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8 **Malaria and Dengue mosquito vectors from Lao PDR show a lack of the *rd1* mutant allele**
9 **responsible for cyclodiene insecticide resistance**

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25

26 **Abstract**

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

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44 **Keywords**

45 dieldrin, GABA receptor, insecticide resistance, Laos, mosquito

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48 Introduction

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

55 An alanine to serine mutation in the second transmembrane domain (TM2) of RDL (referred
56 to here as A296S) has been found to underlie resistance to several insecticides including picrotoxin
57 and cyclodienes (Ffrench-Constant et al. 1993, Buckingham et al. 2017). Mutation at A296, either to
58 serine or another amino acid such as asparagine or glycine, has since been associated with insecticide
59 resistance in various species ranging from pests afflicting domesticated animals (the cat flea
60 *Ctenocephalides felis* (Rust et al. 2015)), pests afflicting livestock (the horn fly *Haematobia irritans*
61 (Domingues et al. 2013)), crop pests (e.g. the planthopper *Laodelphax striatellus* (Nakao 2017)), and
62 mosquito disease vectors (the malaria vector *Anopheles gambiae* (Du et al. 2005)). In several cases,
63 other mutations in *Rdl* have been observed (Feyereisen et al. 2015, Taylor-Wells and Jones 2017). For
64 instance, T345 was detected in dieldrin-resistant *An. gambiae* (Taylor-Wells et al. 2015), V327I in *An.*
65 *funestus* (Wondji et al. 2011) whilst V327I and T345S were identified in *An. sinensis* (Yang et al. 2017).
66 These mutations were found in addition to a mutation at A296 highlighting this TM2 site as a useful
67 marker for detecting target site resistance. Analysis of mosquitoes from different countries have
68 recorded a notable prevalence of mutations at A296. For example, *An. funestus* mosquitoes from
69 Burkina Faso, West Africa, were found to be resistant to dieldrin and possessed the A296S mutation
70 (Wondji et al. 2011). Other studies, measuring the prevalence of mutations at A296 to predict the
71 extent of resistance in varying countries noted 100% incidence of the mutation in 33 *An. sinensis*
72 mosquitoes from Nanning, China (Yang et al. 2017) whereas 11% of 154 *Anopheles* mosquitoes of

73 various species from Indonesia were found to have either the A296S or A296G mutation (Asih et al.
74 2012). Bioassays showed that *Aedes albopictus* (Skuse) (Diptera: Culicidae) mosquitoes from Malaysia
75 had low level resistance to dieldrin and 62% of 82 *Ae. albopictus* mosquitoes were recorded to possess
76 the A296S mutation (Low et al. 2015). It has been suggested that the persistence of cyclodienes in the
77 environment or the still continued use of these insecticides, such as in crop protection, may be
78 underlying the surprisingly high incidence of the *Rdl* mutation in mosquitoes (Wondji et al. 2011, Asih
79 et al. 2012) although further studies are required to prove that this is the case.

80 Lao PDR (here after Laos) is a landlocked country in South-East Asia, which mostly consists of
81 mountainous ranges, forests, plateaux and highlands through which rivers cut through. In Laos, 170
82 mosquito taxa have been officially reported (Motoki et al. 2019) and mosquito-borne diseases such as
83 malaria and dengue fever, which are spread by *Anopheles* and *Aedes* mosquitoes respectively, present
84 important health issues (Khampapongpane et al. 2014, Souris et al. 2017). As of September 2019,
85 27,904 dengue cases, including 54 deaths, were reported in Laos (World Health Organization 2019)
86 and between 2010 and 2017 the incidence of malaria varied between 9,336 and 48,071 presumed and
87 confirmed cases (World Health Organization 2018). In Laos, vector control relies mainly on the use of
88 pyrethroid insecticides (larvicides, thermal fogging, indoor residual spraying, and impregnated bed-
89 nets). Dieldrin was previously used for agricultural purposes in the 1950's and 60's in very low
90 quantities compared to neighbouring countries as Laos was agriculturally self-sufficient. This
91 insecticide was banned in Laos in 1992 as was the case for other organochlorine insecticides used for
92 Public Health against malaria such as DDT (Ministry of Natural Resources and Environment Pollution
93 Control Department 2016). A recent study implemented in the Mekong region determining the
94 presence of Persistent Organic Pollutants (POPs) in wetlands showed that dielrdrin was detected at
95 low concentrations in several provinces of Laos ranging from the northern to the southern parts of
96 the country (Tran et al. 2014). Another insecticide acting on the GABA receptor, endosulfan, was found
97 in higher quantities in the same areas of the country (Tran et al. 2014). This insecticide was used to
98 protect coffee trees in the 2000's (Committee for the Planning and Investment Lao People's

99 Democratic Republic Japan International Cooperation Agency 2005) whilst, more recently, the use of
100 endosulfan in banana plantations in the north of Laos (i.e. Bokeo province) was reported (Ghosh
101 2016). The utilization of insecticides such as endosulfan may select for *Rdl* mutations in mosquitoes
102 that would present cross-resistance and therefore hamper the efficacy of different insecticides to be
103 used for vector control in the future, an example of which is fipronil (Kolaczinski and Curtis 2001,
104 Davari et al. 2007). Indeed, recent studies implemented in Laos showed that insecticide resistance to
105 pyrethroids and organophosphates has developed in malaria secondary vectors and in the two dengue
106 vectors, *Ae. aegypti* (Linnaeus) (Diptera: Culicidae) and *Ae. albopictus*, highlighting the growing need
107 for alternatives to currently used insecticides (Marcombe et al. 2017, Marcombe et al. 2018, Tangena
108 et al. 2018).

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

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115 **Materials and Methods**

116 **Collection of mosquito samples and identification of species and subspecies**

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

123 For collecting *Anopheles* species, indoor and outdoor human landing catch and cow bait
124 collections were used (Marcombe et al. 2017). The genus of collected adult mosquitoes were
125 morphologically identified as *Anopheles* species or species group/complex in a field laboratory, using
126 microscopes and appropriate identification keys for Southeast Asian Anopheline (Rattanarithikul et al.
127 2006). Identification was carried out at the Institut Pasteur du Laos (IPL) and the Center for
128 Malariology, Parasitology and Entomology by entomologist staff onsite. After identification,
129 mosquitoes of the same species were stored in RNAlater® or in silica gel in labelled 1.5 ml tubes and
130 stored at -20°C for subsequent laboratory analysis. Female *An. Dirus* (Diptera: Culicidae), *An. minimus*
131 (Theobald) and *An. maculatus* (Theobald) complex mosquitoes (primary malaria vectors) as well as
132 *An. aconitus* s.l. (Diptera: Culicidae), *An. annularis* s.l. and *An. barbirostris* group mosquitoes
133 (secondary malaria vectors) were cut into two parts to separate the head plus thorax from the
134 abdomen and legs. DNA was extracted from the head and thorax using the cetyl trimethyl ammonium
135 bromide (CTAB) method (Murray and Thompson 1980). An allele-specific multiplex assay (AS-PCR)
136 examining the ITS-2 region of the DNA (Walton et al. 1999, Garros et al. 2004, Walton et al. 2007) was
137 used for molecular detection of sibling species within the dirus complex, minimus complex and
138 maculatus complex assemblages. For *Aedes* sp. mosquitoes, larval and pupal collections were made
139 in the field (rural and urban areas) and about fifty sampling places (tires, jars, freezers, buckets, toilets,

140 vases, cups etc.) per each location were required to produce sufficient number of specimens for
141 colonization in the laboratory. All samples were brought back to the laboratory at IPL and maintained
142 under controlled conditions (27 ± 2 °C and $80 \pm 10\%$ relative humidity) with a diet of powdered cat
143 food for rearing until adults (F1 generation). After adult identification using morphological keys,
144 mosquitoes were separated by species and were kept for breeding following standardized techniques
145 (Marcombe et al. 2014). Resulting eggs were kept for the adult bioassays. The insecticide susceptible
146 reference strain (*Ae. aegypti* USDA) was used as a control for the different tests. The USDA laboratory
147 population originated from the Center for Medical, Agricultural, and Veterinary Entomology,
148 Gainesville, FL, U.S.A and has undergone continuous colonization for 40 years at Kasetsart University,
149 Bangkok, Thailand (Chuaycharoensuk et al. 2011). This strain was colonized at IPL before experiments
150 were carried out for this study.

151 **Insecticide resistance bioassays**

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

163 **PCR amplification of mosquito Rdl TM2**

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

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181 Results

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

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

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

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199 Discussion

200 Due to the high resistance levels against pyrethroids detected in *Aedes* and to a lesser extent in
201 *Anopheles* in Laos (Marcombe et al. 2017, Marcombe et al. 2018, Tangena et al. 2018),
202 recommendations to Public Health authorities of the country were made to use alternative
203 insecticides belonging to different insecticide families. Because of potential cross resistance between
204 dieldrin and fipronil (Kolaczinski and Curtis 2001, Davari et al. 2007), which is an insecticide authorized
205 for agricultural purpose in Laos (Lao People's Democratic Republic Ministry of Agriculture and Forestry
206 2017) and thus a potential insecticide to be use for Public Health (Hoppe et al. 2016, Poche et al. 2017),
207 detection for dieldrin resistance prior to application of fipronil is recommended.

208 We therefore report here the first study on the insecticide resistance status of *Ae. aegypti* and
209 *Ae. albopictus* against dieldrin in Laos. All the populations tested throughout the country were
210 susceptible to dieldrin. In agreement with this, we found that none of the 497 *Aedes* mosquitoes
211 tested had a mutation at A296 of *Rdl* and only one of the 294 *Anopheles* mosquitoes (*An. maculatus*
212 from Attapeu) was shown to be heterozygous for the A296S mutation. This is in contrast to many of
213 the other studies investigating the prevalence of insecticide resistance mutations in mosquitoes from
214 a variety of countries. As summarised in Table 5, in thirteen of the twenty nine samples shown,
215 mutation at A296 in *Rdl* of over 50% of mosquitoes tested were observed and only two of the samples
216 showed a complete absence of the mutation (excluding the present study). This is surprising
217 considering that the use of cyclodienes to control mosquitoes has been banned (Tantely et al. 2010).
218 It has been suggested that the persistence of the *Rdl* mutation may be due to either mosquitoes being
219 exposed to the agricultural use of insecticides that target the GABA receptor, to cyclodienes still
220 present in the environment, or to the illegal use of cyclodienes (Tantely et al. 2010, Wondji et al. 2011,
221 Antonio-Nkondjio et al. 2017). Another possibility may be that substitution of A296 is a polymorphism
222 that is present irrespective of whether there is insecticide use and thus would commonly occur in
223 mosquito populations (Ffrench-Constant and Bass 2017). Our finding of no A296 mutations in *Aedes*

224 mosquitoes and only in 0.34% of *Anopheles* species suggests that this is not the case instead pointing
225 towards the substitution of A296 being a result of selection pressure such as exposure to insecticides.
226 In line with this, studies have indicated that mutation at A296 results in a fitness cost. For example,
227 male *Anopheles* mosquitoes that were homozygous for the A296S mutations showed reduced mating
228 success compared to heterozygote-resistant males indicating that the *Rdl* mutation has a detrimental
229 effect on the ability of mosquitoes to mate (Platt et al. 2015).

230 It remains to be determined whether the high prevalence of the mutant *Rdl* allele in
231 mosquitoes from any of the several countries studied (Table 5) is maintained by mutations elsewhere
232 in the genome (Ffrench-Constant and Bass 2017). For instance, a 2La chromosomal inversion, which
233 was found to be associated with dieldrin resistance in *An. gambiae* (Brooke et al. 2000), may suppress
234 cross-over of *Rdl* (located in chromosome 2L) ensuring that *Rdl* mutations are preserved even though
235 they confer a fitness cost. However, surveys found a reduction in the prevalence of *Rdl* mutations in
236 *An. funestus* collected over several years in Cameroon (Table 5 (Menze et al. 2016)) suggesting that in
237 at least this case the lack of using cyclodienes has resulted in *Rdl* reverting to wild-type (Menze et al.
238 2018).

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

248

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255 **Authors' contributions**

256 SMar, SMai, SN and PTB supervised mosquito collections in Laos, rearing and laboratory tests. SMar
257 analysed insecticide resistance tests and contributed to writing the manuscript. SMar, SC, PT, NP, KL,
258 PL, NX and SN collected, identified and performed insecticide resistance tests. ZR, PJS, OHA, JF, AEJ,
259 SK, WA, CB, HK, JH, RL, NMP, MS, NB, NCK, SL and GS extracted DNA from mosquitoes, amplified exon
260 7 of *Rdl* and analysed sequence data. AKJ conceived the idea for the project, analysed sequence data
261 and contributed to writing the manuscript. All authors read and approved the final manuscript.

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439 **Tables**

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

Mosquito species	Province	District	Village	Latitude¹	Longitude¹
<i>Aedes</i> 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	18.125733	102.665011
			Phailom	18.057037	102.774993
	Xayaboury	Xayaboury	Taling	17.784729	101.170521
Xayaboury			19.26457	101.71184	
<i>Anopheles</i> 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

441 ¹GPS coordinates

442 *IPL strain collected at the Institut Pasteur du Laos

443

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

Species	Forward Primer	Reverse Primer	Size bp
<i>Ae. aegypti</i>	1 st PCR: atgtatcttcttaatttctctc ¹	1 st PCR: aacacaaacacgaaagagactg ¹	
<i>Ae. albopictus</i>	Nested PCR: ttaccaaatatatgccaactaac ¹	Nested PCR: atttgtacaagtagcaaatagtg ¹	428
<hr/>			
<i>An. hyrcanus</i>			
<i>An. kochi</i> (Donitz) (Diptera: Culicidae)			
<i>An. minimus</i>	cactaaagcaaggaatcaaagc ²	caacgaaactaacagagtacag ²	435
<i>An. nivipes</i> (Theobald) (Diptera: Culicidae)			
<i>An. philippinensis</i> (Ludlow) (Diptera: Culicidae)			
<i>An. maculatus</i>	ccatccggattaattgtaac ³	cagcagactggcaaatacc ³	210

446 Sequences are shown 5'-3'.

447 ¹Sequence based on the *Rdl* gene of *Ae. aegypti* available at VectorBase
 448 (<https://www.vectorbase.org/>).

449 ²Sequence based on the *Rdl* gene of *An. gambiae* available at VectorBase
 450 (<https://www.vectorbase.org/>).

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

452

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

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

455

456

457

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

Species	Province	SS	RS	RR
<i>Ae. aegypti</i>	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
<i>Ae. albopictus</i>	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
<i>An. hyrcanus</i>	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
<i>An. kochi</i>	Khammouane	5	0	0
	Sekong	11	0	0
<i>An. maculatus maculatus</i>	Luang Prabang	8	0	0
	Phongsaly	30	0	0
<i>An. maculatus rampae</i>	Attapeu	30	1	0
	Saravane	5	0	0
<i>An. maculatus sawadwongporni</i>	Attapeu	3	0	0
	Luang Prabang	17	0	0
	Phongsaly	9	0	0
<i>An. minimus aconitus</i>	Phongsaly	7	0	0
	Vientiane Province	20	0	0
<i>An. minimus minimus</i>	Vientiane Province	27	0	0
<i>An. minimus pampanai</i>	Attapeu	3	0	0
<i>An. nivipes</i>	Vientiane Province	2	0	0
<i>An. philippinensis</i>	Vientiane Province	2	0	0
	TOTAL	790	1	0

459 SS indicates homozygous for wildtype (A296). RS and SS indicate heterozygous or homozygous,

460 respectively, for a mutation at 296.

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

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

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

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

463 ¹This is the frequency of the *Rdl* resistant allele.

464

465

466 **Figure Legends**

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

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

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

477