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Understanding evolutionary paradigm of knockdown resistance in mosquitoes by analyzing DNA sequence polymorphisms in the voltage-gated sodium channel in *Culex quinquefasciatus*

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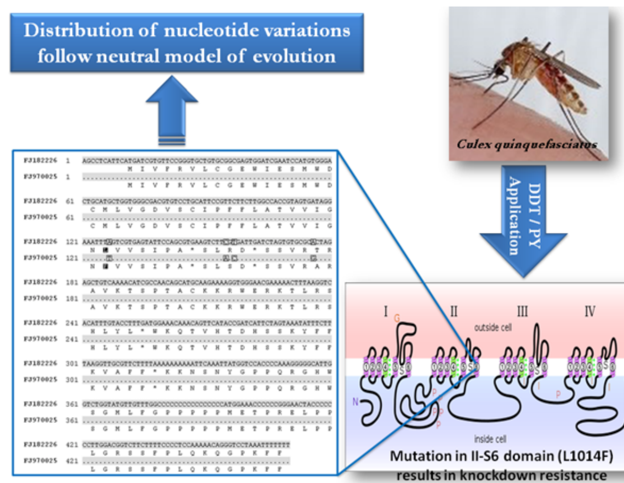
Graphical Abstract



Abstract: The Voltage Gated Sodium Channel (*VGSC*) is critical for binding of different insecticides and plays a key role in insecticide resistance. The insect sodium channel consists of four homologous domains (I to IV), each containing six transmembrane segments (S1 to S6). An important mechanism of resistance to DDT and pyrethroids is termed knockdown resistance (*kdr*), caused by a single nucleotide polymorphism in the IIS6 domain of sodium channels. We analyzed the polymorphisms, nucleotide diversity, and phylogenies in the *vgsc*-IIS6 gene in *Culex quinquefasciatus* from India. We analyzed the neutral model/hypothesis to infer if natural selection is acting upon the *vgsc* gene. Tajima's D, Fu and Li's D* and F* and Fu's Fs test were performed to determine whether the distribution of nucleotide variation within the samples was consistent with the neutral model. We theorized that the evolutionary pattern of intra-population distribution of variability in *vgsc* gene is consistent with the neutral expectation.

Keywords: Knockdown resistance; insecticide resistance; molecular evolution; neutral theory; natural selection; India

Introduction

Culex quinquefasciatus is the principal vector of bancroftian filariasis on the Indian subcontinent. Control of this vector has relied extensively on application of insecticides[1]. Because of continued and/or indiscriminate use of insecticides, increased resistance, especially against DDT has been observed in this part of the world[2].

DDT and pyrethroids share a similar target site, the para-type voltage-gated sodium channel (*vgsc*). It alters the normal functions of the sodium channel. The prolonged channel opening causes increased nerve impulse transmission, leading to paralysis and death of the insect[3, 4]. The transmembrane structure of para-type VGSC consists of four internally homologous domains (I–IV), each having six transmembrane helices (S1–S6). Extensive research has shown that kdr or kdr-like mechanisms have resulted in mutations in sodium channels[5]. Mutations in the domain-II region of the channel are commonly responsible for insecticide resistance. A single nucleotide polymorphism (SNP) (A to T) in the S6 hydrophobic transmembrane segment of domain-II of *vgsc* confers insensitivity to pyrethroid and DDT. This resistance mechanism is known as knockdown resistance (kdr) and has been reported in many insects, such as *Musca domestica*[6], *Blattella germanica*[7] and *Heliothis virescens*[8, 9]. Mosquitoes with the kdr phenotype display a high level of resistance to both pyrethroids and DDT[10-17].

Insecticide-binding simulation studies of *vgsc* with DDT and pyrethroids showed that most of the mutations conferring insecticide resistance were located in the domain-II of the *vgsc* gene[18, 19]. However, information regarding the evolutionary pattern of this gene and knowledge of its phylogenetic lineage are not available for *Culex* mosquitoes. In *Anopheles gambiae*, there is evidence for the multiple origin of kdr mutation in Africa[20]. In *Culex*, there is no published study so far on polymorphisms, nucleotide, and/or genetic diversity and phylogeny of *vgsc* associated with the molecular evolutionary pattern.

We, therefore, analyzed the polymorphisms and nucleotide diversities in the *vgsc*-IIS6 gene to gain insight into the evolutionary forces at work in the insecticide-binding domain of the *vgsc* gene conferring resistance to a population of *Cx. quinquefasciatus* from northeastern India. This is the first study presenting the data of DNA polymorphisms in the *vgsc* gene in relation with the molecular evolutionary pattern of kdr traits in the most important disease vector *Cx. quinquefasciatus*. Given the scale of the resistance problem, this study is not only of academic interest, but also crucial for devising appropriate resistance-management strategies.

Materials and Methods

Mosquito population and bioassays

In order to carry out the tests for selection based on allele frequencies, especially with relatively small sample size, it is important to assemble random samples from a population rather than ascertained samples. We have assembled random samples of adult *Cx. quinquefasciatus* from foothill areas of Assam, northeastern India (26°48′43.0″N, 92°35′39.3″E; 26°51′47.4″N, 92°33′48.7″E; 26°51′48.4″N, 92°32′27.6″E; 26°50′45.3″N, 92°35′33.2″E). We considered these mosquitoes as a single population, because the collection sites are relatively close to each other. Insecticide susceptibility assays were performed on wild caught adult female mosquitoes using the WHO adult bioassay kit[21]. Mortality was determined 24 hours post-exposure. Resistance status of wild populations was compared to a susceptible reference strain of *Cx. quinquefasciatus*, known as S–Lab, which was collected from Tezpur city of Assam and was reared for more than 10 years in the insectary of Defence Research Laboratoey (Tezpur) and considered to be > 90% susceptible to DDT and > 98% to other insecticides. We used the WHO criteria for evaluating resistance or susceptibility in the mosquito population[22].

Gene Amplification and Sequencing of PCR products

In this study, we amplified and sequenced IIS6 domain of *vgsc* gene from a population of Cx. *quinquefasciatus* collected from northeastern India. Genomic DNA from individual adult female *Cx. quinquefasciatus* mosquitoes was extracted using the SDS extraction and ethanol precipitation method as described previously[23]. The DNA samples were air-dried and suspended in 100 μl of nuclease free water. We designed two primers, Cq1 (5'GTGGAACTTCACCGACTTC 3') and Cq2 (5' GCAAGGCTAAGAAAAGGTTAAG 3') to amplify a fragment of the IIS6 domain of the *vgsc* gene, reported to contain the kdr mutation site. The PCR reaction was carried out according to Sarkar et al.[17]. PCR reactions were replicated twice, each experiment conducted on a different day.

PCR products were purified using the QIAquick PCR purification kit (Qiagen). A total of 100 PCR samples, 50 numbers each from knockdown resistant and knockdown susceptible phenotypes were sequenced on both strands using an ABI automated DNA sequencer. Sequences were analyzed using BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences were submitted to the National Center for Biotechnology

Information Open Reading Frame Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/) to determine the coding regions and introns.

Sequence analysis and polymorphism detection

Single nucleotide polymorphisms in IIS6 domain of *vgsc* were detected as sequence differences in multiple alignments using CLUSTALW (http://align.genome.jp/). Electrophoregrams were visually inspected using BioEdit (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html) and heterozygotes were identified[24]. SNPs were identified as transitions or transversions in coding and non-coding regions. Nucleotide diversity, polymorphisms, divergence, gene flow, and genetic differentiations among the populations studied were analyzed using DnaSP 5.00.02 (http://www.ub.es/dnasp)[25].

Analysis of Data and Molecular Evolutionary Theories

Most intra-population (i.e., the single population tested for neutrality in this study) data analyses – including estimates of DNA polymorphisms, nucleotide diversity (π) number of segregating sites (S), number of haplotypes (h), haplotype diversity (Hd) – were performed among 100 sequences of the *vgsc* gene by subdividing the gene into functional domains (exons and introns). The data were analyzed using DnaSP 5.00.02. We analyzed the neutral model/hypothesis (alternatively known as the null model; states that the vast majority of evolutionary changes at the molecular level is caused by random drift of selectively neutral mutants[26]), to infer if natural selection is acting upon the analyzed *vgsc* gene. Tajima's D test[27], Fu and Li's D* and F*[28] tests and Fu's Fs test[29] were performed to determine whether the distribution of nucleotide variation within the samples was consistent with the neutral model. Tajima's D test statistics is defined as the standardized difference between two estimators of the population mutation rate parameter θ (= 4 Nμ for an autosomal region of diploid individuals; where N is the effective population size, and μ is the per-gene or per-site per-generation mutation rate). One estimator based on the number of segregating sites and other on the average number of pairwise nucleotide differences, which should be equal under the neutral mutation model and should differ when natural selection affects the genomic region. Fu and Li's D* test compares two estimators of the population mutation rate parameter θ, based on the differences between the number of singletons (mutations appearing only once among the sequences), and the total number of mutations[28]. Fu and Li's F* test statistics is based on the differences between the number of singletons and the average number of nucleotide differences between pairs of sequences[28, 30, equation 10]. The Fs test statistic[29, equation 1] is based on the haplotype (gene) frequency distribution for a given value of θ derived from the average number of pair wise nucleotide differences[31, equations 19-21]. We performed Coalescent simulations to estimate confidence intervals and exact P-values (10000 interactions) for Tajima's D, Fu and Li's D* and F* test, nucleotide diversity, linkage disequilibrium, number of haplotype and haplotype diversity test. These simulations were conditioned on the observed number of segregating sites (S), and thus population size is not a factor.

Results and Discussion

Bioassays were carried out using 4% DDT and mortalities were recorded after 24 hour exposure. The mortality of *Cx. quinquefasciatus* with DDT ranged from 11.9% to 41.25% whereas mortality in S–Lab insects was estimated at 91.2%. This bioassay result suggests that *Cx. quinquefasciatus* population tested is highly resistant to DDT across all study sites. The details of insecticide resistance and/or susceptibility status, and detoxifying enzyme profiles of this population were described in our other publication[2].

Detection of kdr mutation in wild population of Culex quinquefasciatus

We randomly sampled 50 DDT-resistant and 50 susceptible mosquitoes in search of polymorphisms in the insecticide-binding segment of *vgsc* gene. We found two distinct sequence variants (i.e., knockdown susceptible or kds and knockdown resistance or kdr) after sequencing 100 samples. These two distinct sequence variants were submitted in GenBank under accession number **FJ182226** and **FJ970025**. Figure 1 displays the sequence alignment of the knockdown-susceptible and knockdown resistant population, which confirms the presence of the polymorphic site at position 127 (TTA to TTT) that induces the substitution of leucine to phenylalanine in resistant mosquitoes, as previously reported by others in the same species [14, 15]. However, we did not find any A to C mutation as reported by Wondji et al.[15]

Neutrality tests and evolutionary pattern of knockdown resistance

The sequences of the *vgsc* gene consisted of one exon and one intron region. We found four parsimony variable sites (127, 155, 157, and 176) in these sequences; out of these one was found in the exon region (A127T, kdr mutation) and three in the intron-2 region (Table 1). Polymorphic sites in the intronic sequences co-segregate with the kdr allele. Statistical analysis of polymorphism for the entire sequenced region and each functional domain of the *vgsc* gene are summarized in Table 1. In *vgsc*, nucleotide diversity in the exon (π/site=0.0033) and intron (π/site=0.0038) was found to be almost similar when compared with the entire gene (π/site=0.0037). However, we observed a higher θ/site value for the intron region in comparison to the exon and entire

sequence (Table 1). This is probably due to higher mutation rate per nucleotide per generation in the intronic region.

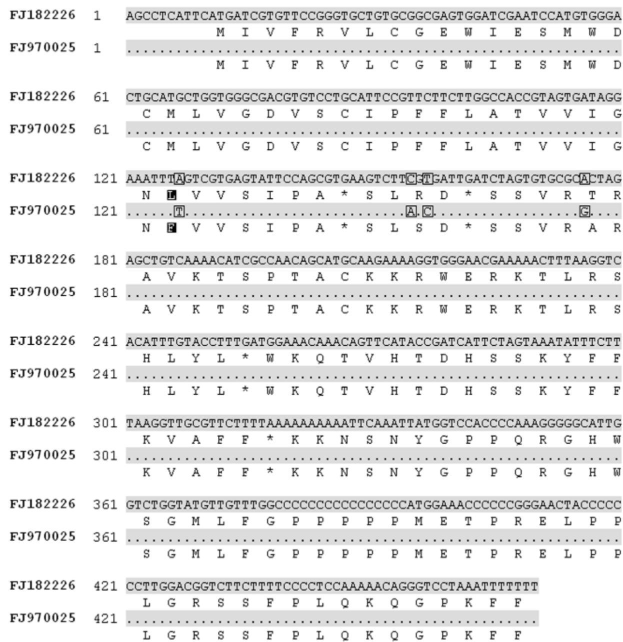


Figure 1. Alignment of nucleic acids and corresponding amino acids of IIS6 domain of the *para*-type sodium channel gene from two representative sequences of knockdown susceptible (**FJ182226**) and resistant (**FJ970025**) strain of *Culex quinquefasciatus*. Note the A to T change at nucleotide 127 and leucine (L) to phenylalanine (F) at amino acid at 39 in the resistant strain. Alignment also displays four polymorphic sites: at position 155 (C-A), 157 (T-C) and 176 (A-G) in the intron-2 region.

The polymorphic sites in different intronic regions of the *vgsc* gene vary depending on the mosquito species. We observed three polymorphic sites in intron-2 of *Cx. quinquefasciatus* whereas Pinto et al.[20] reported eight polymorphic sites in intron-1 of the *An. gambiae* population that explain the distinctive pattern of polymorphisms in different intronic regions of *vgsc* in different populations.

We performed Fisher's exact test for independence between sites (with Bonferroni correction) and Kelly's ZnS to test linkage disequilibrium[32]. Only parsimony informative polymorphic sites were considered for the analysis and results are presented in Table 1; there is no evidence of significant nonrandom association between nucleotide variants at different polymorphic sites (P > 0.1). Recombination per gene in *vgsc* domain-II was very low (R=0.001). The minimum number of recombination events R_M=0. In the presence of non-significant linkage disequilibrium and very low recombination, it is not unexpected that the estimates of haplotype number and corresponding haplotype diversity for *vgsc* do not deviate from the neutral expectations [number of haplotype, h=3; haplotype diversity, Hd=0.537 (±0.09) with P=0.316] (Table 1 and 2). Nucleotide variations in synonymous and nonsynonymous sites in *vgsc* are presented in Table 3. We found no singleton and synonymous mutation (Table 3).

One method for revealing the influence of selection on a sequence is by examining the distribution of segregating variants at that locus in a population and testing this distribution against the neutral model[33]. We have used the D statistics[27, 28] to examine the hypothesis that all substitutions at the locus are neutral. Several possibilities were explored: using the whole region sequenced, only the coding region, or

Table 1. Polymorphism statistics and molecular evolutionary parameters for the *vgsc* -IIS6 domain in *Culex quinquefasciatus* in northeastern India.

	S^1	h^2	Hd^3 (±SD)	π^4/site	ZnS^5	K^6 (π/gene)	θ^7/site (±SD)	Tajima's *D*	Fu & Li's *D**	Fu & Li's *F**	Fu's *Fs*
Exon region (Length[8]: 145)	1	2	0.485 (0.064)	0.0033	NA[9]	0.485	0.0019 (0.00189)	1.335 NS	0.635 NS	0.942 NS	1.392 NS
Intron region (Length[8]: 325)	3	2	0.416 (0.09)	0.0038	1.00NS	1.247	0.0038 (0.00384)	1.332 NS	0.992 NS	1.252 NS	3.714 NS
Full length (Length[8]: 470)	4	3	0.537 (0.09)	0.0037	0.83NS	1.732	0.0023 (0.00117)	1.623 NS	1.096 NS	1.436 NS	2.876 NS

[1]*S*, number of segregating sites. [2]*h,* number of haplotype. [3]*Hd*, haplotype (gene) diversity, equivalent to expected heterozygosity; SD, standard deviation. [4]π, nucleotide diversity, the mean number of nucleotide differences per site between two sequences. [5]*ZnS,* linkage disequilibrium statistic (Kelly, 1997, equation 3). [6]*K*, The average number of nucleotide differences (Tajima, 1989, equation A3). [7]θ, Theta (per site) from *S*. [8]Length of functional domains. [9]NA, Not Applicable; because in the exon only one polymorphic site is present, so no pairwise comparison. NS, Not Significant (P > 0.05, 0.1).

only the intron-2. Where appropriate, the significance of the test or parameter has been verified by coalescent simulation (10000 repeats) (Table 2). The detailed results of neutrality tests including coalescent simulation are presented in Table 1 and Table 2. Upon analyses of segregating variation, no statistics (D, F* or Fs) provide evidence for a significant departure from neutral evolution. The power of neutrality test is limited in the presence of recombination[34], but because recombination was very low, this may explain the lack of significant departure from neutrality. Overall, the evolutionary pattern of intra-population distribution of variability in the domain-II of the *vgsc* gene is consistent with the neutral expectation; hence there is no evidence that positive Darwinian selection has recently influenced, or is currently influencing, nucleotide variation in this region of the genome. Hence, there is a possibility that knockdown resistance (*kdr*), associated with polymorphism in domain-II of the *vgsc* gene, may be caused by random drift of selectively neutral mutants. Should we then conclude that selection did not affect the nucleotide variation in *vgsc* and evolution of the *kdr* trait is largely due to genetic drift rather than natural selection? The present data are obviously not sufficient to tell apart the various scenarios that could have led to the present structure, because information at many independent loci is required to make strong inference on past demographic factors that could affect the selection processes.

Table 2. Results of coalescent simulations and test of the neutral model/hypothesis

Parameters	Observed value	95% CI	P [1]
Number of haplotype (NHap)	3.00	2.00–5.00	0.21
Haplotype diversity (*Hd*)	0.537	0.256–0.784	0.32
Nucleotide diversity (π/gene)	1.732	0.446–1.866	0.95
Theta-W (from S) (θ/gene)	1.097	0.274–3.841	0.33
Linkage Disequilibrium (ZnS)	0.83	0.007–1.00	0.96
Tajima's *D*	1.623	-1.878–1.856	0.95
Fu & Li's *D**	1.096	-1.763–1.095	1.00
Fu & Li's *F**	1.436	-2.00–1.49	0.96
Fu's *Fs*	2.876	-2.71–3.17	0.96

[1] Estimation of probability of obtaining lower values than the ones observed

Table 3. Nucleotide variation in synonymous and non-synonymous sites in *vgsc* gene of *Culex quinquefasciatus* (Nei & Gojobori, 1986, equations 1–3).

	Synonymous sites	Non synonymous sites	Synonymous and Noncoding (Silent sites)
S	0	1	3
π	NA	0.0047	0.0034
θw	NA	0.0027	0.0023

On the other hand, the neutral theory does not rule out the role of natural selection in certain scenarios of adaptive evolution - such patterns are expected from neutrality. Hence, the molecular evolution of the *vgsc* domain may be dominated by selectively neutral evolution of segregating variants (polymorphic sites) at genomic level, but at the phenotypic level, changes in knockdown resistance were probably dominated by natural selection rather than sampling drift.

Our observations provoked a hypothesis of possible evolutionary pattern of kdr allele in *Cx. quinquefasciatus*. Further critical study with whole *vgsc* sequences is needed to address this problem. At this point, we have no means to claim any definite evolutionary mechanisms controlling knockdown resistance in mosquito by using the data from the present study. We also suspect that the evolutionary puzzle regarding the origin of knockdown resistance in mosquitoes can be addressed successfully by thorough analysis of intronic polymorphism in the entire *vgsc* gene between samples from different parts of the world. The study of a molecular evolutionary pattern of the insecticide-binding domain of the *vgsc* gene in *Cx. quinquefasciatus*, described here, has important academic implications and opens a scope for a future study with a holistic approach to identify definite evolutionary forces at work in knockdown resistance.

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