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Malaria and Dengue mosquito vectors from Lao PDR show a lack of the *rdl* mutant allele responsible for cyclodiene insecticide resistance

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Abstract

The gamma-aminobutyric acid (GABA) receptor, RDL, plays important roles in neuronal signalling and is the target of highly effective insecticides. A mutation in RDL, commonly A296S, underlies resistance to several insecticides such as cyclodienes. Even though the use of cyclodienes has been banned, the occurrence of mutations substituting A296 is notably high in mosquitoes from several countries. Here we report a survey investigating the prevalence of the *Rdl* mutant allele in mosquitoes from Laos, a country where mosquito-borne diseases such as malaria and dengue fever are health concerns. *Anopheles* and *Aedes* mosquitoes were collected from twelve provinces in Laos. Adult bioassays on *Ae. aegypti* (Linnaeus) (Diptera: Culicidae) and *Ae. albopictus* (Skuse) showed that all the populations tested were susceptible to dieldrin (4%) following WHO protocols. Exon 7 from a total of 791 mosquitoes was sequenced to identify the amino acid encoded for at 296 of RDL. Only one of these mosquitoes, *Anopheles maculatus rampae* (Diptera: Culicidae) from Attapeu, carried the mutant allele being heterozygous for A296S. We therefore found a general lack of the *Rdl* mutant allele indicating that mosquitoes from Laos are not exposed to insecticides that act on the GABA receptor compared to mosquitoes in several other countries. Identifying the prevalence of the *Rdl* mutation may help inform the potential use of alternative insecticides that act on the GABA receptor should there be a need to replace pyrethroids in order to prevent/manage resistance.

Keywords

dieldrin, GABA receptor, insecticide resistance, Laos, mosquito

Introduction

The insect γ-aminobutyric acid (GABA) receptor, RDL (resistant to dieldrin), is the target of highly effective insecticides such as cyclodienes (e.g. dieldrin), phenylpyrazoles (e.g. fipronil) and isoxazolines (e.g. fluralaner) (Buckingham et al. 2017). It is a member of the Cys-loop ligand-gated ion channel superfamily (Jones 2018) and plays many important roles in the nervous system, examples of which are regulation of aggression (Yuan et al. 2014), sleep (Liu et al. 2014) and food consumption (Cheung and Scott 2017).

An alanine to serine mutation in the second transmembrane domain (TM2) of RDL (referred to here as A296S) has been found to underlie resistance to several insecticides including picrotoxin and cyclodienes (Ffrench-Constant et al. 1993, Buckingham et al. 2017). Mutation at A296, either to serine or another amino acid such as asparagine or glycine, has since been associated with insecticide resistance in various species ranging from pests afflicting domesticated animals (the cat flea *Ctenocephalides felis* (Rust et al. 2015)), pests afflicting livestock (the horn fly *Haematobia irritans* (Domingues et al. 2013)), crop pests (e.g. the planthopper *Laodelphax striatellus* (Nakao 2017)), and mosquito disease vectors (the malaria vector *Anopheles gambiae* (Du et al. 2005)). In several cases, other mutations in *Rdl* have been observed (Feyereisen et al. 2015, Taylor-Wells and Jones 2017). For instance, T345 was detected in dieldrin-resistant *An. gambiae* (Taylor-Wells et al. 2015), V327I in *An. funestus* (Wondji et al. 2011) whilst V327I and T345S were identified in *An. sinensis* (Yang et al. 2017). These mutations were found in addition to a mutation at A296 highlighting this TM2 site as a useful marker for detecting target site resistance. Analysis of mosquitoes from different countries have recorded a notable prevalence of mutations at A296. For example, *An. funestus* mosquitoes from Burkina Faso, West Africa, were found to be resistant to dieldrin and possessed the A296S mutation (Wondji et al. 2011). Other studies, measuring the prevalence of mutations at A296 to predict the extent of resistance in varying countries noted 100% incidence of the mutation in 33 *An. sinensis* mosquitoes from Nanning, China (Yang et al. 2017) whereas 11% of 154 *Anopheles* mosquitoes of various species from Indonesia were found to have either the A296S or A296G mutation (Asih et al. 2012). Bioassays showed that *Aedes albopictus* (Skuse) (Diptera: Culicidae) mosquitoes from Malaysia had low level resistance to dieldrin and 62% of 82 *Ae. albopictus* mosquitoes were recorded to possess the A296S mutation (Low et al. 2015). It has been suggested that the persistence of cyclodienes in the environment or the still continued use of these insecticides, such as in crop protection, may be underlying the surprisingly high incidence of the *Rdl* mutation in mosquitoes (Wondji et al. 2011, Asih et al. 2012) although further studies are required to prove that this is the case.

Lao PDR (here after Laos) is a landlocked country in South-East Asia, which mostly consists of mountainous ranges, forests, plateaux and highlands through which rivers cut through. In Laos, 170 mosquito taxa have been officially reported (Motoki et al. 2019) and mosquito-borne diseases such as malaria and dengue fever, which are spread by *Anopheles* and *Aedes* mosquitoes respectively, present important health issues (Khampapongpane et al. 2014, Souris et al. 2017). As of September 2019, 27,904 dengue cases, including 54 deaths, were reported in Laos (World Health Organization 2019) and between 2010 and 2017 the incidence of malaria varied between 9,336 and 48,071 presumed and confirmed cases (World Health Organization 2018). In Laos, vector control relies mainly on the use of pyrethroid insecticides (larvicides, thermal fogging, indoor residual spraying, and impregnated bed-nets). Dieldrin was previously used for agricultural purposes in the 1950's and 60's in very low quantities compared to neighbouring countries as Laos was agriculturally self-sufficient. This insecticide was banned in Laos in 1992 as was the case for other organochlorine insecticides used for Public Health against malaria such as DDT (Ministry of Natural Resources and Environment Pollution Control Department 2016). A recent study implemented in the Mekong region determining the presence of Persistent Organic Pollutants (POPs) in wetlands showed that dielrdrin was detected at low concentrations in several provinces of Laos ranging from the northern to the southern parts of the country (Tran et al. 2014). Another insecticide acting on the GABA receptor, endosulfan, was found in higher quantities in the same areas of the country (Tran et al. 2014). This insecticide was used to protect coffee trees in the 2000's (Committee for the Planning and Investment Lao People's

Democratic Republic Japan International Cooperation Agency 2005) whilst, more recently, the use of endosulfan in banana plantations in the north of Laos (i.e. Bokeo province) was reported (Ghosh 2016). The utilization of insecticides such as endosulfan may select for *Rdl* mutations in mosquitoes that would present cross-resistance and therefore hamper the efficacy of different insecticides to be used for vector control in the future, an example of which is fipronil (Kolaczinski and Curtis 2001, Davari et al. 2007). Indeed, recent studies implemented in Laos showed that insecticide resistance to pyrethroids and organophosphates has developed in malaria secondary vectors and in the two dengue vectors, *Ae. aegypti* (Linnaeus) (Diptera: Culicidae) and *Ae. albopictus*, highlighting the growing need for alternatives to currently used insecticides (Marcombe et al. 2017, Marcombe et al. 2018, Tangena et al. 2018).

In this study, the insecticide resistance levels of the vectors against dieldrin and the prevalence of RDL A296 mutations in *Anopheles* and *Aedes* mosquitoes from Laos was investigated in order to provide information to the Public Health authorities in the country for the potential use of alternative insecticides that act on the GABA receptor.

Materials and Methods

Collection of mosquito samples and identification of species and subspecies

Ethical clearance for *Anophele*s sp. collection was obtained from Lao PDR Council of Medical Science National Ethics Committee (authorization No033/NECHR, 05/07/2013). Each person collecting mosquitoes signed an informed consent form and received a Japanese Encephalitis vaccination (IMOJEV®MD, GPO-MBP Co., Ltd). Twenty-five villages from twelve provinces in Laos were selected for the study (Figure 1, Table 1). The collection areas were geo-referenced using a Global Positioning System (Table 1).

For collecting *Anopheles* species, indoor and outdoor human landing catch and cow bait collections were used (Marcombe et al. 2017). The genus of collected adult mosquitoes were morphologically identified as *Anopheles* species or species group/complex in a field laboratory, using microscopes and appropriate identification keys for Southeast Asian Anopheline (Rattanarithikul et al. 2006). Identification was carried out at the Institut Pasteur du Laos (IPL) and the Center for Malariology, Parasitology and Entomology by entomologist staff onsite. After identification, mosquitoes of the same species were stored in RNAlater® or in silica gel in labelled 1.5 ml tubes and stored at -20°C for subsequent laboratory analysis. Female *An. Dirus* (Diptera: Culicidae), *An. minimus* (Theobald) and *An. maculatus* (Theobald) complex mosquitoes (primary malaria vectors) as well as *An. aconitus* s.l. (Diptera: Culicidae), *An. annularis* s.l. and *An. barbirostris* group mosquitoes (secondary malaria vectors) were cut into two parts to separate the head plus thorax from the abdomen and legs. DNA was extracted from the head and thorax using the cetyl trimethyl ammonium bromide (CTAB) method (Murray and Thompson 1980). An allele-specific multiplex assay (AS-PCR) examining the ITS-2 region of the DNA (Walton et al. 1999, Garros et al. 2004, Walton et al. 2007) was used for molecular detection of sibling species within the dirus complex, minimus complex and maculatus complex assemblages. For *Aedes* sp. mosquitoes, larval and pupal collections were made in the field (rural and urban areas) and about fifty sampling places (tires, jars, freezers, buckets, toilets, vases, cups etc.) per each location were required to produce sufficient number of specimens for colonization in the laboratory. All samples were brought back to the laboratory at IPL and maintained under controlled conditions (27 ± 2 ºC and 80 ± 10% relative humidity) with a diet of powdered cat food for rearing until adults (F1 generation). After adult identification using morphological keys, mosquitoes were separated by species and were kept for breeding following standardized techniques (Marcombe et al. 2014). Resulting eggs were kept for the adult bioassays. The insecticide susceptible reference strain (*Ae. aegypti* USDA) was used as a control for the different tests. The USDA laboratory population originated from the Center for Medical, Agricultural, and Veterinary Entomology, Gainesville, FL, U.S.A and has undergone continuous colonization for 40 years at Kasetsart University, Bangkok, Thailand (Chuaycharoensuk et al. 2011). This strain was colonized at IPL before experiments were carried out for this study.

Insecticide resistance bioassays

Adult bioassays were run using filter papers treated with a diagnostic dose of 4% dieldrin for *Ae. aegypti* and *Ae. albopictus* following WHO protocols to detect resistant mosquitoes (World Health Organization 2016). WHO test kits were used to measure mortality resulting from tarsal contact with treated filter papers. Four replicates of 25 non-blood-fed female mosquitoes (2–5 days of age) were maintained for 60 minutes at 27 ± 2°C and a relative humidity of 80 ± 10% in holding tubes. The mosquitoes were then subjected to dieldrin for 60 minutes in exposure tubes that were placed vertically under subdued light. Mosquitoes were kept in similar conditions of temperature and humidity for 24 hours after exposure after which mortality was recorded. According to WHO criteria, a population was considered resistant if mortality after 24 h is under 90% whilst resistance was suspected with mortality between 90 and 98%. With mortality over 98%, the population was deemed susceptible.

PCR amplification of mosquito Rdl TM2

Genomic DNA was extracted from individual mosquitoes using 250 μl Trizol (Fisher Scientific, Loughborough, UK) following the manufacturer's protocol. 2 μl of extracted DNA (not at a specific concentration) was used as template for PCR to amplify exon 7 of *Rdl*, as has been performed in previous studies (Wondji et al. 2011, Asih et al. 2012), using primers listed in Table 2 and the Q5® High-Fidelity PCR Kit (New England Biolabs, Ipswich, MA, USA). For *Anopheles* mosquitoes, one round of PCR was performed whereas for *Aedes* a nested approach was required to generate enough DNA to be visualised by agarose gel electrophoresis. In the nested reaction, a final dilution of 1 in 5000 of the first PCR reaction was used as template for the second nested PCR reaction. Agarose gel electrophoresis was performed to verify success of PCR products with expected sizes of amplification products given in Table 2. PCR products were purified using the Monarch® PCR & DNA Cleanup Kit (New England Biolabs, Ipswich, MA, USA) before being sequenced at SourceBioscience (available online: https://www.sourcebioscience.com/). Sequences were aligned with either *An. gambiae Rdl* (Accession number KX431144) or *Ae. aegypti Rdl* (U28803) using Global Align available at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence chromatograms were visualised using Chromas (available online: https://technelysium.com.au/wp/chromas/).

Results

The results of the adult bioassays are shown in Table 3. The susceptible insecticide reference strain *Ae. aegypti* (USDA) showed full susceptibility to dieldrin. All the populations tested, *Ae. aegypti* and *Ae. albopictus*, were also fully susceptible to dieldrin (i.e. 100% mortality) with the use of 4% impregnated paper.

Exon 7 was amplified from 791 individual mosquitoes representing two *Aedes* species and ten *Anopheles* subspecies taken from twelve provinces in Laos (Table 4). To our knowledge this is the first report of *Rdl* sequence from *An. hyrcanus* (Pallas) (Diptera: Culicidae) (submitted with Accession number MF977812). The sequences showed high level of conservation in the nucleotide sequences with only one base difference between *Aedes* and *Anopheles* species (Figure 2). Despite this difference, the deduced amino acid sequence remained the same for all mosquito species.

Out of the 791 mosquitoes tested, 790 were wildtype for A296 (gca) (Figure 3a, Table 4). The sequence chromatogram of the remaining mosquito, *An. maculatus rampae* (Diptera: Culicidae) from the Attapeu province, showed mixed peaks indicating the mosquito is heterozygous for alanine or serine at 296 (gca or tca) (Figure 3b). A repeat of the PCR from this *An. maculatus rampae* mosquito confirmed the presence of the heterozygous mutation.

Discussion

Due to the high resistance levels against pyrethroids detected in *Aedes* and to a lesser extent in *Anopheles* in Laos (Marcombe et al. 2017, Marcombe et al. 2018, Tangena et al. 2018), recommendations to Public Health authorities of the country were made to use alternative insecticides belonging to different insecticide families. Because of potential cross resistance between dieldrin and fipronil (Kolaczinski and Curtis 2001, Davari et al. 2007), which is an insecticide authorized for agricultural purpose in Laos (Lao People's Democratic Republic Ministry of Agriculture and Forestry 2017) and thus a potential insecticide to be use for Public Health (Hoppe et al. 2016, Poche et al. 2017), detection for dieldrin resistance prior to application of fipronil is recommended.

We therefore report here the first study on the insecticide resistance status of *Ae. aegypti* and *Ae. albopictus* against dieldrin in Laos. All the populations tested throughout the country were susceptible to dieldrin. In agreement with this, we found that none of the 497 *Aedes* mosquitoes tested had a mutation at A296 of *Rdl* and only one of the 294 *Anopheles* mosquitoes (*An. maculatus* from Attapeu) was shown to be heterozygous for the A296S mutation. This is in contrast to many of the other studies investigating the prevalence of insecticide resistance mutations in mosquitoes from a variety of countries. As summarised in Table 5, in thirteen of the twenty nine samples shown, mutation at A296 in *Rdl* of over 50% of mosquitoes tested were observed and only two of the samples showed a complete absence of the mutation (excluding the present study). This is surprising considering that the use of cyclodienes to control mosquitoes has been banned (Tantely et al. 2010). It has been suggested that the persistence of the *Rdl* mutation may be due to either mosquitoes being exposed to the agricultural use of insecticides that target the GABA receptor, to cyclodienes still present in the environment, or to the illegal use of cyclodienes (Tantely et al. 2010, Wondji et al. 2011, Antonio-Nkondjio et al. 2017). Another possibility may be that substitution of A296 is a polymorphism that is present irrespective of whether there is insecticide use and thus would commonly occur in mosquito populations (Ffrench-Constant and Bass 2017). Our finding of no A296 mutations in *Aedes* mosquitoes and only in 0.34% of *Anopheles* species suggests that this is not the case instead pointing towards the substitution of A296 being a result of selection pressure such as exposure to insecticides. In line with this, studies have indicated that mutation at A296 results in a fitness cost. For example, male *Anopheles* mosquitoes that were homozygous for the A296S mutations showed reduced mating success compared to heterozygote-resistant males indicating that the *Rdl* mutation has a detrimental effect on the ability of mosquitoes to mate (Platt et al. 2015).

It remains to be determined whether the high prevalence of the mutant *Rdl* allele in mosquitoes from any of the several countries studied (Table 5) is maintained by mutations elsewhere in the genome (Ffrench-Constant and Bass 2017). For instance, a 2La chromosomal inversion, which was found to be associated with dieldrin resistance in *An. gambiae* (Brooke et al. 2000), may suppress cross-over of *Rdl* (located in chromosome 2L) ensuring that *Rdl* mutations are preserved even though they confer a fitness cost. However, surveys found a reduction in the prevalence of *Rdl* mutations in *An. funestus* collected over several years in Cameroon (Table 5 (Menze et al. 2016)) suggesting that in at least this case the lack of using cyclodienes has resulted in *Rdl* reverting to wild-type (Menze et al. 2018).

It is concluded that the presence of mutations at *Rdl* A296 can signify current exposure to insecticides targeting the GABA receptor. Our finding of only one A296S mutation indicates that mosquitoes in Laos are exposed to lower levels of these insecticides compared to other countries in Southeast Asia such as Indonesia and Malaysia (Asih et al. 2012, Low et al. 2015). Further studies are required to determine whether the mutation found in *An. maculatus rampae* is indicative of a higher prevalence of *Rdl* mutations in Attapeu and therefore exposure to insecticides acting on the GABA receptor in this province. Knowledge of the occurrence of mutations in *Rdl* may help inform the future use of insecticides in Laos should alternatives to pyrethroids be desired in order to manage insecticide resistance.

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Authors' contributions

SMar, SMai, SN and PTB supervised mosquito collections in Laos, rearing and laboratory tests. SMar analysed insecticide resistance tests and contributed to writing the manuscript. SMar, SC, PT, NP, KL, PL, NX and SN collected, identified and performed insecticide resistance tests. ZR, PJS, OHA, JF, AEJ, SK, WA, CB, HK, JH, RL, NMP, MS, NB, NCK, SL and GS extracted DNA from mosquitoes, amplified exon 7 of *Rdl* and analysed sequence data. AKJ conceived the idea for the project, analysed sequence data and contributed to writing the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1. List of *Aedes* sp. and *Anopheles* sp. populations collected in Laos from 2014 to 2018.

Mosquito species	**Province**	**District**	**Village**	**Latitude**[1]	**Longitude**[1]
Aedes sp.	Attapeu	Samakheexay	Xaysa-art	14.484109	106.501415
	Bokeo	Huayxai	Huayxai	20.27032	100.41376
	Borlikhamxay	Paksan	Paksan	18.37134	103.66586
	Champasak	Pakse	Pakse	15.12267	105.80289
	Khammouane	Mahaxay	Mahaxay	17.41078	105.19927
	Luang Namtha	Luang Namtha	Luang Namtha	21.00633	101.40792
	Luang Prabang	Luang Prabang	Khomkhuang	19.902775	102.156213
			Thatnoy	19.531432	102.075364
			Thongchaleun	19.887366	102.132352
	Saravane	Lakhonepheng	Lakhonepheng	15.485507	105.403469
	Vientiane Capital	Sisattanak	Kao-gnot*	17.962684	102.615035
			Suanmone	17.919145	102.621941
		Xaithany	Oudomphon	18.125733	102.665011
			Phailom	18.057037	102.774993
	Xayaboury	Xayaboury	Taling	17.784729	101.170521
			Xayaboury	19.26457	101.71184
Anopheles sp.	Attapeu	Sanamxay	Hadoudomxay	14.45668	106.367272
	Bokeo	Paktha	Hadsa	19.92268	100.581479
	Borlikhamxay	Khamkeut	Phameung	18.11425	104.80229
	Khammouane	Gnommalath	Koutphadang	17.63663	105.177948
	Luang Prabang	Pakseng	Sopjak	20.13477	102.558343
	Phongsaly	Bountai	Boulykao	21.33778	102.082469
	Vientiane Province	Feuang	Na-ang	18.55996	101.973886
	Sekong	Lamam	Lavynoy	15.27291	106.697478

	Saravane	Toomlarn	Katao	15.95187	106.352853

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[1] GPS coordinates

*IPL strain collected at the Institut Pasteur du Laos

Table 2. Primers used in PCR to amplify exon 7 from genomic DNA of mosquitoes. Size of amplification products are also provided.

Species	**Forward Primer**	**Reverse Primer**	**Size bp**
Ae. aegypti *Ae. albopictus*	1st PCR: atgtatcttcttctaatttctctc[1] Nested PCR: ttaccaaatatatgccaactaac[1]	1st PCR: aacacaaacacgaaagagactg[1] Nested PCR: atttgtacaagtagcaaatagtg[1]	428
An. hyrcanus *An. kochi* (Donitz) (Diptera: Culicidae) *An. minimus* *An. nivipes* (Theobald) (Diptera: Culicidae) *An. philippinensis* (Ludlow) (Diptera: Culicidae)	cactaaagcaaggaatcaaagc[2]	caacgaaactaacagagtacag[2]	435
An. maculatus	ccatccggattaattgtaatc[3]	cagcagactggcaaatacc[3]	210

Sequences are shown 5'-3'.

[1] Sequence based on the *Rdl* gene of *Ae. aegypti* available at VectorBase (https://www.vectorbase.org/).

[2] Sequence based on the *Rdl* gene of *An. gambiae* available at VectorBase (https://www.vectorbase.org/).

[3] Sequence based on partial coding sequence of *Rdl* from *An. maculatus* (accession number JN690017).

Table 3. Resistance status of adult *Aedes* to dieldrin (4%) according to WHO criteria (World Health Organization 2016).

Province	**Species**	**N tested**	**Mortality after 24h (%)**	**Status**
Bokeo	*Ae. aegypti*	100	100	Susceptible
	Ae. albopictus	100	100	Susceptible
Borlikhamxay	*Ae. aegypti*	100	100	Susceptible
	Ae. albopictus	100	100	Susceptible
Champasak	*Ae. aegypti*	100	100	Susceptible
	Ae. albopictus	100	100	Susceptible
Khammouane	*Ae. aegypti*	99	100	Susceptible
	Ae. albopictus	100	100	Susceptible
Luang Namtha	*Ae. aegypti*	100	100	Susceptible
	Ae. albopictus	100	100	Susceptible
Xayaboury	*Ae. aegypti*	100	100	Susceptible
	Ae. albopictus	100	100	Susceptible
Vientiane Capital	*Ae. aegypti*	100	100	Susceptible
	Ae. albopictus	100	100	Susceptible
USDA (reference)	*Ae. aegypti*	100	100	Susceptible

458 **Table 4.** Frequency of mutations at RDL 296 in mosquitoes from different provinces in Laos.

Species	**Province**	**SS**	**RS**	**RR**
Ae. aegypti	Bokeo	30	0	0
	Borlikhamxay	25	0	0
	Champasak	30	0	0
	Khammouane	23	0	0
	Luang Prabang	74	0	0
	Luang Namtha	24	0	0
	Saravane	11	0	0
	Vientiane Capital	56	0	0
	Xayaboury	41	0	0
Ae. albopictus	Attapeu	2	0	0
	Bokeo	20	0	0
	Borlikhamxay	22	0	0
	Champasak	30	0	0
	Khammouane	28	0	0
	Luang Namtha	23	0	0
	Luang Prabang	11	0	0
	Vientiane Capital	28	0	0
	Xayaboury	19	0	0
An. hyrcanus	Attapeu	8	0	0
	Bokeo	28	0	0
	Borlikhamxay	16	0	0

	Luang Prabang	16	0	0
	Phongsaly	11	0	0
	Saravane	5	0	0
	Sekong	15	0	0
	Vientiane Province	15	0	0
An. kochi	Khammouane	5	0	0
	Sekong	11	0	0
An. maculatus maculatus	Luang Prabang	8	0	0
	Phongsaly	30	0	0
An. maculatus rampae	Attapeu	30	1	0
	Saravane	5	0	0
An. maculatus sawadwongporni	Attapeu	3	0	0
	Luang Prabang	17	0	0
	Phongsaly	9	0	0
An. minimus aconitus	Phongsaly	7	0	0
	Vientiane Province	20	0	0
An. minimus minimus	Vientiane Province	27	0	0
An. minimus pampanai	Attapeu	3	0	0
An. nivipes	Vientiane Province	2	0	0
An. philippinensis	Vientiane Province	2	0	0
	TOTAL	**790**	**1**	**0**

SS indicates homozygous for wildtype (A296). RS and SS indicate heterozygous or homozygous, respectively, for a mutation at 296.

Table 5. Frequency of mosquitoes from various countries with mutations at A296 (heterozygous or homozygous) in *Rdl*.

Country	**Species**	**No. Studied**	**Frequency (%)**	**Reference**
Benin	*An. funestus*	25	32	(Wondji et al. 2011)
Burkina Faso	*An. funestus*	25	68	(Wondji et al. 2011)
Burkina Faso	*An. gambiae*	94	97	(Kwiatkowska et al. 2013)
Burkina Faso	*An. gambiae*	183	97	(Platt et al. 2015)
Cameroon	*An. funestus*	25	88	(Wondji et al. 2011)
Cameroon	*An. funestus*, collected 2006	50	88	(Menze et al. 2016)
Cameroon	*An. funestus*, collected 2012	50	58	(Menze et al. 2016)
Cameroon	*An. funestus*, collected 2015	50	15	(Menze et al. 2016)
Cameroon	*An. funestus*	92	16	(Menze et al. 2018)
China, Guangxi	*An. sinensis*	240	93	(Yang et al. 2017)

Democratic Republic of the Congo	*An. gambiae*	33	3	(Nardini et al. 2017)
Republic of the Congo	*An. gambiae*	33	70	(Koekemoer et al. 2011)
Ghana	*An. funestus*	78	53	(Riveron et al. 2016)
Indonesia	*Anopheles*, various	154	11	(Asih et al. 2012)
Laos	*Ae. aegypti*	314	0	This study
Laos	*Ae. albopictus*	183	0	This study
Laos	*Anopheles*, various	294	0.34	This study
Malawi	*An. funestus*	25	0	(Wondji et al. 2011)
Malawi	*An. funestus*	38	11	(Riveron et al. 2015)
Malaysia	*Ae. albopictus*	82	62	(Low et al. 2015)
Mozambique	*An. funestus*	25	0	(Wondji et al. 2011)
Nigeria	*An. funestus*	92	98	(Djouaka et al. 2016)
Reunion	*Ae. albopictus*	48	40[1]	(Tantely et al. 2010)

Reunion	*Cx. pipiens*	275	56[1]	(Tantely et al. 2010)
Tanzania	*An. arabiensis*	534	89	(Mahande et al. 2012)
Turkey	*Cx. pipiens*, spring 2012	375	15	(Taskin et al. 2016)
Turkey	*Cx. pipiens*, autumn 2012	375	17	(Taskin et al. 2016)
Turkey	*Cx. pipiens*, spring 2013	375	13	(Taskin et al. 2016)
Uganda	*An. funestus*	20	15	(Wondji et al. 2011)

[1] This is the frequency of the *Rdl* resistant allele.

Figure Legends

Figure 1. Map showing provinces from where mosquitoes were collected. Created with SimpleMappr, http://www.simplemappr.net.

Figure 2. DNA sequence alignment of *Rdl* encoding for TM2 in several mosquito species collected in Laos. Sequences in black shading are completely conserved in all the mosquito species whilst grey indicates conservation in the majority of species tested. The corresponding amino acid sequence is shown at the top and residues in TM2 are boxed. A296, mutation of which is associated with insecticide resistance, is circled.

Figure 3. Sequence chromatograms of DNA encoding exon 7 in *Rdl* of *An. maculatus rampae* from Attapeu. **a** Wildtype sequence encoding for alanine at 296. **b** Heterozygous mutation encoding for either alanine or serine at 296.