studies have described the over-production of detoxification enzymes in resistant populations.

Liu et al. (2011) identified similar genes and reached similar conclusions.

Determination of these molecular targets will lay the foundation for the future development of molecular marker-based detection methods.

Tian et al. (2001) and Liu et al. (2007) used SSH and microarray methods to identify many such genes in Cx.

In the past decade, a detailed study of chymotrypsin, ribosomal protein L39, ribosomal protein L22 and opsin genes established the correlation between mosquito resistance and gene expression levels (Tan et al., 2007; Yang et al., 2008b; He et al., 2009; Sun et al., 2012).

However, there was no correlation between the expression of chitinase and resistance to the other insecticides.

The genomic resources and knowledge developed by this study will contribute to better understanding molecular mechanisms that govern development of insecticide resistance.

pipiens pallens and Cx.

Some previous studies have found that the higher the level of insecticide resistance in mosquito populations the lower their fitness in terms of hatching, survival, emergence and spawning rates (Bourguet et al., 1997; Berticat et al., 2002,2008; Agnew et al., 2004).

Cytochrome P450 monooxygenases (CYPs for genes), carboxylesterases (ESTs) and glutathione S-transferases (GSTs) are common metabolic enzymes in mosquitoes.

Our analysis leads us to believe that these eight CYPs are the key enzymes responsible for metabolizing pyrethroids in the Cx.

Negative correlation means that gene expression decreases with increasing resistance.

pipiens quinquefasciatus indicate that esterase is highly expressed in the midgut, dermis, malpighian tubules, salivary glands and other tissues in resistant mosquitoes.

The mechanisms involved in insecticide resistance in mosquitoes have been extensively studied over the past decade.

Our results provide the first evidence that certain mosquito genes are involved in insecticide resistance.

In the study, data on a total of 17,757 genes is obtained.

quinquefasciatus genome sequence contains 18.9 thousand universal protein-coding genes.

Therefore, the generation of insecticide resistance through metabolic enzymes may both promote and inhibit the spread of the vector-borne diseases.

For example, ribosomal protein (RP) is a widespread RNA-binding protein in animals.

Our results support these findings to a certain extent.

From the results of sequencing and GO analysis, profound physiological changes had been found in the resistant mosquitoes, showing up- or down-regulation of genes.

In the study of Reid et al. (2012), some proteases from Cx.

A correlation coefficient greater than 0.9 means strong correlation between gene expression and resistance.

Pathogen defense-related immune genes play an even more important role in the transmission of mosquito-borne pathogens.

The Cx.

However, increased cytochrome P450 increase increases the oxidation of cells which is not conducive to the survival of pathogens (Dimopoulos et al., 2002).

Recent years, high throughput sequencing was gradually used in the exploration of resistant mechanism in Aedes aegypti (David et al., 2010), Anopheles gambiae (Bonizzoni et al., 2012) and Cx.

Such genes have a significant effect on mosquitoes' physiological functions.

In the cellular component category, many genes belonging to the plasma membrane and ribosome were negatively correlated with cyhalothrin resistance.

To adapt to this physiological change, chitinase expression must also increase so that mosquitoes can successfully complete molting, eclosion and related processes such as peritrophic membrane regeneration.

Consequently only hundreds, rather than thousands, of genes have been studied, most of which are detoxification enzyme genes, such as the P450 and esterase gene.

pipiens Complex.

The relationship between the expression of these genes and mosquito resistance is still unknown.

Pyrethroid-resistant mosquitoes protect their tissues from oxidative injury by elevating glutathione S-transferase enzyme, which may be conducive to pathogen survival (Vontas et al., 2001; McCarroll et al., 2000).

There were also some genes that we previously thought would be positively correlated with resistance but found to be negatively correlated with it.

The majority of genes with this level of correlation were those coding for enzymes involved in metabolic processes and the process of digestion, such as glycerate kinase carboxypeptidase and chymotrypsin, and those involved in reproductive growth and development, such as chitinase and sulfhydryl oxidase.

GO analysis of resistance related genes

Meanwhile, the results obtained under a biological process category were similar.

The number of genes in the physiological processes of signal transduction, multicellular organismal development, protein modification process, anatomical structure

morphogenesis, translation and cytoskeleton organization, negatively associated with cyhalothrin resistance far exceeded the number negatively associated with resistance to other insecticides.

Results for the molecular function and biological process category were similar.

The percentage of genes correlated with three pyrethroid resistance accounted for about 5% (4.6–6.1% in cellular; 5.6–6.9% in intracellular) in the cellular and intracellular gene function category, whereas that of genes correlated with propoxur and dichlorvos resistance only accounted for about 2% (1.4–1.7% in cellular; 2.1–2.4% in intracellular) in the category.

Resistance to all five insecticides was, however, also negatively correlated to 252, 145, 1085, 69 and 349 gene expression levels, respectively; i.e., the greater the resistance the lower the level of gene expression.

GO analysis produced several interesting results.

For example, in the plasma membrane of cellular component category, more than 3% of the genes were negatively correlated with pyrethroid resistance, 10.1% in the case of cyhalothrin resistance.

This is significantly less than the number of genes associated with pyrethroid resistance and therefore may be related to high pyrethroid resistance in these mosquitoes.

Resistance (LC50) to deltamethrin, beta-cypermethrin, cyhalothrin, propoxur and dichlorvos was positively correlated to 1090, 1047, 1317, 368 and 340 gene expression levels, respectively; i.e., the greater the resistance the higher the level of gene expression.

There were 33, 32, and 23 functional groups, respectively, in each of the three main categories (biological process, molecular function, and cellular component) of the GO classification (Fig. 2).

To facilitate the analysis of gene expression, all predicted resistance-related genes were assigned to different functional categories using Blast2GO (version 2.3.5) (http://www.blast2go.org/).

Presumably for this reason, 48 (14.1%) of genes which function in mosquitoes structural molecule activity were shown negatively correlated with cyhalothrin resistance.

It indicated that the higher the mosquitoes resistance to cyhalothrin coincide with the lower level of expression in plasma membrane genes and ribosome genes.

Binding (GO: 0005488), 1049 genes, and cell part (GO: 0044464), 960 genes, were the dominant sub-categories in the main categories of molecular function and cellular component, respectively.

This affected the physiological function of mosquitoes to some extent.

The results of GO analysis (Fig. 2 and Supplementary Tables 2,3) indicate that, under a cellular component model, the percentage of resistance-related genes in both the cellular and intracellular gene function categories were associated with resistance level to different insecticide type.

Moreover, 37 of 110 (33.6%) ribosome genes, such as 28S ribosomal protein S24, 30S ribosomal protein S8, 40S ribosomal protein S2–S23 and 60S ribosomal protein L3–L44, were significantly correlated with genes for cyhalothrin resistance (Table 4).

Metabolic process (GO: 0008152), with 440 genes, was the dominant sub-category in the main category of biological process.

The annotations were verified manually and integrated using Gene Ontology (GO) classification (http://www.geneontology.org).

Illumina sequencing and alignment to the reference genome

The remaining 16.30% of reads were unmatched (Table 1) because the software only matches reads that align entirely within exonic regions (reads from exon–exon junction regions will not match).

We sequenced the cDNA libraries, and generated 11,579,627–12,482,038 sequence reads, each of which was 42–50 bp in length, encompassing 18.45 Gb of sequence data (Table 3).

The RNA-Seq method generates absolute information, rather than relative gene expression measurements, thereby avoiding many of the inherent limitations of microarray analysis.

pipiens quinquefasciatus reference genome database using SOAPaligner/soap2 software (set to allow up to two base mismatches).

An average of 83.70% of the total reads matched to either unique (76.83%) or multiple (6.88%) genomic locations.

The sequence reads were aligned to the Cx.

Each population was represented by approximately 12 million reads, a tag density sufficient for the quantitative analysis of gene expression.

An immediate application of our transcriptome sequence data included gene expression profiling of different populations.

This method was used to analyze variations in gene expression in different mosquito populations with high or low insecticide resistance.

Introduction

Before recent developments in mosquito genetics, high throughput screening using the above methods was thought to have achieved very good results.

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 advent of suppression subtractive hybridization (SSH) and microarray platforms has allowed research on the genetic basis of resistance in the Cx.

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).

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.

The mosquitoes Culex pipiens pallens Coquillett and Cx.

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).

Using this approach we identified hundreds of genes that are up-regulated in insecticide resistant mosquitoes.

We hypothesize that many gene expression patterns have become altered in Culex mosquitoes in response to selection for insecticide resistance.

These mosquitoes are widespread globally and especially common in tropical and temperate regions (Cupp et al., 2011; Fonseca et al., 2004).

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.

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.

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.

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).

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).

In addition, many other proteins with unknown functions have been identified in other insects.

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.

Insecticide resistance in mosquitoes is a very complex phenomenon.

Highly resistant populations of both species have been reported in several Chinese provinces (Liu et al., 2013).

pipiens species complex to progress from the analysis of a small number of candidate genes to high-throughput genetic profiling.

The identification of these genes play a very important role for more detailed investigation of the molecular mechanisms involved in insecticide resistance in mosquitoes.

Analysis of mRNA has provided significant insights into the molecular basis of insecticide resistance (Hemingway et al., 2004; Ffrench-Constant et al., 2004).

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).

Consequently, knowledge of the genetic basis of insecticide resistance was limited to this small proportion of the total genome.

Mapping reads to the reference genome and annotated genes

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.

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 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.

The insert between paired reads was set as 1 base-5 kilobases, allowing them to span introns of various sizes in the genome.

Mosquito larval bioassays

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.

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.

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).

Each concentration of insecticide was tested on 30 larvae.

To assess the susceptibility of Cx.

pipiens pallens and Cx.

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 experiments were repeated three times and larval mortality recorded 24 h after treatment.

LC50 values were calculated using the probit analysis program of Schoofs and Willhite (1984).

Each concentration of insecticide diluted in 1 ml acetone was applied onto the water surface with an automatic pipette.

Larval mortality was determined by dividing the number of dead larvae by the total tested.

All analytical grade of insecticides were diluted to five–seven concentrations with acetone.

As a measure of resistance we calculated the resistance ratio (RR) (Orshan et al., 2005), which was the ratio of the estimated LC50 of larvae of the wild-caught F1 generation to that of larvae of the susceptible strain.

P value (significant at $P < 0.05$) was performed to determine if the correlation was significant.

Mosquito strains

pipiens pallens and Cx.

Larvae of Cx.

Larvae were flash-frozen at −70 °C after insecticide exposure trials.

Bioassays for insecticide resistance were conducted on the larvae of this F1 generation and the results compared to those of a susceptible strain of Cx.

Collected larvae were transported to the laboratory and reared to adulthood.

pipiens pallens that had been maintained in a laboratory for more than 30 years without exposure to insecticides.

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.

Normalized expression levels of genes from RNA-Seq and GO analysis

The expression level of a gene from RNA-Seq was normalized by the RPKM (reads per kilo bases per million reads) value.

The cutoff value for determining gene transcriptional activity was determined based on a 95% confidence interval for all RPKM values for each gene.

Blast2GO was also used for the GO functional enrichment analysis of certain genes.

ERANGE software (version 4.0) (http://woldlab.caltech.edu/gitweb/) was used to calculate normalized gene locus expression levels by assigning reads to their site of origin and counting them.

We obtained the GO terms for each gene using Blast2GO (version 2.3.5) (http://www.blast2go.org/) with the default parameters.

This was done by performing Fisher's exact test with a robust FDR correction to obtain an adjusted P-value between test gene groups and the whole genome annotation.

Overlap analysis of genes associated with resistance to three pyrethroid insecticides

One hundred and thirty-seven genes displayed a >50% negative correlation with resistance.

The overlap between genes associated with resistance to three different pyrethroids was investigated (Fig. 3).

The results show that the highest degree of overlap, >75%, was in the 858 genes associated with deltamethrin and beta-cypermethrin resistance.

The least correlation, either positive or negative, was found between genes involved in beta-cypermethrin and cyhalothrin resistance.

In contrast, there was little overlap between the genes involved in deltamethrin and cyhalothrin, or beta-cypermethrin and cyhalothrin, resistance.

This is consistent with the lack of cross-resistance found, other than that between deltamethrin and beta-cypermethrin.

Preparation of cDNA libraries for RNA-Seq

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).

pipiens pallens population and five Cx.

The yield and purity of the extacted RNA were assessed by determining its absorbance (Abs) at 260 and 280 nm.

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.

pipiens quinquefasciatus population) was extracted using the TRIzol reagent according to the manufacturer's protocol (Invitrogen, Burlington, ON, Canada).

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).

RNA was only used if its Abs260 nm/Abs280 nm ratio was >1.8.

Total RNA of 30 mosquito larvae of each population (seven Cx.

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.

Selection of genes significantly associated with resistance

Genes with correlation coefficients of more than 0.9 are considered to be significantly associated with resistance and shown in Tables 8 and 9.

These were comprised of 13 deltamethrin resistance related genes, 11 beta-cypermethrin resistance related genes and 58 cyhalothrin resistance related genes.

In addition, deltamethrin resistance was correlated with the previously identified H/ACA ribonucleoprotein complex genes, and beta-cypermethrin resistance with the glycerate kinase peptidoglycan recognition protein and sodium-dependent phosphate transporter genes.

In contrast, the genes associated with cyhalothrin resistance were completely different; most are involved in enzyme production and some were not previously known to be involved in resistance.

Six genes were identical with respect to deltamethrin and beta-cypermethrin, three of which were chitinase, sulfhydryl oxidase 1 and odorant-binding protein OBPjj7a.

Sensitivity status to insecticides

pipiens quinquefasciatus from Xiuying, Longhua, Qiongshan and Meilan had a high level of resistance to three pyrethroid insecticides.

However, no similar relationship was found for resistance to the other insecticides.

Larvae of mosquitoes collected from all sample sites showed varying levels of resistance to the five insecticides tested (Table 1).

Cx.

There was a positive correlation between resistance to beta-cypermethrin and deltamethrin among populations, i.e., populations with high resistance to beta-cypermethrin tended to also be highly resistant to deltamethrin ($P < 0.05$) (Table 2).

Average resistance ratios (RR) to beta-cypermethrin, deltamethrin, propoxur, dichlorvos and cyhalothrin were 41.8 (0.8–168.3), 52.6 (1.1–227.9), 4.4 (2.6–7.4), 9.6 (1.2–15.2) and 256.9 (1.0–924.1), respectively.

However, all populations had relatively low resistance to propoxur.

Statistical analysis of relationship between insecticide resistance and gene expression

P value (significant at $P < 0.05$) was performed to determine if the correlation was significant.

The level of significance of each test was adjusted to take into account the number of tests performed using the Bonferroni correction (Rice, 1989).

To assess levels of gene expression and insecticide resistance in mosquito populations, the relationship between the RPKM of each gene and the LC50 value of each insecticide in each mosquito population was analyzed using SAS Software 6.22 (SAS Institute Inc.).

Coefficients of Pearson correlation were calculated between the RPKM (reads per kilo bases per million reads) of each gene and the corresponding resistance ratio for each insecticide in each mosquito population.

Transcripts related to general insecticide detoxification and target proteins

Cuticle protein is often considered one of the main factors conferring resistance to insecticide penetration and we found that certain cuticle protein genes were associated with beta-cypermethrin, cyhalothrin, and propoxur resistance.

We also analyzed the degree of overlap of genes that regulate deltamethrin, beta-cypermethrin and cyhalothrin resistance related enzymes, such as cytochrome P450 (CYP), esterase (EST) and glutathione-s-transferase (GST) genes (Fig. 4).

For example, there are a total of nearly 200 cytochrome P450 genes in Cx.

Acetylcholinesterase and GABA receptor expression levels were also negatively correlated with cyhalothrin and dichlorvos resistance.

Some genes related to insecticide resistance were indentified by blasting Cx.

One unexpected result was that the genes associated with several cytochrome P450, carboxylesterases and ABC transporters were negatively correlated with resistance (Table 6).

However, unique resistance-associated metabolic enzyme genes are also involved in resistance to these insecticides.

In pairwise comparisons between deltamethrin and beta-cypermethrin, deltamethrin and cyhalothrin and beta-cypermethrin and cyhalothrin, the degree of overlap was almost identical.

The results show that eight CYP, two GST and one EST gene are related to resistance to these three insecticides.

Although the results identified some gene families already known to be correlated with insecticide resistance, such as cytochrome P450, carboxylesterases and GSTs genes, the number of genes associated with resistance was not as many as we had anticipated (Table 5).

These results indicate that expression of the target gene for resistance can be negatively regulated, that is, the level of gene expression decreases with increasing resistance.

However, several other cuticle protein genes were negatively associated with resistance.

pipiens transcriptomes against the VectorBase nucleotide database.

However, sodium channel gene expression levels were negatively correlated with cyhalothrin resistance.

Four of nine CYP genes are associated with deltamethrin and beta-cypermethrin resistance, and two of nine CYP genes and a GST gene are associated with cyhalothrin resistance.

pipiens complex mosquitoes but we identified only 19, 16, 29, 5 and 24 genes is positively associated with deltamethrin, beta-cypermethrin, cyhalothrin, propoxur and dichlorvos, respectively.

An interesting result was that there was a significant correlation between the expression level of the pyrethroid targeted sodium channel in deltamethrin and beta-cypermethrin resistance related genes and resistance to these two insecticides.

However, there are still a number of metabolic genes, such as ABC transporters, catalase, superoxide dismutase and NADH dehydrogenase, associated with resistance.

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