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Sodium Channel Genes and Their Differential Genotypes at the L-to-F *Kdr* Locus in the Mosquito *Culex quinquefasciatus*

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Abstract

The *para*-type sodium channel in insects is the primary target of pyrethroid and DDT insecticides. However, modifications in the target protein structure such as point mutations or substitutions, resulting from single nucleotide polymorphisms (SNP), cause insensitivity of the insect's nervous system to pyrethroids and DDT and, in turn, result in insecticide resistance. Among these mutations, substitution of leucine to phenylalanine (L to F) in the 6th segment of domain II (IIS6) has been clearly associated with pyrethroid and DDT resistance in many insect species, including mosquitoes. Here, multiple copies of the sodium channel gene were identified in the mosquito *Culex quinquefasciatus* by Southern blot analysis and polymerase chain reaction (PCR) analysis. Two genomic DNA fragments of the mosquito sodium channel gene (509 bp and 181 bp) were detected by a single PCR primer pair. Sequence analysis indicated the lack of an intron sequence in the 181 bp sodium channel fragment. Single nucleotide polymorphism (SNP) analysis revealed a strong correlation among the frequencies of L-to-F allelic (T) expression at the RNA level, the frequencies and resistance allele (T) at the L-to-F site of the 509 bp genomic DNA fragment, which did include an intron sequence, and the levels of insecticide resistance. Taking together, this study, for the first time, not only revealed multiple copies of the sodium channel gene presented in the *Culex* mosquito genome but also suggested that the one with the intro sequence may be a functional copy of the sodium channel gene in the *Culex* mosquitoes. .

Keywords

Sodium channel; multiple copies; insecticide resistance; *Culex quinquefasciatus*

Introduction

Voltage-gated sodium channels are responsible for the depolarization phase of action potentials in membranes of neurons and in all types of excitable cells [1]. The *para*-type sodium channel in insects is the primary target of pyrethroid and DDT insecticides [2]. Pyrethroids and DDT deliver their toxic, insecticidal effects primarily by binding onto the sodium channel, altering its gating properties, and keeping the sodium channel open for

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unusually long times. This prolongs the flow of the sodium current, which in turn elevates and extends the depolarizing phase of the action potential of the neuron membrane and thus initiates repetitive discharges and prevents the repolarization phase of the action potentials [3]. However, modifications in the sodium channel structure such as point mutations or substitutions resulting from single nucleotide polymorphisms [SNP] can dramatically lower sensitivity to DDT and pyrethroids in the sodium channels of the insect's nervous system by reducing or even eliminating the binding affinity of the insecticides to proteins [3], thus diminishing the toxic effects of the insecticides and conferring insecticide resistance [4,5]. Reduced target-site sensitivity of sodium channels is known to be one of the major mechanisms of pyrethroid resistance and is referred to as knockdown resistance (*kdr*) [6].

An increasing body of evidence from molecular and pharmacological studies has pointed to the involvement of point mutations in voltage-gated sodium channels in *kdr* and *kdr*-like resistance in many medically or agriculturally important pest species, including the mosquito [4,5,7–9]. Among these *kdr* mutations, substitution of leucine to phenylalanine [L to F], histidine [L to H], or serine [L to S] in the 6th segment of domain II (IIS6) resulting from a single nucleotide polymorphism, which in the case of mosquitoes is the change from codon TTA to TTT) has been clearly associated with resistance to pyrethroids and DDT. This L to F *kdr* mutation was first detected in pyrethroid-resistant house flies and cockroaches, but has now also been found in many other pyrethroid-resistant insect species, although other *kdr* mutations appear to be unique to specific species in many insect species [10–13]. Recently, our group investigated the frequency of alleles (A or T) at the L-to-F locus at genomic DNA and RNA levels within the same individuals of different mosquito strains of *Culex quinquefasciatus* bearing different resistant phenotypes [14,15]. In the earlier study we generated a 341 bp PCR fragment, where the L-to-F mutation resides, from the mosquito sodium channel genomic DNA but found no correlation between the L-to-F allele (T) at the genomic DNA level and the level of permethrin resistance. However, there was a strong correlation between the prevalence of the L-to-F allelic (T) expression at the RNA level and the level of resistance [15]. Interestingly, we detected no introns in this 341 bp sodium channel Genomic DNA region, in contrast to previous reports [16]. We therefore proposed that multiple copies of the sodium channel gene may be present in the genome of *Cx. quinquefasciatus*. Building on our previous research, the current study was designed to characterize the genomic organization and allelic expression at the L-to-F site of the sodium channel gene in *Cx. quinquefasciatus*.

Materials and Methods

Mosquitoes

Five strains of *Cx. quinquefasciatus* were used in this study: 2 field collected strains HAmCq^{G0} and MAmCq^{G0} from Madison County and Mobile County, Alabama, respectively [17]; two permethrin selected resistant strains HAmCq^{G8} and MAmCq^{G6}, which were the 8th and the 6th generation permethrin-selected offspring of HAmCq^{G0} and MAmCq^{G0}, respectively [15,18]; and a susceptible strain S-Lab, provided by Dr. Laura Harrington (Cornell University).

Southern blot analysis

Genomic DNA was extracted from the mosquitoes as described by Xu et al. [14]. Genomic DNA (40 µg) was digested completely with 260 units *Eco*RI or *Hind*III in a 170 µl solution. Samples were precipitated with ethanol and then re-suspended in TE buffer prior to electrophoresis on 0.8% agarose gels in 0.5× TBE, after which they were transferred to Nytran membranes (Schleicher and Schuell, Keene, NH, USA). To optimize the detection of a single band rather than multiple bands per sodium channel gene, the probe design met the

following 2 criteria: (1) lack of internal *Eco*RI and *Hind*III restriction cutting sites and (2) inclusion within a single exon. The mosquito *para* type sodium channel cDNA fragments were amplified using a primer pair: KDR S1S (5' GACAACGTGGACCGCTTCCCGGACA 3')/KDR AS1S (5' ACGACAAAAT TTCCTATCACTACGGTG 3'). The cDNA fragment was labeled with [α -³²P] dCTP using a High Prime Labeling Kit (Roche, Indianapolis, IN) and hybridized with blots using QuickHyb solution (Stratagene, La Jolla, CA).

Amplification of *para*-type sodium channel gene fragments from genomic DNA and RNA of *Culex* mosquitoes

Males and females of each mosquito population had their genomic DNA and RNA extracted for each experiment. Three replications were performed, each on a different day, for a total of 50 individual mosquitoes from each population. The sodium channel cDNA fragments were amplified by a primer pair KDR S#8 (5'-ATTTCATCATCGTGGCCCTTTCGC-3') and KDR AS#1 (5'-TTGTTTCGTTTCGTTGTCGGCTGTG-3'), which generated amplicons that spanned the intron/exon boundaries but did not amplify genomic DNA. This approach eliminated any influence due to genomic DNA contamination. The sodium channel genomic DNA fragments were amplified using a primer pair KDR S14 (5' GGAAGTTACCGACTTCATGCACTC 3') and KDR AS1 (5' GACAAAAGCAAGGCTAAGAAAAGG 3'). The primers were designed based on exonic sequences that would not generate products in RNA samples without a reverse transcription step.

SNP determination for the *kdr* allele in *Culex* mosquitoes

The frequency and heterozygosity of the *kdr* allele in mosquitoes were investigated further by SNP determination using an ABI Prism SNaPshot Multiplex Kit and analyzed on the ABI Prism® 3100 Genetic Analyzer using Genemapper software according to the manufacturer's instructions (A&B Applied Biosystems) [15,19]. The primer Cx_SNP2 (5' GCCACCGTAGTGATAGGAAATTT 3') used for SNP determination was designed according to the sequence immediately upstream of the *kdr* allele. Three replications of the SNP determination were carried out with different preparations of the PCR templates. To confirm whether the PCR products used for the SNP determination were in fact the *para*-type sodium channel gene fragments, PCR products from both the cDNA and genomic DNA of each individual were sequenced at least once each.

Results

Identification of multiple sodium channel genes in the genome of *Culex* mosquitoes

To examine the number of copies of the *para*-type sodium channel gene in the genome of *Culex* mosquitoes, genomic DNA isolated from S-Lab and HAMCq^{G8} was subjected to Southern blot analysis with the sodium channel cDNA probe generated according to a single exon sequence (exon 20, according to Saavedra-Rodriguez et al. [20]) of mosquito sodium channel gene using a primer pair: KDR S1S /KDR AS1S. Two genomic DNA bands with molecular sizes of approximately 4 and 2 kb were detected in pyrethroid resistant HAMCq^{G8} mosquitoes when mosquito genomic DNAs were digested with either *Eco*R I or *Hind* III (Fig. 1). Digestion of genomic DNA of S-Lab with either *Eco*R I or *Hind* III resulted in 3 genomic DNA bands with approximately molecular sizes of ~5, 2, and 1.5 kb or ~4, 2.5, and 2 kb, respectively (Fig. 1). The different numbers and sizes of genomic DNA molecules detected in HAMCq^{G8} and S-Lab may be due to polymorphisms at the restriction enzyme cutting site(s) between these two strains. These results, reported here for the first time, strongly suggest that multiple, or at least two, sodium channel genes are present in the genome of the mosquito *Cx. quinquefasciatus*.

Sequencing Analysis of Multiple Sodium Channel Genes in Mosquitoes

Two genomic DNA products (509 bp and 181 bp) were generated by PCR from all 50 individuals in each of the five mosquito strains tested, ranging from susceptible (S-Lab) through intermediate (HAmCq^{G0} and MAmCq^{G0}) to highly resistant (HAmCq^{G8} and MAmCq^{G6}), when only a single primer pair, KDR S14/KDR AS1, was used. The PCR products of both 509 bp and 181 bp from 5 individual samples of each strain were then sequenced and an analysis of the results indicated that the 509 bp PCR product of the sodium channel gDNA was indeed the mosquito sodium channel gene sequence, with an intron sequence near the L-to-F site (Fig. 2) as previously reported in mosquitoes [16]. The 181 bp PCR product did not include the intron sequence (Fig. 3), which was consistent with the previous report by Xu et al. [14,15]. This discovery provided not only strong support for our Southern blot analyses, which indicated that there were at least two copies of the sodium channel gene present in the genome of the mosquito *Cx. quinquefasciatus*, but also suggested an explanation for the controversial results reported by Martinez-Torres et al. [16] and Xu et al. [14,15], namely that each of the studies identified only one of the two copies of the sodium channel gene - one with an intron near the L-to-F site [16] and the other without the intron [14,15].

Comparison of L-to-F *kdr* allelic variation at the genomic DNA and RNA levels of *Culex* mosquitoes

The frequency of alleles (A or T) at the L-to-F *kdr* locus at genomic DNA (i.e., two DNA fragments) and RNA levels was investigated in 50 individuals from each of the HAmCq^{G8}, MAmCq^{G6}, HAmCq^{G0}, MAmCq^{G0} and S-Lab strains. We first assessed the RNA expression variation of the A or T alleles at the L-to-F *kdr* locus. A strong correlation between the prevalence of the L-to-F allelic expression at the RNA level and the level of susceptibility or resistance to permethrin was identified in all 5 mosquito strains. All 50 individuals in the susceptible S-Lab strain expressed susceptible allele A at the L-to-F *kdr* locus of the sodium channel gene at the RNA level, resulting in a codon TTA encoding Leu corresponding to the susceptibility in this strain to permethrin. In contrast, in the highly resistant *Culex* strains of HAmCq^{G8} and MAmCq^{G6} with multiple generations of permethrin selection in the laboratory after collection from the field, 84 and 100% of the individuals, respectively, showed monoallelic expression of the T allele resulting in a change from Leu to Phe (Table 1), which strongly correlated with the high level of resistance in these two populations. Furthermore, there was a clear pattern of allelic variation in individuals of the two field collected parental strains HAmCq^{G0} and MAmCq^{G0} (Table 1), with frequencies ranging from 0.02 and 0.04, respectively, for individuals that expressed only the susceptible allele A, through 0.48 and 0.38 for those that expressed both alleles, and 0.50 and 0.58 for those that expressed the *kdr* T allele. The allelic variation pattern for the L-to-F *kdr* locus in these two field collected mosquito populations strongly correlated with their intermediate levels of resistance [17], which agrees with the findings of the previous study by Xu et al. [15].

We next compared the frequency of alleles (A or T) at the L-to-F *kdr* site at the genomic DNA level of both 181 and 509 bp gDNA fragments in all five mosquito populations. Different genotypes for the L-to-F *kdr* site were exhibited among individuals in the same population for all 5 of the mosquito populations, including individuals that were heterozygous for both susceptible (A) and resistance (T) alleles or homozygous for either the A or T allele, which is consistent with the results of the earlier study [15]. No correlation was found between the frequency of L-to-F allelic variation at the L-to-F site of the 181 bp sodium channel DNA fragment, the prevalence of the L-to-F allelic expression at the RNA level and the level of permethrin resistance in the 5 mosquito populations (Table 1), which is also consistent with the findings reported by Xu et al. [15].

In contrast, there was a strong correlation between the frequency of allelic variation at the L-to-F site of the 509 bp genomic DNA fragment (with the intron), the prevalence of L-to-F allelic expression at the RNA level and the level of permethrin resistance in all five mosquito populations. In the S-Lab strain, all the individuals were genotype homozygous for the susceptible A allele at the L-to-F site, which corresponded to their L-to-F allelic expression at the RNA level; all the individuals expressed the susceptible allele A (Table 1), commensurate with their level of susceptibility to permethrin. In the highly resistant HAMCq^{G8} and MAMCq^{G6} populations, 84% and 100%, respectively, of the individual mosquitoes were genotype homozygous for the resistance T allele at the L-to-F site, which was strongly correlated with the frequency of their L-to-F allelic expression at the RNA level (Table 1) and their levels of resistance to permethrin. The intermediate susceptibility populations, HAMCq^{G0} and MAMCq^{G0}, had individuals that were homozygous for the susceptible A allele (28 and 42%, respectively), heterozygous for both (A/T) alleles (48 and 42%), and homozygous for resistance (T) alleles (24 and 16%) (Table 1), which, again, strongly correlated with the frequency of their L-to-F allelic expression at the RNA level and their intermediate level of resistance. The data from the field parental mosquitoes HAMCq^{G0} and MAMCq^{G0} thus clearly represent a transitional status between susceptible and highly resistant through their intermediate genotype (509 bp DNA fragment) and expression condition for the L-to-F *kdr* locus, which closely corresponded to their relatively low levels of resistance.

Discussion

Voltage-gated sodium channels belong to a superfamily of ion channels that play an important function in membrane excitability [23] and are responsible for the depolarization phase of action potentials in membranes of neurons and in all types of excitable cells. Molecular characterization has revealed that 9 voltage-gated sodium channel genes and 1 related sodium channel gene are present in the mammalian sodium channel gene family [21–23]; 8 sodium channel genes have been documented in zebra fish [24]; and 6 sodium channel genes have been cloned from electric fish [25]. Multiple sodium channel genes have also been characterized in some invertebrate species; for example, *Hirudo medicinalis* (leech) and *Holocynthia roretzi* (ascidia) have 4 and 2 characterized sodium channel genes, respectively [26–28]. Compared to the fairly well defined multiple vertebrate sodium channel genes, it appears only one sodium channel gene, homologous to *para* (currently *DmNa_v*) of *Drosophila melanogaster*, that has been well characterized in insects [29] encodes the equivalent of the α -subunit of the mammalian sodium channels. A putative sodium channel gene, DSC1, has been described in *D. melanogaster* [30] and homologous genes were later found in other insect species. A more recent study by Moignot et al. [31] revealed two sodium channel cDNAs, a *para*-like sodium channel and a novel sodium channel that is evolutionarily closely related to the *para*-type sodium channel, present in the cockroach *Periplaneta Americana*. This suggests a possibility that insects may also have multiple sodium channels in their genomes.

The current study has, for the first time, revealed multiple (at least two) *para*-type sodium channel genes in the genome of the mosquito *Cx. quinquefasciatus*. Comparison of L-to-F *kdr* genotypes in two *para*-type sodium channel genes (509 bp and 181 bp PCR fragments) in 5 mosquito strains with their corresponding transcriptional expression and their levels of resistance revealed a strong correlation between the frequency of the resistant allele (T genotype at the L-to-F site of the 509 bp DNA fragment [with an intron sequence]), the frequency of L-to-F allelic (T) expression at the RNA level, and the levels of resistance. Nevertheless, no-correlation among genotype, transcriptional expression, and resistance phenotype was identified for the 181 bp sodium channel DNA fragment lacking the intron sequence. Yet, with the first glance, these results strongly suggest that the sodium

channel gene that includes the intron sequence might be a functional copy of the sodium channel gene for transcription and the translation of the sodium channel gene and protein in mosquitoes, and is thus involved in the insecticide resistance of the mosquito *Cx. quinquefasciatus*.

Recent studies have suggested that pseudogenes are present in the genome, accompanied by a loss of function [32]. Pseudogenes can be generated by either direct DNA duplication or retrotransposition. A pseudogene resulting from the latter pathway is referred to as a processed pseudogene (or retro-pseudogene). Processed pseudogenes arise from reverse-transcription of mRNA and re-integration into the genome. In the human genome, it has been proposed that processed pseudogenes are generated from reverse transcribing spliced mRNAs into cDNAs using the reverse transcriptase from the long interspersed nuclear element (LINE) followed by re-integration into the human genome [33,34]. About 8000 processed pseudogenes have been reported in the human genome through exhaustive sequence comparisons with known human proteins [34]. The characteristics of processed pseudogenes include the absence of introns, the presence of flanking direct repeats, and a 3'-polyadenylation tract [32]. Thus, whether the non-intron *para*-type sodium channel gene identified in the current study is a pseudogene or a functional gene with a post transcriptional regulation [15] remains a subject for further investigation.

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Research Highlights

Our study is the first report of multiple *para*-type sodium channel genes in the mosquito *Cx. quinquefasciatus*. Only the L-to-F *kdr* genotypes in the *para*-type sodium channel gene with the intron sequence were consistent with the expression of their transcripts and levels of resistance. This research highlights the complexity of sodium channel gene expression and its role in the pyrethroid resistance of insects and provides a new framework with which to study the role of sodium channels, channel gene expression regulation, and their importance in the insecticide resistance of insects.

Figure 1.

Southern blot analysis of the sodium channel gene in the genome of *Cx. quinquefasciatus*. Genomic DNAs (40 µg each) from HAmCq^{G8} and S-Lab mosquitoes were digested with *EcoRI* and *HindIII*. The membranes were hybridized with ³²P-labelled mosquito sodium channel gene fragment generated from a single exon (exon 20).

Figure 2.

Polymerase chain reaction (PCR) amplification of para-type sodium channel gene fragments from genomic DNA of *Culex* mosquitoes. Sodium channel genomic DNA fragments were amplified from individual mosquitoes using a single primer pair KDR S14/KDR AS1. The primers were designed based on exonic sequences that would not generate products in RNA samples without a reverse transcription step.

A comparison of the frequency of alleles (A or T) at the L-to-F *kdr* site for genomic DNA (two DNA fragments) and RNA levels of *Cx. quinquefasciatus*

Table 1

Strain	N ^b	Phenotype	Template source	Individual allele ^c (frequency)		
				TTA	TTA/T	TTT
S-Lab	54	Susceptible	gDNA w/o intron	4 (0.07)	43 (0.8)	7 (0.13)
			gDNA w/ intron	54 (1.0)	0	0
			cDNA	54 (1.0)	0	0
HAmCq ^{G0a}	54	100-fold resistance	gDNA w/o intron	1 (0.02)	26 (0.48)	27 (0.5)
			gDNA w/ intron	15 (0.28)	26 (0.48)	13 (0.24)
			cDNA	14 (0.26)	25 (0.46)	15 (0.28)
HAmCq ^{G8}	50	3,100-fold resistance	gDNA w/o intron	0	28 (0.56)	22 (0.44)
			gDNA w/ intron	0	8 (0.16)	42 (0.84)
			cDNA	0	2 (0.04)	48 (0.96)
MAmCq ^{G0a}	24	40-fold resistance	gDNA w/o intron	1 (0.04)	9 (0.38)	14 (0.58)
			gDNA w/ intron	10 (0.42)	10 (0.42)	4 (0.16)
			cDNA	10 (0.42)	10 (0.42)	4 (0.16)
MAmCq ^{G6}	24	583-fold resistance	gDNA w/o intron	1 (0.04)	4 (0.17)	19 (0.79)
			gDNA w/ intron	0	0	24 (1.0)
			cDNA	0	0	24 (1.0)

^a G0: The parental insects were collected directly from the field and the numeral indicates the generation (s) of selection with permethrin

^b The total number of tested mosquitoes

^c The nucleotides in the L-to-F *kdr* locus that changed in the genotypes are underlined