Journal of the American Mosquito Control Association, 39(1):000–000, 2023

SCIENTIFIC NOTE

DETECTION OF TARGET SITE MUTATIONS IN THE ACETYLCHOLINESTERASE AND VOLTAGE-GATED SODIUM CHANNEL IN FIELD POPULATIONS OF *CULEX QUINQUEFASCIATUS* AND *CX. TRITAENIORHYNCHUS* FROM SOUTHERN SICHUAN REGION OF CHINA

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ABSTRACT. *Culex quinquefasciatus* and *Cx. tritaeniorhynchus* are 2 dominant disease vectors in Neijiang City, Sichuan Province, China. Although there is evidence of confirmed resistance against insecticides in mosquito vectors, nothing is known about the existing insecticide resistance-conferring mutations in *Cx. quinquefasciatus* and *Cx. tritaeniorhynchu* in this region so far. In this study, the G119S mutation in the acetylcholinesterase (AChE) was detected in *Cx. quinquefasciatus* at a very low frequency (0.9%) with no resistant homozygotes being observed. Two resistance mutations in the voltage-gated sodium channel (VGSC) (L1014F and L1014S) were found in *Cx. quinquefasciatus* with frequencies of 88.7% and 8.3%, respectively. By contrast, the AChE F455W mutation was found to be fixed (with a frequency of 100%) in 3 of the 5 studied populations, with an overall frequency being 98.1%. In addition, 1 resistance-conferring VGSC mutation (L1014F) was detected with an overall frequency of 15.2% in *Cx. tritaeniorhynchus*. These results indicate that the well-recognized insecticide resistance-conferring mutations in both AChE and VGSC are present in the 2 *Culex* species in Neijiang. The contrasting patterns in the frequency of resistance alleles indicate that species-customized strategies of insecticide resistance management should be considered for the 2 species.

KEY WORDS AChE G119S, AChE F455W, insecticide resistance, VGSC L1014F, VGSC L1014S

For the past several decades, insecticide-based management of vector mosquitoes has been the major measure for prevention and control of mosquito-borne diseases. The most common insecticides used for mosquito control are pyrethroids targeting the voltage-gated sodium channel (VGSC) and organophosphates (OPs) and carbamates (CBs) targeting the acetylcholinesterase (AChE, encoded by the *ace* gene). However, the prolonged use of these insecticides has led to the development of insecticide resistance in numerous mosquito species worldwide (Liu 2015). To ensure that vector control strategies are sustainable, regular monitoring and effective management of insecticide resistance are required. Understanding the mechanisms governing the development of resistance is a crucial prerequisite for effective vector control programs.

One major mechanism of insecticide resistance is insecticide insensitivity caused by mutations in insecticide target sites. The Gly-119-Ser (G119S) substitution in AChE and the Leu-1014-Phe (L1014F) mutation in VGSC are the 2 mutations commonly detected in mosquitoes (Liu 2015). Interestingly, G119S has not been reported in *Culex tritaeniorhynchus* Giles; instead, the F455W mutation leading to insensitivity of AChE to OP and CB has been identified in *Cx. tritaeniorhynchus* from Japan (Nabeshima et al. 2004), India (Misra and Gore 2015), and China (Wu et al. 2016). Moreover, the occurrence of 2 novel concomitant mutations L932F and I936V in VGSC was reported in a Brazilian strain of *Cx. tritaeniorhynchus* (Sugiura et al. 2021), and L1014S was newly identified in *Cx. quinquefasciatus* Say from India (Rai and Saha, 2022).

Neijiang City is located in the southeast of Sichuan Basin of China with a subtropical humid monsoon climate. *Culex quinquefasciatus* and *Cx. tritaeniorhynchus* are 2 of the most abundant mosquito species in Neijiang. However, nothing was known about the resistance status and involved genetic mutations in the 2 mosquito species in this region before this study. The aim of the present study was thus to investigate the presence and distribution frequency of AChE and VGSC mutations in *Cx. quinquefasciatus* and *Cx. tritaeniorhynchus*. These efforts should help health authorities and pest control operators in developing molecular methods for resistance monitoring and inform more effective vector control programs.

Culex quinquefasciatus and *Cx. tritaeniorhynchus* were sampled in 5 counties of Neijiang City in July 2021. The DNA fragments of the *ace* and *vgsc* genes containing the codons encoding amino acid residue 119 of *Cx. quinquefasciatus* AChE, 455 of *Cx.*

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Table 1. Genotypic frequencies of the AChE G119S and VGSC L1014F/S mutations in 5 populations of *Culex quinquefasciatus* (%).

Population[1]	G119S[2]				VGSC1014[2]						
	n[3]	GG	GS	HWE[4]	n[3]	LL	LF	FF	FS	SS	HWE[4]
SZ	31	100	0	N/I	28	0	7.1	82.1	7.1	3.6	0.128
DX	32	96.9	3.1	N/I	30	0	3.3	86.7	3.3	6.7	0.0045
ZZ	15	93.3	6.7	N/I	13	0	0	92.3	7.7	0	N/I
WY	11	100	0	N/I	10	10	0	90	0	0	0.0526
LC	22	100	0	N/I	21	0	4.8	76.2	4.8	14.2	0.0018
Total	111	98.2	1.8	N/I	102	1	3.9	84.3	4.9	5.9	

[1] SZ, Shizhong (29°32′42″N, 105°05′23″E); DX, Dongxing (29°39′00″N, 105°01′11″E); LC, Longchang (29°32′61″N, 105°31′04″E); WY, Weiyuan (29°31′36″N, 104°39′40″E); ZZ, Zizhong (29°46′24″N, 104°51′24″E).
[2] Genotypes.
[3] n, sample size.
[4] HWE, Hardy–Weinberg equilibrium. The values are the P value calculated by the probability test. N/I, no information.

tritaeniorhynchus AChE, and 1014 of *Cx. quinquefasciatus* and *Cx. tritaeniorhynchus* VGSC were amplified by polymerase chain reaction (PCR), using the primer pairs described by Yanola et al. (2015), Wu et al. (2016), and Jiang et al. (2017). The PCR mixture in a total volume of 30 µl contained 15 µl 2 × EZ3 HIFI PCR MasterMix (Dakewe Biotech Co., Ltd, China), 12.5 µl ddH_2O, 0.75 µl for each pair primer (10 µM), and 1 µl gDNA template. The thermal cycle program for *Cx. quinquefasciatus ace* and *vgsc* and *Cx. tritaeniorhynchus ace* was the same as an initial denaturation at 95°C for 25 min, followed by 35 cycles at 95°C for 10 sec, 55°C for 15 sec, 72°C for 30 sec, and 72°C for 5 min. The PCR condition for *Cx. tritaeniorhynchus vgsc* gene was 95°C for 3 min, 35 cycles at 95°C for 25 sec, 55°C for 25 sec, 72°C for 30 sec, and 72°C for 5 min.

Individual genotypes were identified by Sanger sequencing of PCR products. The genotype of heterozygotes with 2-site variations in the codon of interest was phased by sequencing of 3 to 5 clones. The DNA Sanger sequencing was performed by SinoGenoMax (Beijing, China). Hardy–Weinberg equilibrium (HWE) of genotypes for each locus in each population was calculated by exact probability test using the online software GENEPOP version 7.5 (https://genepop.curtin.edu.au/). The level of significance was statistically significant when a P value was <0.05.

A nucleotide variation (G to A) at the first nucleotide of codon 119 of the *Cx. quinquefasciatus ace1* gene was detected. The nucleotide variation resulting in G119S (GGC to AGC) mutation was detected in 2 of the 5 locations (Table 1). Two genotypes (GGC (G/G), and GGC/AGC (G/S) were observed. The dominant genotype was the wild homozygote with an overall frequency of 98.2%, while the frequency of resistance allele (119S) was 0.9%.

Nucleotide polymorphisms were identified at the second (T/C) and the third nucleotide (A/T) of codon 1014 of the *Cx. quinquefasciatus vgsc* gene. These variations could render 2 amino acid replacements, i.e., L1014F (TTA to TTT) and L1014S (TTA to TCA). Five genotypes (TTA (L/L), TTT/TTA (L/F), TTT (F/F), TCA (S/S), and TTT/TCA (F/S)) were detected (Table 1). From 102 individuals, only 1 was wild homozygote, and the frequency of the wild allele (1014L) was low (2.9%). The predominant genotype was 1014F/F homozygote, which was detected in all 5 locations with a frequency over 76%. In comparison, the 1014S/S homozygotes were present in 3 of the 5 populations with frequencies ranging from 3.6% to 14.2%. Overall, a very high frequency of the 1014F allele (88.7%) was detected (Table 1). A significant deviation from the HWE was observed in 2 (DX and LC) of the 5 populations due to heterozygote deficiency, probably a consequence of pyrethroid selection. By contrast, the other populations were in the HWE.

By sequencing a fragment of an exon region of the *Cx. tritaeniorhynchus ace2* gene, 2 nonsynonymous polymorphic sites resulting in F455W (TTT to TGG) mutation were identified. The frequency of the resistance allele 455W was extremely high (98.1%) with more than 96% individuals being 455W homozygotes. Genotype frequencies were detected in conformity to HWE in SZ and LC, while the resistant 455W mutation was fixed in the other 3 populations (Table 2).

A variation (A to T) was observed at the third nucleotide of codon 1014 of the *Cx. tritaeniorhynchus vgsc* gene, leading to the amino acid substitution L1014F (TTA to TTT). All 3 possible genotypes (TTA (L/L), TTT (F/F), TTT/TTA (L/F)) were detected (Table 2). The predominant genotype was the wild 1014L/L homozygote with an overall frequency of 72.3%. The resistance allele 1014F was present in the form of heterozygote in all 5 locations, while the F/F homozygote was observed in 2 of 5 populations with a frequency below 10%. The overall allele frequencies for 1014L and 1014F were 84.8% and 15.2%, respectively. All *vgsc* genotypes agreed with the Hardy–Weinberg equilibrium (Table 2).

Our results revealed that the genetic mutation and frequency of the resistance-conferring AChE mutations are different in the 2 species. In *Cx.*

Table 2. Genotypic frequencies of the AChE F455W and VGSC L1014F mutations in 5 populations of *Culex tritaeniorhynchus* (%).

Population[1]	F455W[2]				VGSC1014[2]				
	n[3]	FW	WW	HWE[4]	n[3]	LL	LF	FF	HWE[4]
SZ	25	8	92	1	25	60	40	0	0.5418
DX	22	0	100	N/I	21	71.4	23.8	4.8	0.4483
ZZ	20	0	100	N/I	24	79.2	20.8	0	1
WY	20	0	100	N/I	22	68.2	22.7	9.1	0.1789
LC	16	12.5	87.5	1	20	85	15	0	1
Total	103	3.9	96.1		112	72.3	25	2.7	

[1] SZ, Shizhong (29°38′59″N, 105°04′37″E); DX, Dongxing (29°39′00″N, 105°01′11″E); LC, Longchang (29°32′07″N, 105°31′18″E); WY, Weiyuan (29°31′36″N, 104°39′40″E); ZZ, Zizhong(29°46′24″N, 104°51′24″E).
[2] Genotypes.
[3] n, sample size.
[4] HWE, Hardy–Weinberg equilibrium. The values are the P value calculated by the probability test. N/I, no information.

quinquefasciatus, the resistance allele (119S) had a very low frequency (0.9%) with no resistant homozygote being observed in the 5 investigated populations. Similar results were reported in field populations of *Cx. quinquefasciatus* in West Africa and Malaysia (Djogbenou et al. 2008, Low et al. 2013). The low frequency may suggest G119S is selected against because of the high fitness cost caused by the substitution of the glycine residue in the oxyanion hole at the active site gorge (Raymond et al. 2001). By contrast, the F455W mutation was fixed in 3 of the 5 *Cx. tritaeniorhynchus* populations. Similarly, F455W fixation was also observed in 12 populations of *Cx. tritaeniorhynchus* exhibiting high levels of resistance (>100-fold) to OP and CB collected from 4 other provinces of China (Wu et al. 2016).

Unsurprisingly, the L1014F was detected in both *Cx. quinquefasciatus* and *Cx. tritaeniorhynchus* collected from Neijiang. In comparison, the frequency of the resistant 1014F allele was much lower (15.2%) in *Cx. tritaeniorhynchus* than that in *Cx. quinquefasciatus* (88.7%). A similar frequency (10–30%) of the 1014F allele was previously reported in 12 *Cx. tritaeniorhynchus* populations from 4 other provinces of China (Wu et al. 2016). Notably, another mutation (L1014S) that was recently identified in *Cx. quinquefasciatus* from India (Rai and Saha, 2022) was detected in *Cx. quinquefasciatus* in Neijiang (Table 1). Compared with the L1014F, the frequency of the L1014S was much lower (8.3 % vs 88.7%) than that of the classic L1014F mutation.

Taken together, it can be determined that a big difference in the frequency of resistance-conferring target mutations in field populations of the 2 *Culex* species in Neijiang exists. In *Cx. quinquefasciatus*, a very high frequency of the VGSC mutation (1014F+1014S = 97%), and a very low occurrence of the G119S mutation (0.9%) were observed. By contrast, low abundance of 1014F (∼ 15%) and high prevalence (with a frequency >98%) of 455W were detected in the *Cx. tritaeniorhynchus* populations. These observations suggest that the VGSC mutation at the position 1014 is a notable mechanism in pyrethroid resistance in *Cx. quinquefasciatus*, while the F455W mutation contributes significantly to resistance to organophosphates and carbamates in *Cx. tritaeniorhynchus*. Although factors that shape such a contrasting pattern in allele frequency remain unclear, differences in the profiles of resistance mutations between the 2 *Culex* mosquitoes highlight the need of focused control interventions to each species in Neijiang.

The present study was an effort to understand the possible genetic mutations that can result in target-site insensitivity in *Cx. quinquefasciatus* and *Cx. tritaeniorhynchus*. The involvement of other possible mechanisms (e.g., metabolic resistance) remains to be investigated. Our data demonstrated the presence of multiple target-site mutations, which may pose a significant challenge in chemical pest control. Therefore, regular monitoring of phenotypic resistance is highly recommended to better inform insecticide usage in this region.

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