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Detection of pyrethroid resistance mutations and intron variants in the voltage-gated sodium channel of *Aedes (Stegomyia) aegypti* and *Aedes (Stegomyia) albopictus* mosquitoes from Lao People's Democratic Republic

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Abstract

In Lao People's Democratic Republic, *Aedes aegypti* (Linnaeus 1762) and *Aedes albopictus* (Skuse 1894) mosquitoes (Diptera: Culicidae) are vectors of arboviral diseases such as dengue. As the treatment for these diseases is limited, control of the vectors with the use of pyrethroid insecticides is still essential. However, mutations in the voltage-gated sodium channel (*vgsc*) gene giving rise to pyrethroid resistance are threatening vector control programs. Here, we analysed both *Ae. aegypti* and *Ae. albopictus* mosquitoes, which were collected in different districts of Laos (Kaysone Phomvihane, Vangvieng, Saysettha and Xaythany), for *vgsc* mutations commonly found throughout Asia (S989P, V1016G and F1534C). Sequences of the *vgsc* gene showed that the F1534C mutation was prevalent in both Aedes species. S989P and V1016G mutations were detected in *Ae. aegypti* from each site and were always found together. In addition, the mutation T1520I was seen in *Ae. albopictus* mosquitoes from Saysettha district as well as in all *Ae. aegypti* samples. Thus, mutations in the *vgsc* gene of *Ae. aegypti* are prevalent in the four districts studied indicating growing insecticide resistance throughout Laos. Constant monitoring programmes and alternative strategies for controlling Aedes should be utilized in order to prolong the effectiveness of pyrethroids thereby maximizing vector control.

KEYWORDS
Aedes, deltamethrin, insecticide resistance, Kdr, permethrin, pyrethroid, vector control

INTRODUCTION

Aedes aegypti and *Aedes albopictus* mosquitoes are vectors of various viral diseases such as yellow fever, Zika, chikungunya and dengue that have colonized almost all continents and are still expanding geographically (Lwande et al., 2020). As a consequence, the global incidence of dengue has grown dramatically in recent decades and about half of the world's population is now at risk (World Health Organization, 2022). In the absence of drugs and vaccines, managing these diseases is heavily reliant on vector control, such as the use of insecticides (Wilson et al., 2020). However, overreliance on these insecticides has resulted in the emergence of resistance in many countries throughout the world, which threatens to undermine efforts to control dengue and other viruses carried by *Aedes* mosquitoes (Smith et al., 2016). Understanding and monitoring the extent of insecticide resistance is crucial in informing the optimal use of vector control strategies thereby minimizing disease outbreaks.

In Lao People's Democratic Republic (PDR; hereafter referred to as Laos), a land-locked country located in the middle of the Indochinese peninsula, dengue fever is a major national health problem where outbreaks have been regularly declared (Calvez et al., 2020). Since the early 2000s, pyrethroid insecticides such as deltamethrin and permethrin have been used in Laos for the control of adult mosquitoes. In 2019, Marcombe et al. reported that several populations of adult *Ae. aegypti* taken from different regions of Laos were highly resistant to permethrin and populations from Attapeu and Vientiane Capital were also resistant to deltamethrin (Marcombe et al., 2019). In line with this, resistant mosquitoes possessed V1016G and F1534C mutations (amino acid numbering used throughout is according to the *Musca domestica* sodium channel protein) in the voltage-gated sodium channel (*vgsc*) gene that are associated with target-site resistance (Du et al., 2016; Marcombe et al., 2019; Smith et al., 2016). The V1016G and F1534C mutations, as well as S989P, have been commonly observed in pyrethroid-resistant Aedes mosquitoes throughout Asian countries (Fan et al., 2020) including China (Li et al., 2015), Indonesia (Wuliandari et al., 2015), Malaysia (Leong et al., 2019), Myanmar (Kawada et al., 2014), Saudi Arabia (Al Nazawi et al., 2017), Sri Lanka (Fernando et al., 2018), Taiwan (Chung et al., 2019), Thailand (Yanola et al., 2011) and Vietnam (Kasai et al., 2019).

Recently, S989P + V1016G or S989P + V1016G + F1534C mutations were found to be significantly associated with resistance to deltamethrin in *Ae. aegypti* from Xaythany located in Vientiane Capital (Shimono et al., 2021). Analysis of *Ae. albopictus* taken from either Luang Prabang province or Vientiane Capital found that these mosquitoes were not resistant to deltamethrin nor permethrin with one exception being a group of mosquitoes taken from Kao-gnot village, in Vientiane Capital, which showed suspected resistance to permethrin (Tangena et al., 2018). The presence of *vgsc* target site mutations associated with pyrethroid resistance in *Ae. albopictus* from Laos has yet to be measured. The S989P and V1016G mutations are located in domain II of the VGSC protein whilst F1534C is present in domain III (Du et al., 2016). In this study, sequences of domains II and III were examined to further investigate the prevalence of *vgsc* mutations in *Ae. aegypti* from Savannakhet, Vientiane and Vientiane Capital provinces as well as in *Ae. albopictus* from Vientiane province and Vientiane Capital.

MATERIALS AND METHODS

Collection of mosquito samples

Mosquito collections were carried out in three provinces of Laos (Vientiane Capital, Vientiane Province and Savannakhet; Table 1 and Figure 1). *Aedes* sp. mosquitoes at larval and pupal stages were collected in rural and urban areas in different sampling containers (buckets, cups, fridges, jars, tires, toilets, vases, etc.). All samples were brought back to the laboratory at the Institut Pasteur du Laos and maintained under controlled conditions for rearing until adults (F1 generation) following previously described standardized techniques (Marcombe et al., 2019). After adult identification using morphological keys (Rattanarithikul et al., 2006), mosquitoes were separated by species and were kept for breeding. Female mosquitoes from each population were stored in desiccated tubes at −80°C and sent to Oxford Brookes University for molecular analyses.

Detection of mutations in the voltage-gated sodium channel

Genomic DNA was extracted from individual mosquitoes (191 *Ae. aegypti* and 81 *Ae. albopictus*) using 250 µl Trizol (Fisher Scientific, Loughborough, UK) following the manufacturer's protocol. With 2 µl of extracted DNA as template, DNA encoding for transmembrane region 6 (TM6) in domain I, transmembrane region TM6 in domain II or TM6 of domain III from the *Ae.* aegypti or *Ae. albopictus vgsc* gene was amplified by polymerase chain reaction (PCR) using the Q5® High-Fidelity PCR Kit (New England Biolabs, Ipswich, MA, U.S.A.). We designed primers to amplify TM6 in domain I, which were 5′-TCTTCGTGGTGTGCAAAACAG-3′ (forward) and 5′-TTCGCTCA CCCGAAGCGC-3′ (reverse) with resulting amplification products being sequenced with the 5′-TCTTCGTGGTGTGCAAAACAG-3′ (forward) oligoneucleotide. For TM6 in domain II, the primers used were 5′-AGACAATGTGGATCGCTTCC-3′ (forward) and 5′-GATATCCGATT GAACGCCTC-3′ (reverse) with resulting amplification products being sequenced using either sense (5′-ACGGTGGAACTTCACCGAC-3′) or antisense (5′-CTTGTTCGTTTCGTTGTCGG-3′) oligonucleotides. For TM6 in domain III, the primers used were 5′-AGTGCGTCGA CAAGAACAAG-3′ (forward) and 5′-CCCTAGGCCGTGGAATAGC-3′ (reverse) with amplification products being sequenced using either sense (5′-ACGAGATCATTCCGGATGTG-3′) or antisense (5′-TT CAGCGGCTTCTTCGAGC-3′) oligonucleotides. The PCR products were purified using the Monarch® PCR & DNA Cleanup Kit (New England Biolabs, Ipswich, MA, U.S.A.) and then sequenced at SourceBioscience (https://www.sourcebioscience.com/). Sequence chromatograms were visualized using Chromas (available online: https://technelysium.com.au/wp/chromas/).

TABLE 1 List of the *Aedes aegypti* and *Aedes albopictus* populations collected and their GPS coordinates

Aedes sp.	Province	District	Village	Strain name	GPS latitude	GPS longitude
Ae. aegypti	Vientiane Capital	Xaythany	Sivilay	VTESVL	18.010516	102.632912
Ae. aegypti/albopictus	Vientiane Capital	Saysettha	Sengsavang	VTESSV	17.995816	102.664895
Ae. aegypti/albopictus	Vientiane	Vangvieng	Viengkeo	VTVV	18.552044	102.165025
Ae. aegypti	Savannakhet	Kaisone	Phomvihane	SVKS	16.324949	104.451839

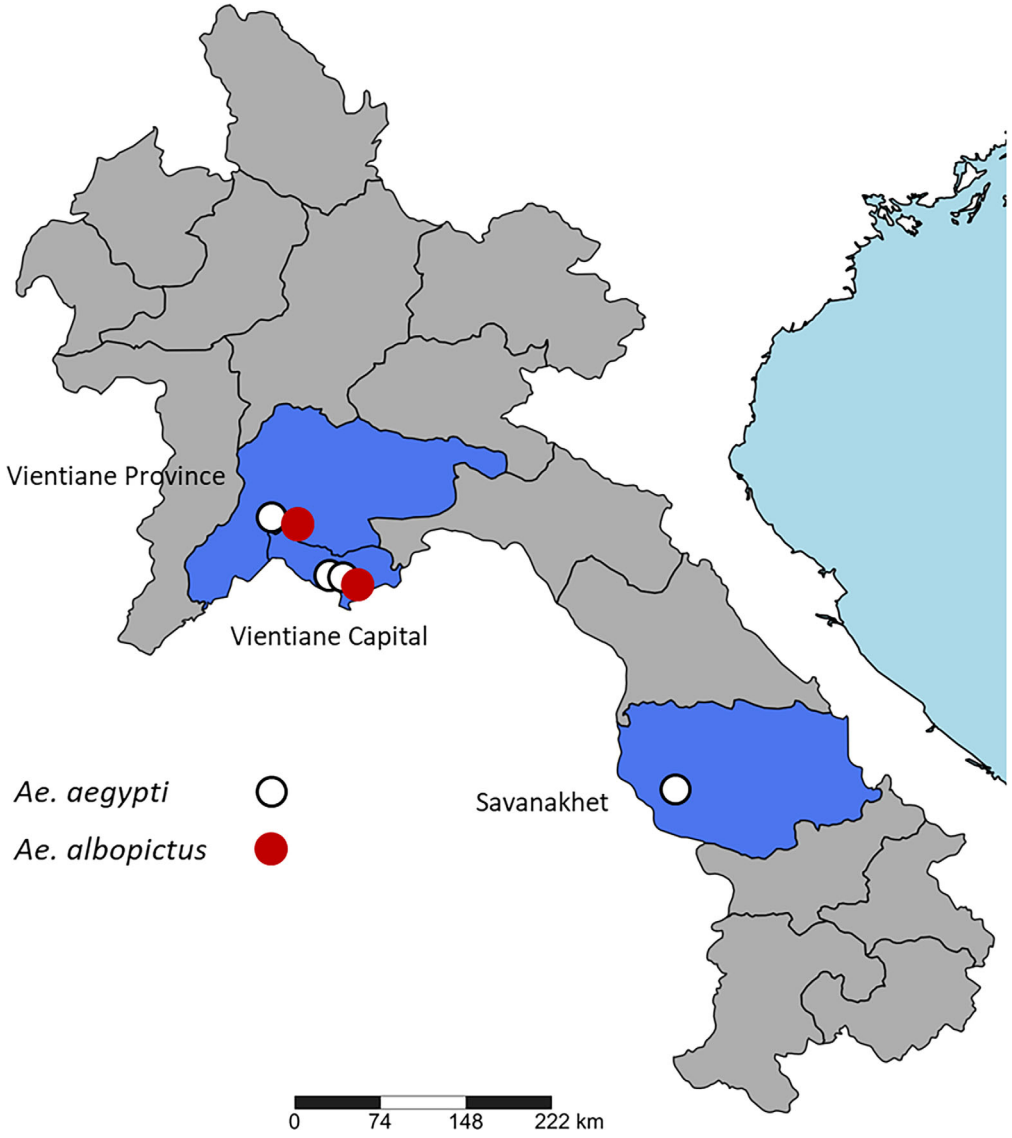

FIGURE 1 Map showing the locations of the mosquito collection sites in Lao PDR. Created with quantum GIS v.3.20.2, https://qgis.org/en/site/

RESULTS

Detection of mutations and intron variants in the voltage-gated sodium channel

Genomic DNA encoding for TM6 in domains II or III of the *vgsc* gene from individual female *Ae. aegypti* mosquitoes taken from the districts of Kaisone Phomvihane (Savannakhet province), Vangvieng (Vientiane province), Saysettha (Vientiane Capital) or Xaythany (Vientiane Capital) were amplified and analysed for mutations associated with insecticide resistance (Du et al., 2016). Also, *vgsc* sequences from *Ae. albopictus* mosquitoes taken from either Vangvieng or Saysettha were analysed. Sequence chromatograms showed no mutation at L982 (Brengues et al., 2003) in the 127 *Ae. aegypti* or 23 *Ae. albopictus*

TABLE 2 Frequencies of mutations in the *vgsc* gene of *Aedes aegypti* and *Aedes albopictus* collected from Kaisone Phomvihane (Savannakhet province, SVKS), Vangvieng (Vientiane province, VTVV), Saysettha (Vientiane capital, VTESVV) or Xaythany (Vientiane capital, VTESVL)

District	Aedes species	V410L	L982W	S989P			I1011M	V1016G			T1520I			I1532T	F1534C		
		VV	LL	SS	SP	PP	II	VV	VG	GG	TT	TI	II	II	FF	FC	CC
SVKS	*aegypti*	12	56	42	12	2	56	42	12	2	39	26	3	68	4	24	40
	Total	12	56	56			56	56			68			68	68		
Mutation frequency		0%	0%	14%			0%	14%			24%			0%	76%		
VTVV	*aegypti*	-	21	7	19	7	33	7	19	7	32	12	0	44	4	29	11
	Total	-	21	33			33	33			44			44	44		
Mutation frequency		-	0%	50%			0%	50%			14%			0%	58%		
VTESVV	*aegypti*	-	32	24	9	3	36	24	9	3	25	7	2	34	1	20	13
	Total	-	32	36			36	36			34			34	34		
Mutation frequency		-	0%	21%			0%	21%			16%			0%	68%		
VTESVL	*aegypti*	-	18	9	6	9	24	9	6	9	29	1	0	30	10	9	11
	Total	-	18	24			24	24			30			30	30		
Mutation frequency		-	0%	50%			0%	50%			2%			0%	52%		
VTVV	*albopictus*	-	11	32	0	0	32	28	0	0	26	0	0	26	4	16	6
	Total	-	11	32			32	28			26			26	26		
Mutation frequency		-	0%	0%			0%	0%			0%			0%	54%		
VTESVV	*albopictus*	-	12	31	0	0	31	31	0	0	30	3	0	33	19	14	0
	Total	-	12	31			31	31			33			33	33		
Mutation frequency		-	0%	0%			0%	0%			5%			0%	21%		

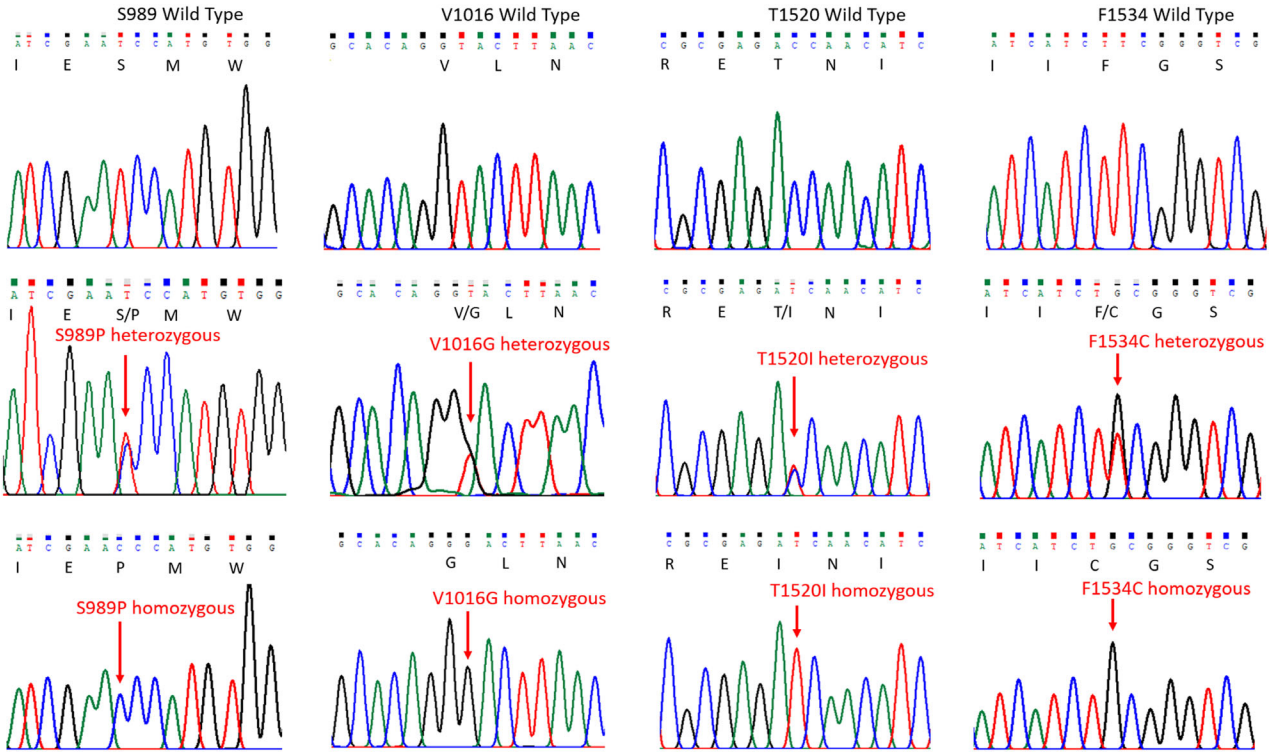

FIGURE 2 Sequence chromatograms showing heterozygous and homozygous mutations in *Aedes* sp. collected in Lao PDR. The amino acid position of the voltage-gated sodium channel (*Musca domestica* numbering) is shown.

mosquitoes analysed (Table 2). Likewise, no mutations were detected at I1011 (Rajatileka et al., 2008) or I1532 (Wei et al., 2021) in all *Ae. aegypti* or *Ae. albopictus* mosquitoes studied. In addition, no mutations were detected at V410 (Haddi et al., 2017) in domain I of the *vgsc* gene in 12 *Ae. aegypti* mosquitoes from Kaisone Phomvihane. However, the mutation F1534C (TTC to TGC; Figure 2), was observed in all groups of mosquitoes with frequencies ranging from 21% to 76% (Table 2). The mutation T1520I (ACC to ATC; Figure 2) was seen in *Ae. albopictus* mosquitoes taken from the Saysettha district (with a frequency of 5%) as well as in *Ae. aegypti* from all four districts (frequencies ranging from 2% to 24%; Table 2). The S989P (TCC to CCC) and V1016G (GTA to GGA) mutations were not detected in *Ae. albopictus* (Table 2) but were found in *Ae. aegypti* from each group (mutation frequencies ranging from 14% to 50%; Table 2 and Figure 2).

In *Ae. aegypti*, 13 different genotypes for VGSC mutations were observed (Table 3). With the exception of one mosquito from Kaisone Phomvihane, all *Ae. aegypti* analysed had at least one mutation. The S989P and V1016G mutations always occurred together, whether heterozygous (mutant and wild-type) or homozygous, and the heterozygous triple mutant, S989P + V1016G + F1534C, was most commonly observed being in 31 of the total of 133 *Ae. aegypti* analysed across the 4 districts. This heterozygous triple mutation was predominant in Saysettha and Vangvieng. For the Kaisone Phomvihane district, the most abundant genotype was the homozygous F1534C mutation whilst this was also the most common genotype seen for mosquitoes from Xaythany along with the homozygous S989P + V1016G double mutant. Interestingly, this homozygous double mutant was not seen at all in mosquitoes from Kaysone Phomvihane, highlighting that mosquitoes from different districts may have varying complements of genotypes. The homozygous S989P + V1016G + F1534C triple mutation was only detected in *Ae. aegypti* from Saysettha at a low frequency of 3%. The T1520I mutation (heterozygous or homozygous) always occurred with at least an F1534C mutation and the heterozygous S989P + V1016G + T1520I + F1534C quadruple mutation was observed in mosquitoes from Kaysone Phomvihane and Vangvieng.

Sequences with the S989P and V1016G mutations always had the intervening intron 20 consisting of 250 bp (Figure 3), which has been previously described as group A, whereas the intron in

TABLE 3 Frequency of genotypes of *vgsc* mutations from adult female *Ae. aegypti* or *Ae. albopictus* mosquitoes collected from Kaisone Phomvihane (Savannakhet province, SVKS), Vangvieng (Vientiane province, VTVV), Saysettha (Vientiane capital, VTESVV) or Xaythany (Vientiane capital, VTESVL).

Species	989	1016	1520	1534	I29	SVKS	VTESVV	VTVV	VTESVL
aegypti	SP	VG	TT	FC	++	13% (6/48)	28% (9/32)	40% (12/30)	18% (4/23)
	SS	VV	TT	CC	++	30% (14/48)	16% (5/32)	7% (2/30)	26% (6/23)
	SS	VV	TI	CC	++	25% (12/48)	16% (5/32)	13% (4/30)	4% (1/23)
	SS	VV	TT	FC	++	8% (4/48)	22% (7/32)	0% (0/30)	4% (1/23)
	PP	GG	TT	FF	++	0% (0/48)	3% (1/32)	10% (3/30)	26% (6/23)
	PP	GG	TT	FC	++	2% (1/48)	3% (1/32)	10% (3/30)	14% (3/23)
	SP	VG	TI	FC	++	6% (3/48)	0% (0/32)	13% (4/30)	0% (0/23)
	SS	VV	TI	FC	++	4% (2/48)	6% (2/32)	3% (1/30)	0% (0/23)
	SP	VG	TT	FF	++	4% (2/48)	0% (0/32)	3% (1/30)	4% (1/23)
	SS	VV	II	CC	++	4% (2/48)	3% (1/32)	0% (0/30)	0% (0/23)
	SP	VG	TT	CC	++	2% (1/48)	0% (0/32)	0% (0/30)	4% (1/23)
	PP	GG	TT	CC	++	0% (0/48)	3% (1/32)	0% (0/30)	0% (0/23)
	SS	VV	TT	FF	++	2% (1/48)	0% (0/32)	0% (0/30)	0% (0/23)
albopictus	SS	VV	TT	FF	−−	–	48% (14/29)	15% (3/20)	–
	SS	VV	TT	FC	+−	–	24% (7/29)	50% (10/20)	–
	SS	VV	TT	CC	++	–	0% (0/29)	25% (5/20)	–
	SS	VV	TT	FC	++	–	7% (2/29)	10% (2/20)	–
	SS	VV	TI	FC	+−	–	7% (2/29)	0% (0/20)	–
	SS	VV	TI	FC	++	–	3.5% (1/29)	0% (0/20)	–
	SS	VV	TT	FC	−−	–	3.5% (1/29)	0% (0/20)	–
	SS	VV	TT	FF	+−	–	3.5% (1/29)	0% (0/20)	–
	SS	VV	TT	FF	++	–	3.5% (1/29)	0% (0/20)	–

Note: i29 marks intron 29 where '+' denotes the 69 bp intron whilst '−' indicates 1 of the 5 intron 29 variants (Figure 4). Grey shading indicates a heterozygous mutation, black shading indicates a homozygous mutation whilst no shading indicates homozygous wild-type. Highest frequencies in each district are underlined.

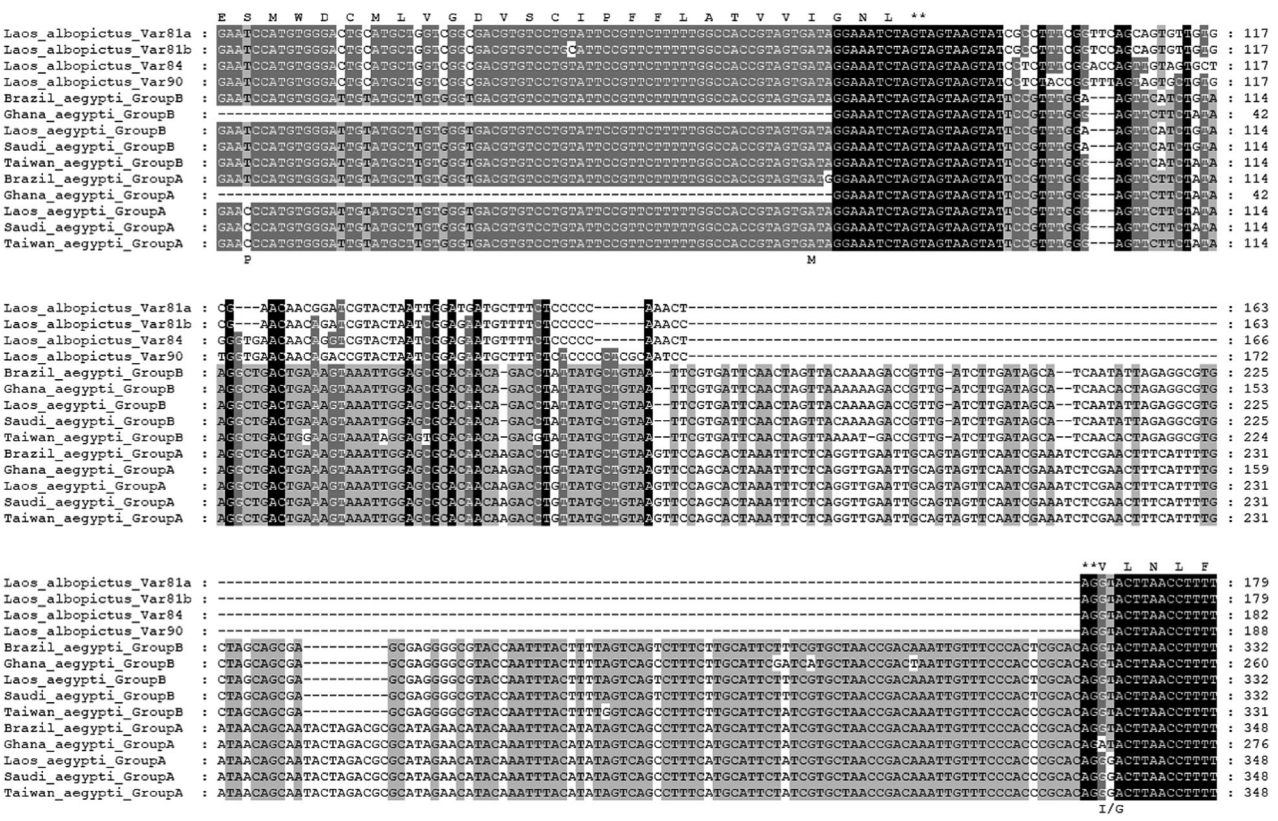

FIGURE 3 Alignment of variable intron 20 sequences and parts of flanking exons found in the *vgsc* gene of *Aedes aegypti* or *Aedes albopictus* collected in Laos. Genomic DNA sequences from *Ae. albopictus* are intron20Var81a (accession no. OM513683), intron20Var81b (OM513684), intron20Var84 (OM513685) and intron20Var90 (OM513686). Sequences from *Ae. aegypti* with S989P + V1016G mutations (group A, OM513681) and without these mutations (group B, OM513682) are shown. Also included in the alignment are group A and group B intron 20 sequences of *Ae. aegypti* from Brazil (Martins et al., 2009), Ghana (Kawada et al., 2016), Saudi Arabia (Fang et al., 2021) and Taiwan (Chung et al., 2019). Black and grey shading indicates degree of conservation. Amino acid regions corresponding to coding regions are shown at the top of the alignment whilst mutated amino acids (989P, 1012M, 1016I and 1016G) found in group A are shown at the bottom. Splice donor and acceptor sites are marked by asterisks. The alignment was constructed using Clustal X2 (Thompson et al., 1997) using default settings and viewed using Genedoc (http://nrbsc.org/gfx/genedoc/index.html).

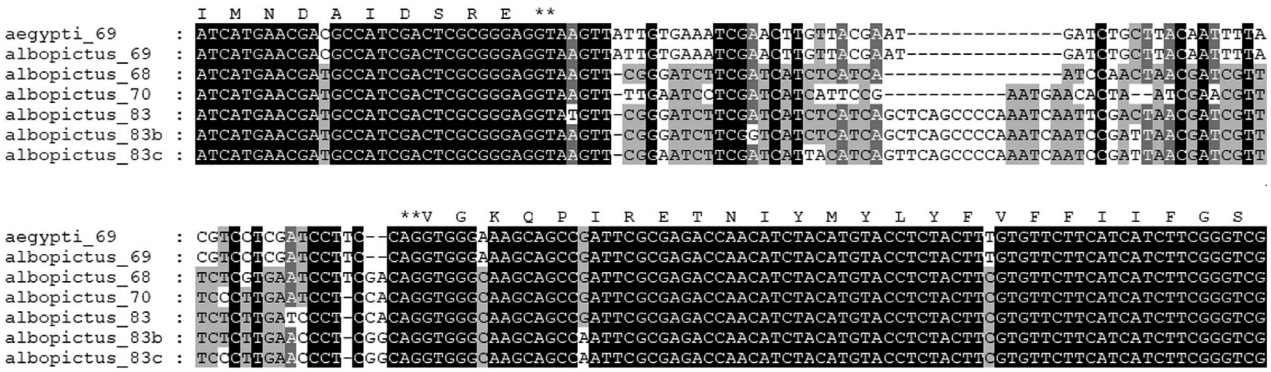

FIGURE 4 Alignment of intron 29 and parts of flanking exons of the *vgsc* gene from *Aedes aegypti* and the variable sequences found for intron 29 found in the *vgsc* gene from *Aedes albopictus* collected in Laos. The alignment includes the only sequence detected in *Ae. aegypti* consisting of 69 bp (accession number MN413379) along with a 69 bp intron in *Ae. albopictus* (MF774494.1) as well as intron29Var68 (MZ622708), intron29Var70 (MZ622709), intron29Var83 (MZ622710), intron29Var83b (MZ622711) and intron29Var83c (MZ622712). Black and grey shading indicates degree of conservation. The amino acid residues encoded by the flanking exons are shown at the top. Splice donor and acceptor sites are marked by asterisks. The alignment was constructed using Clustal X2 (Thompson et al., 1997) using default settings and viewed using Genedoc (http://nrbsc.org/gfx/genedoc/index.html).

sequences without this double mutation was group B consisting of 234 bp (Chung et al., 2019; Fang et al., 2021; Martins et al., 2009). Four different intron 20 sequences were observed in the *Ae. albopictus vgsc* gene, which are considerably shorter than the *Ae. aegypti* intron 20 sequences, consisting of 81, 81, 84 and 90 bp (Figure 3). They have been denoted here as intron20Var81a (Accession no. OM513683), intron20Var81b (OM513684), intron20Var84 (OM513685) and intron20Var90 (OM513686), respectively. No link between any of the *Ae. albopictus* intron20 variants and mutations were detected.

For *Ae. albopictus*, nine genotypes for *vgsc* were observed (Table 3). For mosquitoes from Vangvieng, the heterozygous F1534C mutant was most abundant. The homozygous F1534C mutation was only seen in mosquitoes from Vangvieng whilst the heterozygous T1520I mutation was found only in mosquitoes from Saysettha always together with the heterozygous F1534C mutant.

Six different intron 29 (Chang et al., 2009) sequences were detected in the *Ae. albopictus vgsc* gene whereas only one was seen for *Ae. aegypti* (Table 3, Figure 4). Both *Ae. aegypti* and *Ae. albopictus* possess an intron consisting of 69 bp with the same sequence whilst the remaining five *Ae. albopictus* introns are novel, consisting of 68, 70 bp and three with 83 bp. They have been denoted here as intron29Var68 (accession number MZ622708), intron29Var70 (MZ622709), intron29Var83 (MZ622710), intron29Var83b (MZ622711) and intron29Var83c (MZ622712), respectively. Synonymous mutations were detected in domain III of *Ae. albopictus vgsc* (Figure 4). D1505D (GAC or GAT), G1513G (GGA or GGC) and F1528F (TTT or TTC) have been previously observed in *Ae. albopictus* from West Bengal, India (Chatterjee et al., 2018), and we found a fourth, P1516P (CCG or CCA). These synonymous mutations appear to be linked to the intron 29 variant, for example, D1505 (GAC) was found in the 69 bp sequence whilst D1505 (GAT) was found in the other variants, and P1516 (CCA) was observed in intron29Var83b and intron29Var83c whereas P1516 (CCG) was present in the other variants.

DISCUSSION

In the present study, sequences of domains II and III of the *vgsc* gene from individual adult female *Ae. aegypti* and *Ae. albopictus* mosquitoes from Laos were analysed for the prevalence of mutations associated with pyrethroid resistance. As with previous reports studying *Ae. aegypti* from Laos (Marcombe et al., 2019; Shimono et al., 2021), the F1534C mutation was detected at a high frequency (over 50%, Table 2) in *Ae. aegypti* collected from all four sites, one of which is in Savannakhet (Kaisone district), a province that has not been previously investigated. Indeed, the homozygous F1534C mutation was the most frequent genotype detected for mosquitoes from Kaisone (Table 3). Notable incidence of the F1534C mutation appears to be a consistent trend in *Ae. aegypti* from the neighbouring countries China (average allele frequency 50%; Li et al., 2015), Cambodia (100%; Saingamsook et al., 2017), Myanmar (40%; Naw et al., 2020) and Thailand (63%; Stenhouse et al., 2013 and 62%; Plernsub, Saingamsook, Yanola, Lumjuan, Tippawangkosol, Walton, & Somboon, 2016) as well as other countries such as Costa Rica (100%; Zardkoohi et al., 2020), Cameroon (average 50%; Djiappi-Tchamen et al., 2021), Sri Lanka (average 37%; Ranathunge et al., 2021) and Saudi Arabia (55%; Fang et al., 2021). See Chen et al. (2020) and Fan et al. (2020) for comprehensive summaries of *vgsc* mutations found in *Ae. aegypti* by country (Chen et al., 2020; Fan et al., 2020). The F1534C mutation has been associated with resistance to type I pyrethroids as shown by bioassays where *Ae. aegypti* females surviving permethrin exposure harboured the F1534C mutation but not S989P and V1016G mutations that underlie resistance to type I and type II pyrethroids (Yanola et al., 2011). Heterologous expression of *Ae. aegypti vgsc* in *Xenopus laevis* oocytes also shows that the F1534C mutation alone reduces sensitivity to permethrin but not the type II pyrethroid, deltamethrin (Du et al., 2013).

We observed the T1520I mutation in all *Ae. aegypti* populations tested (Table 2), which never occurred by itself and was always present with at least the F1534C mutation (Table 3). The co-occurrence of T1520I and F1534C mutations has also been reported in *Ae. aegypti* from India (Kushwah et al., 2015) and Myanmar (Naw et al., 2020). Expression of mutant *Ae. aegypti vgsc* in *Xenopus* oocytes showed that alone the T1520I mutation did not alter sensitivity to permethrin but that the double T1520I + F1534C mutant was more resistant to permethrin than just F1534C whilst remaining sensitive to deltamethrin (Chen et al., 2019). This suggests that the addition of T1520I to F1534C may heighten the tolerance of mosquitoes to type I pyrethroids.

The S989P and V1016G mutations were found in all the *Ae. aegypti* populations studied (Table 2). Both mutations were always found together and both were either heterozygous or homozygous (Table 3). This confirms the previous report that S989P and V1016G mutations are present in Laos where the homozygous S989P + V1016G double mutant, which is found mainly in *Ae. aegypti* from Xaythany, was associated with deltamethrin resistance (Shimono et al., 2021 and Table 3). The co-occurrence of both mutations, which confers resistance to pyrethroids (Du et al., 2016), has also been observed in *Ae. aegypti* from several other countries such as Thailand (Stenhouse et al., 2013), Myanmar (Kawada et al., 2014), Singapore (Kasai et al., 2014), China (Li et al., 2015), Indonesia (Wuliandari et al., 2015), Sri Lanka (Fernando et al., 2018), Malaysia (Leong et al., 2019), Taiwan (Chung et al., 2019) and Saudi Arabia (Fang et al., 2021).

In *Ae. aegypti* mosquitoes from Saysettha and Vangvieng, the most prevalent genotype observed was heterozygous for the triple S989P + V1016G + F1534C mutant (Table 3). This genotype was also found in mosquitoes from Kaisone, Xaythany, Pakkading (Borlikhamxay province) and Khounkham (Khammouane Province) but not Thakhek (Khammouane Province; Table 3 and Shimono et al., 2021), demonstrating that it has reached several districts throughout Laos. Crossing experiments to generate *Ae. aegypti* with known *vgsc* genotypes showed that resistance to deltamethrin in heterozygous S989P + V1016G + F1534C mosquitoes were 28 fold when compared to the homozygous S989 + V1016 + F1534

susceptible strain (Plernsub, Saingamsook, Yanola, Lumjuan, Tippawangkosol, Sukontason, et al., 2016). Mosquitoes with the S989P + V1016G double mutation showed 4-fold resistance to deltamethrin demonstrating that the addition of the F1534C mutation heightens pyrethroid resistance (Plernsub, Saingamsook, Yanola, Lumjuan, Tippawangkosol, Sukontason, et al., 2016). In line with this, *Ae. aegypti vgsc* with the S989P + V1016G + F1534C triple mutation expressed in *Xenopus* oocytes was considerably less sensitive to permethrin or deltamethrin than the S989P + V1016G double mutant (Hirata et al., 2014). Therefore, our findings of heterozygous S989P + V1016G + F1534C *Ae. aegypti* in four districts (Table 3) indicate resistance to pyrethroid types I and II in Laos. We found only one *Ae. aegypti*, from Saysettha, to harbour the homozygous S989P + V1016G + F1534C triple mutant (Table 3) and only one mosquito with this genotype was observed from Pakkading and Khounkham (Shimono et al., 2021). *Ae. aegypti* with the homozygous triple mutant were found to be nearly two-fold more resistant to deltamethrin than the heterozygous triple mutant (Plernsub, Saingamsook, Yanola, Lumjuan, Tippawangkosol, Sukontason, et al., 2016). Thus, it is prudent to continue monitoring for the increase in the prevalence of the homozygous S989P + V1016G + F1534C triple mutant, which would signal further selection for pyrethroid resistance.

For the first time, the presence of resistance mutations in the *vgsc* gene in *Ae. albopictus* from Laos was surveyed. S989P and V1016G mutations were not detected, however, F1534C were observed in mosquitoes from both Saysettha and Vangvieng whilst T1520I was only seen in mosquitoes from Saysettha (Table 2). The most prevalent genotype for *Ae. albopictus* from Saysettha was homozygous wild-type with no mutations whilst the second most common genotype was the heterozygous F1534C mutation (Table 3). For Vangvieng, the F1534C heterozygote was the most frequent genotype whilst the second most common genotype was the homozygous F1534C mutation, which may indicate an emerging resistance to type I pyrethroids in this district (Pichler et al., 2019; Yanola et al., 2011). In line with this, *Ae. albopictus* taken from Kao-gnot, a village near our study sites in the Vientiane Capital, showed suspected resistance to permethrin (Tangena et al., 2018). In agreement with bioassays performed on *Ae. albopictus* (Tangena et al., 2018), the lack of S989P and V1016G mutations indicates that *Ae. albopictus* in Laos remain susceptible to deltamethrin. However, the detection of the V1016G mutation in *Ae. albopictus* from Italy and Vietnam (Kasai et al., 2019; Pichler et al., 2019) suggests that these mutations may be selected for with continued use of pyrethroids.

Our study focused on domains II and III of the *vgsc* gene. However, mutations associated with pyrethroid resistance have been found in other domains, such as V410L in domain I of *Ae. aegypti* from America (Granada et al., 2018; Haddi et al., 2017; Saavedra-Rodriguez et al., 2018) and D1763Y in domain IV of *Ae. aegypti* from Taiwan (Chang et al., 2009). An initial analysis did not detect a mutation at 410 in 12 *Ae. aegypti* mosquitoes (Table 2) but it is prudent to analyse sequences of all domains in the future in order to understand more comprehensively the prevalence of target-site resistance in Laos.

We observed intron variants in the *vgsc* genes in both *Ae. aegypti* and *Ae. albopictus*. For *Ae. aegypti* we detected two different sequences for intron 20 with lengths of 250 and 234 bp, respectively, denoted as groups A and B (Martins et al., 2009). It has been noted that there is a link between intron length and the presence of resistance mutations, where the I1011M and the V1016I mutations were found only when there was the longer group A intron in *Ae. aegypti* from Brazil (Martins et al., 2009). We observed the group A intron co-occurring with the S989P and V1016G mutations (Figure 3), and that the F1534C mutation, when without S989P and V1016G, was found with the group B intron, which was also seen in *Ae. aegypti* from Taiwan and Saudi Arabia (Chung et al., 2019; Fang et al., 2021). However, F1534C was found with the group A intron in *Ae. aegypti* from Ghana (Kawada et al., 2016), which may reflect different histories of insecticide usage and mutation events in Asia and Africa (Cosme et al., 2020; Fang et al., 2021).

We found four novel variants for intron 20 in *Ae. albopictus vgsc* (Figure 3), which has a low sequence identity (around 30%) to Groups A and B of *Ae. agypti*. Also, six different sequences ranging in length from 68 to 83 bp were detected for *Ae. albopictus* intron 29, which precedes F1534 (Figure 4). All of the *Ae. albopictus* intron 29 variants occurred in the wild-type *vgsc* lacking mutations in domains II and III and where T1520I and F1534C mutations were detected there did not appear to be an association of particular variants of either intron 20 or intron 29 with resistance mutations. Interestingly, the majority of *vgsc* sequences lacking resistance mutations did not possess the 69 bp intron 29 sequence (Table 3). Further analysis of *Ae. albopictus vgsc* sequences should be conducted to determine whether there are links between intron 20 and 29 sequences and resistance mutations or lack thereof.

Our detection of mutations in the *vgsc* gene of *Ae. aegypti* from the four districts studied highlights previous findings that insecticide resistance in Laos is of concern and is threatening efforts to maintain effective vector control (Marcombe et al., 2019; Shimono et al., 2021). Therefore, integrated vector control approaches and continuous insecticide resistance monitoring programmes are of prime importance in order to control diseases caused by arboviruses in this country. The Lao government recently (2019) adopted a new strategy to deploy alternative larvicides with different modes of action to overcome the spread of temephos resistance in *Ae. aegypti* larvae (Marcombe et al., 2018). Repurposing insecticides originally used in agriculture may provide further vector control tools. For example, the neonicotinoid clothianidin, used in conjunction with deltamethrin, has the potential to prolong the control of the malaria mosquito, *An. gambiae*, which are showing resistance to pyrethroids (Ngufor et al., 2017). A similar strategy could be used for insecticides that act on γ-aminobutyric acid (GABA) receptors, such as fipronil, especially considering that it has been shown that *Aedes* mosquitoes in Laos lack a mutation at A296 in the GABA receptor, resistance to dieldrin (RDL), which underlies insecticide resistance (Marcombe et al., 2020). A combination of fipronil and permethrin has been shown to provide highly effective inhibition of feeding as well as considerable insecticidal efficacy against *Aedes* mosquitoes on dogs for at least four weeks, which may aid in the control of mosquitoes in the vicinity of treated domesticated animals (Fankhauser et al., 2015).

Based on bioassays (Tangena et al., 2018) and the present study, the status of pyrethroid resistance in *Ae. albopictus* from Laos is not of serious concern when compared to that of *Ae. aegypti*. However, there are signs of emerging resistance to type I pyrethroids, in particular in the Vientiane province. A similar situation appears to be occurring on a more global scale, with an increase in *vgsc* mutations observed in *Ae. albopictus* samples that were taken between 2011 and 2018, most notably in China, Greece and Italy (Tancredi et al., 2020). Thus, as with *Ae. aegypti*, constant monitoring programmes and alternative strategies for controlling *Ae. albopictus* should be utilized in order to prolong the effectiveness of pyrethroids against this species thereby maximizing vector control.

AUTHOR CONTRIBUTIONS

Andrew K. Jones and Sebastien Marcombe conceived the idea for the project. Sebastien Marcombe, Phonesavanh Luangamath, Somphat Nilaxay, Vacky Vungkyly, Phoutmany Thammavong and Paul T. Brey supervised mosquito collections in Laos, rearing and laboratory tests. Sebastien Marcombe and Phoutmany Thammavong analysed insecticide resistance tests and contributed to writing the manuscript. Katherine Shimell, Rachel Savage, Edward Howlett, Anne Baby, Mathew King, Josie Clarke, Chloe Jeffries, Josna Jojo, Emily Lacey, Farris Bhatty, Dadirayi Mabika, Andrea Dela Cruz, Cerys Fisher, Milca Mbadu and Iasonas Despiniadis extracted DNA from mosquitoes, amplified domains II and III of *vgsc* and analysed sequence data. Andrew K. Jones analysed sequence data and contributed to writing the manuscript. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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