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Novel insecticide resistance mutations associated with variable PBO synergy in *Anopheles gambiae* s.l. from the Democratic Republic of Congo

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Over-reliance on pyrethroid insecticides in insecticide-treated nets (ITNs) has imposed significant selection pressure for the evolution of insecticide resistance among major malaria vector species. In the Democratic Republic of Congo (DRC), the country with the second highest malaria burden globally, pyrethroid resistance is pervasive, but there is a paucity of information regarding the molecular mechanisms driving resistance. A clear understanding of the specificity of resistance mechanisms to individual insecticides and the likelihood of selecting for cross-resistance mechanisms is crucial for the development of new vector control tools. *Anopheles gambiae* s.l. populations from eight study sites across the DRC were phenotyped for resistance to alpha-cypermethrin, deltamethrin and permethrin, with and without pre-exposure to the synergist piperonyl butoxide (PBO), followed by multiplex amplicon sequencing. Phenotypic pyrethroid resistance and loss of PBO synergy was confirmed in all sites across the DRC. In *An. gambiae* s.s. four non-synonymous SNPs which have been previously associated with insecticide resistance were detected: *gste2*-L119V, *vgsc*-L995F, *vgsc*-L995S and *rdl*-A296G, while three were novel: *gste2*-T154S, *ace1*-N246T and *ace1*-P265L. Nationwide geographical trends in insecticide resistance mutation distribution, prevalence and selection were evident. In the West, near fixation of *vgsc*-L995F and almost complete absence of *vgsc*-L995S was observed, alongside low-moderate frequencies of *rdl*-A296G and *gste2*-L119V. Further East, the converse was apparent. *Gste2*-L119V was significantly associated with resistance to deltamethrin following PBO-pre-exposure, warranting functional validation to determine its putative role in reduced PBO synergy. Furthermore, *gste2*-T154S was implicated in deltamethrin and permethrin resistance but susceptibility to alpha-cypermethrin after PBO pre-exposure. Study findings comprise the most comprehensive overview of the prevalence of genetic markers of *Anopheles* insecticide resistance across the DRC and provide an important baseline for improved malaria vector control and the design of proactive insecticide resistance management strategies. Given the significant scale up in PBO-ITNs, with more than 58% of all ITNs delivered to sub-Saharan Africa in 2023 containing PBO, there is an urgent need to identify novel molecular markers to monitor changes in PBO synergy, which may be predicative of loss of intervention operational efficacy.

Keywords *Anopheles gambiae*, Pyrethroid resistance, PBO synergy, Single nucleotide polymorphisms, Amplicon sequencing, Democratic Republic of Congo

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Malaria remains a significant global health threat, with nearly half of the world's population residing in endemic areas across 83 countries and territories[1]. In 2023, there were an estimated 263 million new malaria cases globally (an increase of 14 million cases compared to 2022), accompanied by 597,000 deaths, with 95% and 96% of all cases and deaths, concentrated in sub-Saharan Africa, respectively[2]. Previously, significant progress in malaria control was accomplished by scaling-up the provision of insecticide-treated nets (ITNs), indoor residual spraying (IRS) and other key diagnostic and treatment measures[1]. In the past two decades, the WHO estimated that 1.5 billion malaria cases and 7.6 million malaria deaths were averted, with ITNs and IRS accounting for 68% and 10% of these achievements, respectively[3]. Pyrethroid insecticides were the exclusive chemical class used on ITNs, with an estimated 2.13 billion pyrethroid ITNs delivered in sub-Saharan Africa between 2004 and 2022[4,5]. While the global malaria mortality rate halved between 2000 and 2015, progress has stalled, and even begun to reverse, in recent years, coinciding with growing pyrethroid resistance, plateauing ITN coverage metrics and exacerbated by disruption during the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic[2]. Selection pressure from multiple sources including decades of agricultural pesticide use, coupled with large-scale distribution of pyrethroid ITNs, sometimes in combination with pyrethroid IRS, inevitably led to a gradual intensification of pyrethroid resistance in major malaria vector species[6–8]. As a result, pyrethroid resistance across sub-Saharan Africa is now ubiquitous and entrenched, with high intensity pyrethroid resistance reported across many countries[9–11]. Mass distribution of pyrethroid-only ITNs continues to further intensify phenotypic resistance, even in established, resistant malaria vector populations[12,13]. Furthermore, the deployment of ITNs containing pyrethroids in combination with the synergist piperonyl butoxide (PBO) may also escalate pyrethroid resistance and diminish the synergist effects of PBO[12].

The Democratic Republic of Congo (DRC) had the second highest global burden of malaria in 2023, accounting for 12.6% and 11.3% of all malaria cases and deaths, respectively[2]. The distribution of ITNs is among the primary methods of malaria prevention in the DRC, with an estimated coverage of 65% in 2019[14]. Mass distribution is conducted on a rotating basis and repeated per province every 3 years or 30 months, based on the durability of ITNs previously given. To maintain high ITN coverage between campaigns, ITNs are also continuously distributed to pregnant women during their first antenatal care (ANC) visit and children at the time of measles vaccination. Between 2011 and 2018, an estimated 134.8 million ITNs were deployed nationwide in the DRC. The program has implemented approximately 20.6 million, 22.5 million, 33 million and 20.3 million ITNs in 2020, 2021, 2022 and 2023, respectively[15]. To optimize the efficacy of interventions, the National Malaria Control Program (NMCP) and its partners have shifted from standard pyrethroid ITNs to PBO and dual active ingredient (AI) ITNs. In the DRC, an estimated 10,249,977 PBO-ITNs in 2022 and 1,056,683 Interceptor G2 ITNs in 2023 were distributed[16]. High ITN coverage in the DRC has imposed significant selection pressure for the evolution of pyrethroid resistance among major malaria vector species, which is now widespread and intense, with reports of *Anopheles (An.) gambiae* sensu lato (s.l.) populations that are capable of surviving exposure to ten times the diagnostic dose of deltamethrin[17]. Pyrethroid resistance may severely jeopardise the efficacy and long-term sustainability of ITNs, threatening to reverse gains in malaria control and elimination[18].

Monitoring the mechanisms of insecticide resistance is vital to ensure that the most effective, contemporary vector control interventions are tactically deployed[19]. Phenotypic insecticide resistance surveillance comprises vector population exposure to the diagnostic dose and intensity doses of public health insecticides and observing immediate and delayed mortality[20]. Whilst phenotypic analysis is important for tracking the spread of insecticide resistance, it provides limited information about the underlying molecular resistance mechanisms. A clear understanding of the specificity of resistance mechanisms to individual insecticides and the likelihood of selecting for cross-resistance mechanisms is crucial for the development of novel vector control tools and the design of proactive insecticide resistance management schemes.

In many malaria endemic countries, the National Malaria Control/Elimination Programmes (NMC/EPs) have incorporated molecular techniques into their insecticide resistance monitoring activities. However, this is usually restricted to endpoint or quantitative polymerase chain reaction (PCR) assays, targeting specific mutations, which preclude the identification of novel mutations that may contribute to resistance phenotypes or be indicative of vector control tool operational failure[21,22]. Amplicon sequencing can be used to characterise genetic diversity within known insecticide target sites, which may then be functionally associated with reduced susceptibility. This technique combines the advantages of next-generation sequencing with the speed and lower costs of conventional PCR-based methods[23].

In the DRC, phenotypic resistance monitoring is widespread across the country, however, there is a paucity of data regarding the molecular mechanisms driving resistance. Previous studies have identified *vgsc*-L995F-*kdr* and *vgsc*-L995S-*kdr* mutations at variable frequencies in *An. gambiae* across the country[24–28], with the former predominating in western and central provinces; a proportion of individuals have also been documented harbouring both *vgsc*-L955F and *vgsc*-L955S alleles[29]. In the DRC, *vgsc*-N1575Y is present at very low prevalence in Nord-Ubangi province[29], *ace1*-G119S, has also been identified in a minority of vectors[30], L43F-CYP4J5 allelic frequencies were moderate in eastern provinces, with evidence for ongoing selection, and finally and the triple mutant haplotype (comprising the point mutation CYP6P4-I236M, the insertion of a partial Zanzibar-like

transposable element and duplication of CYP6AA1) has also been detected at high frequencies[30,31]. Increased mortality following pre-exposure of resistant *An. gambiae* populations to PBO before pyrethroid bioassays also indicates a role for metabolic resistance mechanisms in this species complex in the DRC[27,29], supported by reports of overexpression of CYP6M2 and CYP6P1[32].

This study exploited amplicon sequencing of target regions in four genes, previously implicated in insecticide resistance (*vgsc*, *ace1*, *gste2* and *rdl*), and six genomic regions used for taxonomic species identification (*cox1*, *its1*, *nd4*, *nd5*, *sine200*, *igs*). This study provides the most comprehensive overview to date of the prevalence of genetic markers of insecticide resistance across the DRC and comprises an important baseline for future resistance monitoring studies.

Results

Pyrethroid phenotypic resistance intensity and PBO synergy

WHO tube tests were used to determine resistance prevalence and intensity in 8948 *An. gambiae* s.l. from eight sites across the DRC (Fig. 1). Resistance to the discriminating concentration of each pyrethroid was confirmed in every study site, to all three pyrethroids (Fig. 1, Table S1). High intensity resistance (< 98% mortality to 10x the discriminating concentration) to alpha-cypermethrin was confirmed in all study sites, to deltamethrin in Kalemie, Kapolowe, Katana, Kimpese and Kinshasa, and to permethrin in all sites, except Lisala. Pre-exposure to PBO restored susceptibility to alpha-cypermethrin in Katana and Kimpese, and deltamethrin in Katana and Kinshasa. PBO pre-exposure did not restore full susceptibility to permethrin in any site (Fig. 1, Table S1).

Mosquito species identification

Overall, 99% of *An. gambiae* s.l. identified were *An. gambiae* sensu stricto (s.s.) (1228/1244). *An. arabiensis* was present in Kalemie (1.7%; 2/118) and Katana (2.9%; 4/136), *An. coluzzii* was present in Kinshasa (1.2%; 2/163) and Lodja (1.7%; 2/116). A single *An. gambiae* s.s. – *An. coluzzii* hybrid was found in each of Inongo and Kinshasa (< 1% in both) (Table S2). Only *An. gambiae* s.s. were included in the downstream analysis ($n = 1228$). Preliminary analysis of *its1*, *nd4 and nd5* did not show any significant phylogenetic relationships between samples, likely due to the proximity of study sites, and genetic similarity of mosquitoes; therefore these genes were excluded from further analysis.

Single nucleotide polymorphism (SNP) detection

The average number of sequencing read pairs per sample generated was 45,590 (range: 245 – 178,983). Variant calling for the coding genes (*ace1*, *gste2*, *vgsc*, *rdl* and *cox1*) mapped to the *An. gambiae* PEST 4 reference genome

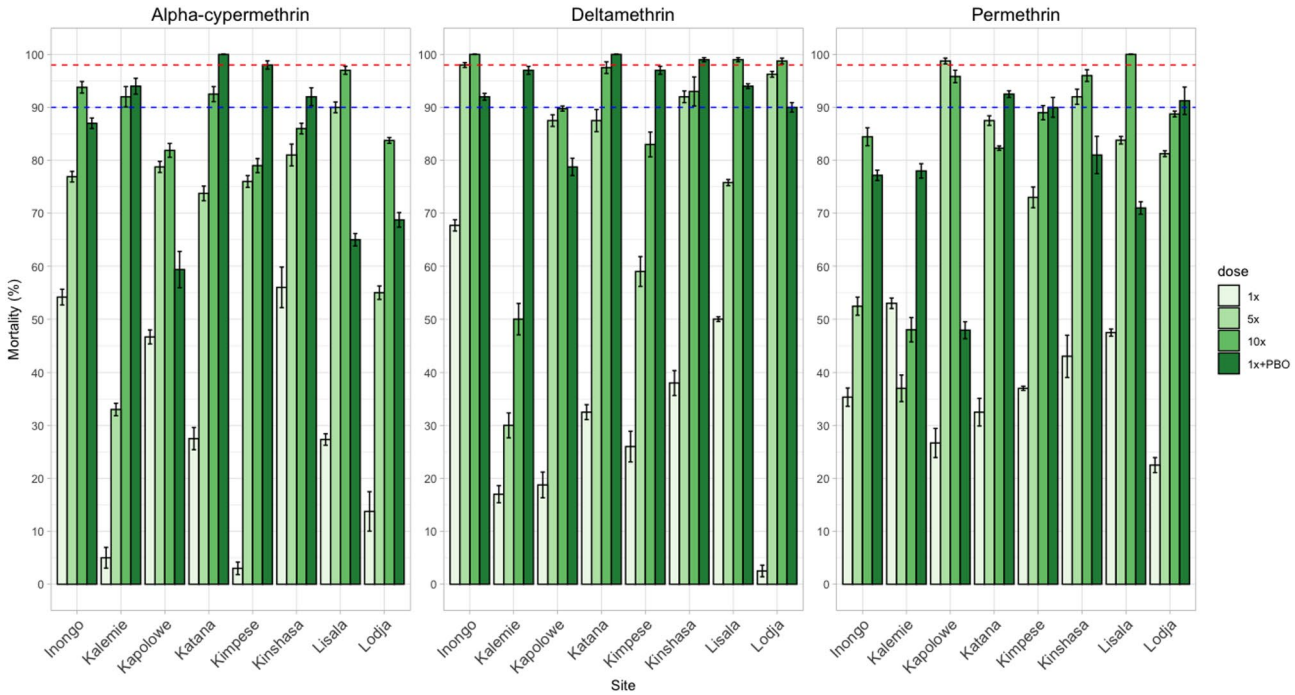

Fig. 1. Resistance intensity of 8948 *An. gambiae* s.l. after exposure to 1, 5, 10 or 1 + PBO x the discriminating concentration of pyrethroid (alpha-cypermethrin, deltamethrin or permethrin) insecticides. Mean mortality rate 24 h after one hour of pyrethroid exposure is shown with 95% confidence intervals (CIs) per insecticide, per dose, per site. Mortality rates below 90% (lower blue line) represent confirmed resistance at the discriminating concentration, while mortality rates between 90 and 98% (upper red line) represent possible resistance. Mortality rates below 98% following exposure to 5x and 10x the discriminating concentration indicate moderate to high and high intensity resistance, respectively.

generated a Variant Call Format (VCF) file with 14,441 variants. A total of 325 variants met the minimum quality threshold of 50 and minimum coverage depth of 10. A total of 280 of these were SNPs, and 255 were present in more than one sample and sequencing pool. These 255 SNPs were used for downstream analysis.

Of the 255 SNPs present in the coding genes, 28 (10.98%) were missense mutations, resulting in an amino acid sequence change. Two (0.78%) mutations changed the amino acid into a stop codon, thereby truncating the protein. One hundred and thirty-seven (53.73%) were silent (synonymous) mutations, resulting in no changes in the amino acid sequence. Fifty-two (20.39%) mutations were in introns. The remaining mutations were in other non-coding regions such as the 3' UTR, the splice region, and upstream gene region (Table S3).

Insecticide resistance mutations

In insecticide resistance genes, there were 20 SNPs present: four in *ace1*, 10 in *gste2*, one in *rdl* and five in the *vgsc*. Four of these mutations have been widely reported to be functionally implicated in insecticide resistance: *vgsc*-L995F ("*vgsc*-west"), *vgsc*-L995S ("*vgsc*-east")[33], *gste2*-L119V and *rdl*-A296S[34]. Across all study sites, *vgsc*-L995F was present at 79.80%, *vgsc*-L995S at 17.79%, *rdl*-A296S at 8.02% and *gste2*-L119V at 5.31%.

The T154S mutation was present in the *gste2* gene at 72.64%, the highest of all the uncharacterised mutations. Two other novel mutations were identified in *ace1* at low frequencies: N265T at 5.03% and P265L at 1.38%. The remainder of the mutations were detected at a frequency below 1%. The full list of non-synonymous SNPs present in insecticide resistance genes are detailed in Table 1.

Allelic frequency of SNPs in different geographical locations across the DRC

Seven non-synonymous SNPs were present at a frequency of more than 1%, and for these allelic frequency was calculated for each study site to provide an overview of geographical variation across the country (Table 2; Fig. 2).

Across all study sites, except Katana, the *vgsc*-L995F mutation was detected at high frequencies (71.15–99.22%), whereas the *vgsc*-L995S mutation was found at comparatively lower frequencies (0.78 – 29.0%). In Katana, the *vgsc*-L995S mutation was dominant (90.57%) and *vgsc*-L995F present at only 4.10%.

The *rdl*-A296G mutation was observed at almost 50% frequency in Kinshasa, by far the highest of all the study sites. *rdl*-A296G was also detected at low frequencies (0.57 – 8.18%) in Inongo, Kimpese, Lisala and Lodja, but was absent in Kalemie, Kapolowe and Katana.

The *gste2*-L119V mutation was present at the highest frequency in Lodja (18.35%), followed by much lower frequencies (< 10%) in Lisala, Kimpese, Kinshasa and Inongo; it was absent in Kalemie, Kapolowe and Katana.

Of the SNPs not previously implicated in insecticide resistance, *gste2*-T154S was the only mutation present in every site. It was observed at the highest frequencies at 51.04% in Lisala and 88.60% in Inongo. The *ace1*-N246T mutation was present at 31.25% and 35.48% in Katana and Inongo, respectively, then at low frequencies (5.00–9.68%) in Kimpese, Lisala and Lodja. It was not detected in Kalemie, Kapolowe and Kinshasa. The *ace1*-P265L mutation was present at low frequencies (1.03–2.68%) in Inongo, Kimpese, Kinshasa, Lisala and Lodja. Allelic frequency breakdown by study site is shown in Table 2; Fig. 2.

Clear geographical trends in insecticide resistance mutation distribution, prevalence and selection were apparent (Table 2; Fig. 2). In the capital region of DRC (Kinshasa) and adjacent province (Kimpese; Kongo Central province), near fixation of *vgsc*-L995F and almost complete absence of *vgsc*-L995S was evident, alongside low-moderate frequencies of *rdl*-A296G and *gste2*-L119V, and high frequencies of *gste2*-T154S; all of which demonstrated significant deviations from Hardy-Weinberg equilibrium (*rdl*-A296G: $x^2 = 3.80$, $p = 0.05$ and $x^2 = 63.83$, $p < 0.001$; *gste2*-T154S: $x^2 = 5.48$, $p = 0.02$; and *gste2*-L119V: $x^2 = 77.55$ $p < 0.001$, in Kinshasa and Kimpese, respectively). By comparison, in northern DRC (Lisala; Mongala province and Inongo; Mai-Ndombe province), increasing frequencies of *vgsc*-L995S and *gste2*-L119V and concomitant diminishing presence of *vgsc*-L995F were observed. In this region there were significant deviations from Hardy-Weinberg equilibrium in Inongo for *rdl*-A296G ($x^2 = 10.25$, $p = 0.001$) and in Lisala for *gste2*-L119V ($x^2 = 15.12$, $p < 0.0001$). In central DRC (Lodja; Sankuru province), high frequencies of *vgsc*-L995F and *gste2*-T154S were detected, with evidence for ongoing selection of *vgsc*-L995F ($x^2 = 31.35$, $p < 0.001$) and *gste2*-T154S ($x^2 = 0.03$, $p < 0.001$). Moving further East, Katana and Kalemie (Sud Kivu and Tanganyika provinces, respectively) displayed divergent insecticide resistance mutation trends, with high frequencies of *vgsc*-L995S and low frequencies of *vgsc*-L995F, in the former, with the converse found in the latter.

Association of phenotypic resistance with genetic mutations

Disaggregating the data by insecticide resistance phenotype, the *vgsc*-L995S mutation was strongly associated with permethrin susceptibility ($p < 0.0001$) and slightly associated with deltamethrin susceptibility ($p = 0.0489$), whilst *vcsg*-L995F was strongly associated with permethrin ($p = 0.0008$) and deltamethrin ($p = 0.004$) resistance. *Gste2*-T145S was significantly associated with susceptibility to PBO + alpha-cypermethrin ($p = 0.0002$) and *gste2*-L119V was slightly associated with PBO + deltamethrin and PBO + permethrin resistance ($p = 0.01$ and 0.07, respectively). *Ace1*-N246T was strongly associated with permethrin resistance ($p = 0.002$) (Table 3).

When considering variation between study sites, the *ace1*-N246T mutation was significantly associated with permethrin susceptibility in Kimpese ($p = 0.003$), present in 8% of resistant, and 50% of susceptible mosquitoes, respectively. *Gste2*-T154S was significantly associated with permethrin and deltamethrin resistance in Lodja ($p = 0.004$) and Katana ($p = 0.047$), respectively, and associated with susceptibility to PBO + alpha-cypermethrin in Lisala ($p = 0.033$) and Kinshasa ($p = 0.083$). *Gste2*-L119V was significantly associated with PBO + deltamethrin resistance in Lisala ($p = 0.021$). *Vgsc*-L995F was significantly associated with deltamethrin resistance in Lodja ($p = 0.032$) and *vgsc*-L995S was significantly associated with permethrin susceptibility in Katana ($p = 0.033$) (Table S4). For most mutations, there was no significant difference in allelic frequency between resistant and susceptible mosquitoes in any study site or insecticide (Table S5).

Mutation Name	Chromosome	Position*	Gene	Reference Allele (S)	Alternative Allele (*R*)	Quality	Mutation	SS (*N*)	SR (*N*)	RR (*N*)	*N* (Total)	Alternative Allele Frequency (%)
D181E	2R	3,491,779	*ace1*	T	A	19061.2	Asp181Glu	778	5	2	801	0.56
N246T	2R	3,491,973	*ace1*	A	C	7696.93	Asn246Thr	469	48	3	536	5.04
P265L	2R	3,492,030	*ace1*	C	T	46160.2	Pro265Leu	832	24	0	872	1.38
A271V	2R	3,492,048	*ace1*	C	T	13643.3	Ala271Val	767	1	0	784	0.06
A185S	3R	28,597,864	*gste2*	C	A	597.113	Ala185Ser	1039	1	0	1056	0.05
Q177E	3R	28,597,888	*gste2*	G	C	193,471	Gln177Glu	1114	15	3	1148	0.91
L175M	3R	28,597,894	*gste2*	A	T	8756.46	Leu175Met	1034	2	0	1052	0.10
I163V	3R	28,597,930	*gste2*	T	C	23767.1	Ile163Val	992	3	0	1011	0.15
T154S	3R	28,597,956	*gste2*	G	C	2.09E+07	Thr154Ser	121	351	654	1142	72.64
L145M	3R	28,597,984	*gste2*	G	T	3946.28	Leu145Met	1034	3	0	1053	0.14
Q135H	3R	28,598,012	*gste2*	C	A	1.50E+07	Gln135His	1010	4	3	1033	0.48
K123N	3R	28,598,048	*gste2*	T	A	129,636	Lys123Asn	1029	9	1	1055	0.52
F120L	3R	28,598,057	*gste2*	G	T	57941.8	Phe120Leu	1030	8	0	1054	0.38
L119V	3R	28,598,062	*gste2*	G	C	1,626,950	Leu119Val	1025	94	14	1149	5.31
L995S	2 L	2,422,651	*vgsc2*	T	C	5,391,390	Leu995Ser	814	170	113	1113	17.79
L995F	2 L	2,422,652	*vgsc2*	A	T	1.99E+07	Leu995Phe	128	162	808	1114	79.80
F1529L	2 L	2,429,622	*vgsc3*	T	C	739.845	Phe1529Leu	683	3	0	702	0.21
S1657I	2 L	2,430,236	*vgsc4*	G	T	306.381	Ser1657Ile	708	1	0	725	0.07
A1746S	2 L	2,430,424	*vgsc4*	G	T	3517.89	Ala1746Ser	741	6	0	763	0.39
A296G	2 L	25,429,236	*rdl*	C	G	1,508,940	Ala296Gly	941	82	46	1085	8.02

Table 1. Amino acid changes caused by non-synonymous SNPs in six insecticide resistance genes in 1228 *Anopheles gambiae* s.s. From the DRC. *Relative to the *Anopheles gambiae* PEST 4 genome[35]. Reference allele: allele in the *An. gambiae* PEST 4 reference genome, alternative allele: mutation, quality: phred-scaled quality score assigned by variant calling software.

Province	Site	*rdl*-A296G	*gste2*-L119V	*vgsc*-L995F	*vgsc*-L995S	*ace1*-N246T	*ace1*-P256L	*gste2*-T154S
Mai-Ndombe	Inongo	2.45*	7.46	91.89	8.22	35.48	1.11	88.60
Tanganyika	Kalemie	0	0	71.15	28.85	0	0	65.46
Haut-Katanga	Kapolowe	0	0	91.36	8.64	0	0	64.20
Sud Kivu	Katana	0	0	4.10	90.57	31.25	0	69.85
Kongo Central	Kimpese	8.18*	0.85*	99.12	0.87	5	2.63	86.36
Kinshasa	Kinshasa	48.41*	4.84	99.22	0.78	0	2.68	90.32*
Mongala	Lisala	3.27	9.10*	85.8	13.54	8.06	1.56	51.04
Sankuru	Lodja	0.57	18.35	94.68*	2.13	9.68	1.03	79.82*

Table 2. Allelic frequency of seven non-synonymous SNPs across eight study sites in the DRC. *Denotes significant deviation from Hardy-Weinberg equilibrium.

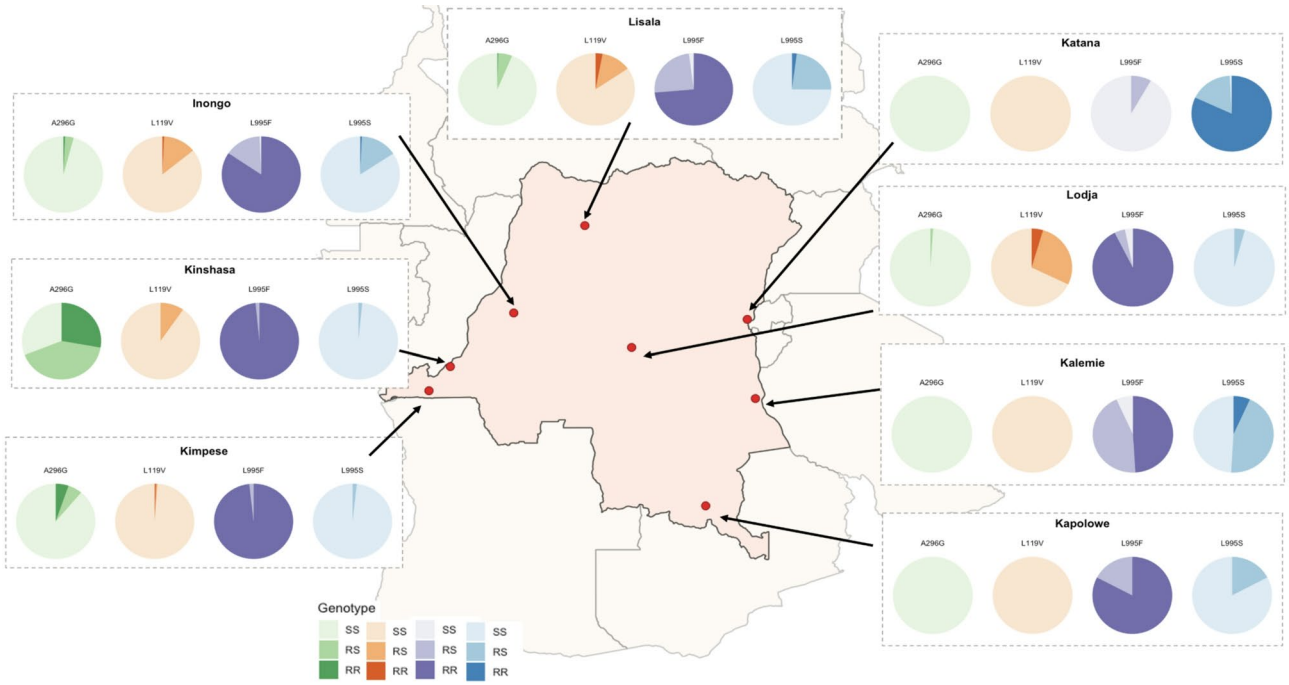

Fig. 2. Frequency of four known resistance genes: *rdl*-A216G (green), *gste2*-L119V (orange), *vgsc*-L995F (purple) and *vgsc*-L995S (blue) in eight sites across the DRC (red dots). Lighter colours represent the homozygous susceptible genotype (SS), whilst darker colours represent the homozygous resistant genotype (RR); intermediate colours represent the heterozygous resistant genotype (RS).

Discussion

Monitoring of insecticide resistance mechanisms is a crucial component of vector control surveillance, for pragmatic vector control intervention deployment and the establishment of effective, prospective insecticide resistance management strategies[19,20]. To our knowledge, this is the most comprehensive amplicon sequencing study that has been conducted in any *Anopheles* malaria vector species. We identified four SNPs that have been previously associated with insecticide resistance in *An. gambiae* s.s.: *rdl*-A296G, *vgsc*-L995F, *vgsc*-L995S, and *gste2*-L119V. A further three new SNPs: *ace1*-N246T, *ace1*-P265L and *gste2*-T154S were present at a frequency of greater than 1% but their putative roles in phenotypic insecticide resistance have yet to be characterised.

The *gst2e* loci had the greatest number of significant SNPs of any of the genetic regions sequenced, relative to its size. *Gste2*-LL19V was present at highest frequency in Lodja, Lisala and Inongo (18%, 9% and 8%, respectively); this mutation has previously been associated with increased permethrin resistance in Burkina Faso and decreased deltamethrin resistance in the DRC. The equivalent *gste2* mutation in *An. funestus*, *gste2*-L119F, is strongly associated with metabolic resistance to DDT[36] and the mutant enzyme directly metabolises permethrin and confers cross-resistance to other pyrethroids[37]. In our study, *gste2*-L119V was significantly associated with resistance to deltamethrin with PBO pre-exposure and there was a non-significant association with resistance to permethrin with PBO pre-exposure. The other *gste2* mutation, T154S, present at 51–90%, was significantly associated with permethrin resistance in Lodja, deltamethrin resistance in Katana, and susceptibility to alpha-cypermethrin with PBO pre-exposure in Lisala and Kinshasa. *Gste2*-T154S has been reported in Guinea (46%),

Mutation	Insecticide	Phenotype	*N*	Freq S (Ref)	Freq *R* (Alt)	Odds Ratio	*P*-value	Mutation Associated With	Protein Domain Location
vgsc-L995S	Permethrin	Alive	213	88.76	11.24	2.07	6.82E-05	Susceptibility	Segment IIS6
		Dead	145	79.25	20.75				
gste2-T154S	PBO + Alpha-cypermethrin	Alive	11	54.55	45.45	10.28	0.000235	Susceptibility	C-terminal
		Dead	20	10.00	90.00				
vgsc-L995F	Permethrin	Alive	266	12.78	87.22	0.55	0.000838	Resistance	Segment IIS6
		Dead	214	21.03	78.97				
ace1-N246T	Permethrin	Alive	132	86.74	13.26	0.37	0.002228	Resistance	α/β hydrolase fold
		Dead	123	94.72	5.28				
vgsc-L995F	Deltamethrin	Alive	161	18.32	81.68	0.54	0.003815	Resistance	Segment IIS6
		Dead	102	29.41	70.59				
gste2-L119V	PBO + Deltamethrin	Alive	13	84.62	15.38	0.11	0.013056	Resistance	C-terminal
		Dead	54	98.15	1.85				
vgsc-L995S	Deltamethrin	Alive	162	81.79	18.21	1.54	0.0489	Susceptibility	Segment IIS6
		Dead	102	74.51	25.49				
gste2-L119V	PBO + Permethrin	Alive	56	91.07	8.93	0.39	0.070069	Resistance	C-terminal
		Dead	109	96.33	3.67				

Table 3. Odds ratios and *p*-values for insecticide resistance mutations, which were significantly associated with *An. gambiae* s.s. phenotype.

Côte d'Ivoire (40%)[23] and the Bijagós Archipelago islands (20%)[38], but has yet to be associated with phenotypic insecticide resistance.

The *vgsc* mutations, *vgsc*-L995F and *vgsc*-L995S, were found at an overall allelic frequency of 80% and 18%, respectively. Katana and Kalemie had the highest frequencies of *vgsc*-L995S (90% and 28%, respectively), and the lowest frequencies of *vgsc*-L995F (4% and 71%, respectively). This is unsurprising given that the *vgsc*-L995S mutation is typically associated with East-African *Anopheles* populations, and Katana and Kalemie are much further east than the other study sites. The spatiotemporal trends in the frequency of the *vgsc*-L995F mutation in the DRC is more similar to that of West African countries, and it is now approaching fixation in parts of the country[39]. In addition to insecticidal interventions used for malaria vector control, selection pressures for these geographical patterns in phenotypic resistance will have been imposed by the use of agricultural pesticides, other public health tools, environmental factors and vector population migration. At country level, *vgsc*-L995F was significantly associated with resistance to deltamethrin, but not permethrin or alpha-cypermethrin. This lack of strong association has been previously reported in *An. gambiae* s.s. from Cote d'Ivoire[40] and *An. gambiae* s.l. from Guinea[41] and study findings highlight the complexities of linking direct insecticide phenotype with genotype. In the DRC, *vgsc*-L995F may be a contributor, alongside concurrent metabolic insecticide resistance mechanisms, rather than a predictor of pyrethroid resistance[39]. At country level, *vgsc*-L995S was significantly associated with susceptibility to permethrin and deltamethrin, contrasting with reports from Tanzania[42,43]. The S form of the mutation may come at a higher fitness cost than the F form of the mutation, and the association with insecticide susceptibility may predominate in populations where the *vgcs*-N1575Y mutation is not present, and further research is required to confirm this.

The *vgsc*-N1575Y mutation was not detected in this study, corroborating other recent regional reports[17,29]. *Vgsc*-N1575Y has only been reported once in the DRC, at a very low allelic frequency (0.011%). This mutation has a strong association with phenotypic resistance to pyrethroids and cross-resistance with DDT[44], and has rapidly spread across West Africa[45,46]. It is therefore imperative that this mutation continues to be screened for during routine molecular entomological surveillance.

The *rdl*-A296G mutation in the GABA receptor has shown to be genetically linked to resistance to the public health insecticide dieldrin[34]. In our *An. gambiae* s.s., *rdl*-A296G was present at almost 50% in Kinshasa, and then at lower frequencies (< 10%) in Inongo, Kimpese, Lisala and Lodja. *Rdl*-A296G is located within a 2La chromosomal inversion, meaning it is highly stable and persists in the absence of selection pressure, despite a potential fitness cost[47]. The deviations from Hardy-Weinberg frequency observed in Kinshasa, Kimpese and Inongo may reflect exposure to agrochemicals which also target the GABA receptor, as well as historical use of dieldrin; insecticides are used for crop protection by farmers across all 26 provinces in the DRC. *Rdl*-A296G was found in a single dieldrin resistant *An. gambiae* s.l. individual from the North-East of the country in 2017 (allelic frequency of 3%)[32]. If novel insecticides which also target the GABA receptor, such as the recently WHO PQT/VCP approved indoor residual spraying (IRS) insecticide, broflanilide[48], are implemented, then it will be important to monitor vector populations carrying the *rdl*-A296G mutation for potential cross-resistance.

Historically, from 1960 to 1990, governmental IRS operations were conducted in Kinshasa and several southern provinces using DDT and dieldrin. More recently, IRS has been implemented by private companies; notably by Tenke Fungurume Mining (TFM) in Lualaba province, Banro Corporation in Maniema and Sud Kivu provinces, Kibali Gold Mines in Haut-Uele province and PPC Barnet Cement in Kongo Central province. Because IRS has been used in focal areas only, compared to mass pyrethroid ITN deployment, *ace1*-P265L was present at low frequencies of between 1% and 3% in Inongo, Kimpese, Kinshasa, Lisala and Lodja. *Ace1*-N246T

was found at 35% and 31% in Inongo and Katana, and then less than 10% in Kimpese, Lisala and Lodja and was strongly associated with permethrin susceptibility in Kimpese.

Our study identified associations between two non-synonymous SNPs in *gste2*, L119V and T154S, and pyrethroid resistance or susceptibility in the presence of PBO pre-exposure. T154S is located within the C-terminal region of GSTe2 which is typically involved in binding hydrophobic substrates, including insecticides. Amino acid substitutions in this region may alter the affinity or orientation of the substrate within the binding pocket, potentially modifying the enzyme's detoxification efficiency and therefore conferring changes in insecticide susceptibility. The WHO recommends that PBO-ITNs are distributed in areas of widespread pyrethroid resistance with underlying metabolic resistance mechanisms[49]. Consequently, in 2023, 58% of ITNs (112.6 million) delivered to sub-Saharan Africa were PBO-ITNs, to improve control of malaria transmitted by pyrethroid-resistant vectors[5]. Currently, there are no DNA-based loci which have been associated with variable responses to PBO pre-exposure in pyrethroid resistant populations, that could be used as early indicators of emerging incomplete PBO synergy. Genetic markers are essential for informing ITN procurement decisions and monitoring the inevitable spread of PBO synergy loss as PBO-ITNs continue to be more widely integrated into vector control campaigns[5,12,19]. Functional validation of *gste2*-L119V is warranted to determine its putative role in reduced PBO synergy, including heterologous expression in *Escherichia coli* to assess enzymatic changes in vitro, as well as RNAi-silencing of this mutation in transgenic *An. gambiae* models to investigate altered PBO + pyrethroid susceptibility[50].

In this study, the use of amplicon sequencing allowed for the identification of novel mutations within insecticide resistance genes, compared with end-point or qPCR which only detect known mutations. The large sample size and sequencing depth resulted in very high-quality scores for all identified mutations. However, we were unable to identify mutations occurring outside of these selected genomic regions. Undertaking whole genome sequencing (WGS) of a subset of samples would have ensured that the genes selected for amplicon sequencing were the most relevant to this population[51–55]. A further limitation of this study is that we did not investigate copy number variants (CNVs), which can have a wide range of effects on gene expression[56,57], or mutations in non-coding regions. While mosquito age and physiological status were standardised across bioassays, study findings should be interpreted acknowledging that phenotypic resistance in WHO tube assays can also be influenced by variable environmental conditions during testing and mosquito holding, which may not directly correlate with presence or absence of specific molecular mutations[58]. Finally, due to relative vector availability, in some bioassays the sample size tested was slightly below the recommended number outlined by WHO guidelines[58].

The partial restoration of pyrethroid susceptibility with pre-exposure to PBO suggests that metabolic-based mechanisms are driving resistance in *Anopheles* populations in the DRC, whilst different associations between the presence of an individual SNP and survival in bioassays suggests that the role of target site mutations in pyrethroid resistance in these populations is likely to be dynamic, site specific and highly variable. Further investigation of the role of metabolic enzyme overexpression in *Anopheles* vector populations in the DRC is strongly encouraged.

Conclusions

Herein we present the most comprehensive report of the prevalence of genetic markers of insecticide resistance in intensely pyrethroid resistant *An. gambiae* s.s. populations across the DRC, characterised using multiplex, next-generation, high-throughput amplicon sequencing. To our knowledge, this study also constitutes the largest amplicon sequencing study that has been performed in any *Anopheles* malaria vector species to date. Local mosquito populations were highly resistant to pyrethroid insecticides, with evidence for loss of PBO synergy in all sentinel sites across the DRC. We identified four non-synonymous SNPs, which were previously associated with insecticide resistance: *gste2*-L119V, *vgsc*-L995F, *vgsc*-L995S and *rdl*-A296G, while three were novel: *gste2*-T154S, *ace1*-N246T and *ace1*-P265L. Of note, *gste2*-L119V was significantly associated with resistance to deltamethrin following PBO-pre-exposure, warranting functional validation to determine its potential role in reduced PBO synergy. This is the first putative DNA-based loci identified in *An. gambiae* s.s. associated with variable PBO response. Given the rapid scale-up of PBO-ITNs across sub-Saharan Africa, heightened molecular surveillance is warranted to identify additional mutations which are predictive of reduced PBO efficacy in wild *Anopheles* populations. Study findings emphasise the importance of using next-generation sequencing technologies to improve our understanding of contemporary molecular insecticide resistance mechanisms, with applications for guiding both local malaria control strategies, as well as improving the design of prospective insecticide resistance management schemes.

Methods

Study sites and sample collection

This study was conducted in 2020 in eight NMCP sentinel sites from eight provinces in the DRC. The targeted sites were Kalemie (−5.913, 29.19), Tanganyika province, Kapolowe (−10.94, 26.94), Haut Katanga province, Katana (−2.225, 28.821), Sud Kivu province, Kimpese (−5.556, 14.44), Kongo Central province, Kingasani (−4.41, 15.41), Kinshasa province, Inongo (−1.919, 18.934), Mai-Ndombe province, Lisala (2.17, 21.49), Mongala province, and Lodja (−3.533, 23.597), Sankuru province. Sites were selected from existing entomological surveillance locations reporting moderate to high pyrethroid resistance in *An. gambiae* s.l. *An. gambiae* s.l. larvae were collected from breeding sites in the radius of 15–30 km from health centers and reared to adulthood in field insectaries (27 ± 3 °C, 75–90% relative humidity and 12:12 h light: dark cycles).

Insecticide susceptibility testing

Susceptibility to three pyrethroids, alpha-cypermethrin, deltamethrin and permethrin, was assessed using World Health Organization (WHO) tube assays, following standard procedures[58]. Bioassays were conducted using 2–5-day old, unfed female *An. gambiae* s.l. For each test 80–100 mosquitoes (20–25 per tube) were exposed for one hour to impregnated papers of one, five and ten times the discriminating concentration of alpha-cypermethrin (0.05%, 0.25%, 0.5%), deltamethrin (0.05%, 0.25%, 0.5%) or permethrin (0.75%, 3.75%, 7.5%). A subset of mosquitoes was pre-exposed to PBO (4%), followed by exposure to the discriminating concentration of a pyrethroid. Two control tubes containing solvent-impregnated papers were run in parallel. Twenty-four hours after exposure, mosquitoes were characterized as resistant (< 90% mortality) or susceptible (> 90% mortality), as per WHO criteria[58], and stored in 1.5 ml Eppendorf tubes containing silica gel. Samples were transported to the London School of Hygiene and Tropical Medicine for molecular analysis.

Sample preparation and sequencing

DNA was extracted from 1244 mosquitoes that had been categorised as resistant or susceptible to alpha-cypermethrin, deltamethrin or permethrin, both with or without PBO pre-exposure (Table S6). Individual mosquitoes were homogenized in a Qiagen TissueLyser II (Qiagen, UK), with sterile 5-mm stainless steel beads for 5 min at 30 Hz and incubated overnight at 56 °C. DNA was extracted using Qiagen DNeasy 96 blood and tissue kits (Qiagen, UK), according to the manufacturer's protocol, with minor modifications; DNA was eluted in 45ul of AE buffer[59].

Samples were prepared for sequencing following the methods of Campos et al.[23] with modifications. Multiplexed PCR assays were performed for the following resistance genes: *voltage-gated sodium channel* (*vgsc*; four fragments), *acetylcholinesterase 1* (*ace1*), *resistance to dieldrin gamma-amino butyric acid receptor* (*rdl*) and *glutathione-S-transferase epsilon 2* (*gste2*). Taxonomic markers were: *internal transcribed spacer 1* (*its1*), *NADH dehydrogenase 4* and *5* (*nd4*, *nd5*), *28 S ribosomal RNA* and *rRNA intergenic spacer region* (*igs*) and *short interspersed elements 200* (*sine*). Gene multiplexing combinations and primer sequences are detailed in Table S7 and Table S8, respectively.

Multiplexed PCR reactions consisted of 10 µl Q5® reaction buffer (New England Biolabs, UK), 1 µl (10mM) dNTPs (New England Biolabs, UK), 1.5 µl (10mM) forward primer, 1.5 µl (10mM) reverse primer, 0.5 µl Q5® High-Fidelity DNA polymerase (New England Biolabs, UK) and 4 µl DNA in a final volume of 50 µl. The following reaction conditions were used: 98 °C for 30 s, 35 cycles of 98 °C for 10 s, 57 °C for 60 s and 72 °C for 1 min, followed by one final elongation step at 72 °C for 2 min.

Amplicons were visualised in 2% E-Gel™ agarose gels with SYBR Safe (Invitrogen, UK), to check for band size and intensity. The addition of unique 8 bp barcodes to the forward and reverse primers enabled samples, as well as genes, to be multiplexed for sequencing. Amplified PCR products from groups of four mosquitoes, each with unique barcodes, were pooled for sequencing and purified with Kappa Pure Beads (Roche, UK) at 0.9x sample volume. Pooled purified PCR products were normalised to a final concentration of 20ng/µl (100 µl final volume) and 250 bp paired end sequencing was conducted on an Illumina MiSeq platform (Illumina, San Diego, CA, USA).

Bioinformatics analysis

Data were analysed using an adapted version of an in-house pipeline (https://github.com/LSHTMPathogenSeqLab/amplicon-seq/tree/main). Raw sequence data were trimmed using trimmomatic v0.39[60] (ILLUMINACLIP:TruSeq3-PE-2.fa:2:30:10:1:true LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:30) and demultiplexed using cutadapt v4.4[61] and a custom python script. Coding genes (*ace1*, *gste2*, *rdl*, *vgsc*, *cox1*) for each sample were merged into a single FASTQ file, whilst non-coding genes remained as separate FASTQ files (*igs*, *sine*, *its1*, *nd4*, *nd5)*. FASTQ files were quality checked using FASTQC v0.11.9[62].

FASTQ files for coding genes were aligned to the *Anopheles gambiae* PEST 4 genome downloaded from Ensemble Metazoa (release 57)[35]. Non-coding genes were aligned to reference sequences obtained from NCBI[63]. Alignment was performed using BWA-mem v0.7.17[64] and mapped sequences were visualised using tablet v1.21.02.08[65]. Summary statistics were generated using samtools v1.18[66]. SNPs and small indels were called using freebayes v1.3.6[67] (haplotype-length 1, min-coverage 30, min-base-quality 30, gvcf, gvcf-dont-use-chunk true).

Variants were filtered using bcftools v1.9[66] and vcftools v0.1.16[68]. High quality variants were identified using filters that included a minimum phred quality of 50 per called base and a minimum depth of 10 reads. Only variants that were present in > 1 sample, and present across two independent pools were retained.

Molecular species identification

An. gambiae s.l. were morphologically identified prior to bioassay testing[69]. The *igs* and *sine* genes were used to identify *An. gambiae* s.l. to species level, using the following script: https://github.com/LSHTMPathogenSeqLab/amplicon-seq/blob/main/scripts/amplicon_species_classifier.py, to distinguished between *An. gambiae* s.s., *An. coluzzii*, *An. arabiensis* and *An. melas.* Species ID was conducted using kmer analysis, a method which extracts and counts short DNA sequences from FASTQ files[70]. The script identified species-specific kmers of 31 bases long in *igs*, *sine* and *gste2* genes. In most samples, there was one species with a predominant number of kmers. For *An. gambiae* s.l. *igs* had the highest number of kmers, then the *sine* gene was used to differentiate between *An. gambiae* s.s. and *An. coluzzii*. A sample was identified as *An. gambiae* s.s. if there were fewer than 1% *An. coluzzii* reads, *An. coluzzii* if more than 10% of *sine* kmers were *An. coluzzii* and a *An. gambiae* s.s. – *An. coluzzii* hybrid if values lay between 1 and 10%[23]. If results appeared discordant, the bam files for *sine* and *igs* genes were visually inspected using tablet v1.21.02.08[65]. VCF files for *igs* and *sine* were also manually inspected to confirm species identification. When aligned with *igs* reference sequences, *An. arabiensis* had a T > C mutation at position

222 and *An. gambiae* s.s. /*An. coluzzii* had a T > C and T > G mutation at positions 134 and 136, respectively. When aligned with the *sine* reference sequence, *An. coluzzii* was heterozygous for T > G, A > G, T > C and T > C mutations at positions 119, 359, 401 and 453, respectively.

Identification of variants in insecticide resistance genes
Variant annotation was conducted for coding genes using SnpEff[71] and data were analysed using R v4.1.13[72]. Only SNPs resulting in a missense mutation and present at an allelic frequency of more than 1% were considered for statistical analysis. Pearson χ^2 and Fisher's exact tests were used to investigate the statistical association between insecticide resistance phenotypes, allelic frequencies, and deviations from Hardy-Weinberg equilibrium. For missense mutations, protein domains were characterised using InterPro[73]. Sequence data generated by this study is available at Sequence Read Archive (SRA) BioProject PRJNA1217448.

Data availability

All datasets are included in this published article and its supplementary information files. Additionally, datasets can be provided directly by the corresponding authors, upon reasonable request. Sequence data generated by this study are available at Sequence Read Archive (SRA) BioProject PRJNA1217448 (Accession numbers: SAMN46488139 – SAMN46492136).

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Author contributions
BP, FA, RMO and LAM designed the study. FA, NB, FW, EM YOS, FN, EZM and EM led the entomology field activities and participated in data collection. BP, CM and LAM performed the molecular assays. BP, HAP, EC, SM, CM, MC, JP and SC performed the bioinformatics analysis. MR, TW and NM provided project funding, laboratory resources and oversight. TC, SBO and MW provided project management support. BP, FA, YPS, SC and LAM were responsible for data interpretation. BP and LAM drafted the manuscript which was revised by all co-authors. All authors read and approved the final manuscript.

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Declarations

Conflict of interest
The authors have no competing interests to declare.

Ethical approval
The work described in this manuscript was determined to be non-human subjects' research by the Centers for Disease Control and Prevention (CDC) Center for Global Health (CDC 2016 – 242, 2016-082). The study was approved by the Ethics Committee of the School of Public Health (University of Kinshasa), and the nationwide testing work was conducted within the remit of the Institut National de Recherche Biomédicale.

Disclaimer
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