

Exploring genetic variation in insecticide resistance loci in *Anopheles gambiae* s.l. populations from Ghana

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Abstract

Malaria remains a major public health burden in Sub-Saharan Africa, with Ghana contributing significantly to global cases and deaths. Control relies heavily on vector control interventions, particularly insecticide-treated nets (ITNs) and indoor residual spraying (IRS), but their effectiveness is increasingly threatened by widespread insecticide resistance in malaria vectors. Knowledge of the molecular markers associated with resistance is important for insecticide resistance management in local vector populations. In this study, a low cost, high throughput multiplex amplicon sequencing approach was used for the molecular screening of insecticide resistance alleles in *vgsc*, *rdl*, *ace1* and *gste2* genes, alongside species identification and genetic diversity loci of *An. gambiae* s.l. populations from Ghana. A total of 330 *Anopheles gambiae* s.l. from 18 study sites were analysed, including a subset of pyrethroid phenotyped mosquitoes. We detected 31 non-synonymous mutations in the target genes, of which 13 have been previously associated with insecticide resistance (7 in *vgsc*, 2 in *rdl*, 1 in *ace1*, and 3 in *gste2*). We identified one SNP, T154S in *vgsc*, which was recently detected at a frequency of 24% in *Anopheles* populations from Guinea-Bissau. An additional 17 novel SNPs were identified, including A1553T and L1680F also in the *vgsc* gene. Phylogenetic and genetic analyses of *ITS2* and *mitochondrial* sequences showed low nucleotide diversity and limited genetic variation, indicating high gene flow across these *An. gambiae* s.l. populations. Overall, this study demonstrates that high-throughput amplicon sequencing provides a cost-effective and scalable method for surveillance of malaria vector populations and can support improved insecticide resistance management and targeted control strategies.

Introduction

Malaria continues to be a major public health issue in Sub-Saharan Africa (SSA) [1]. In 2024, an estimated 263 million cases with 597,000 deaths were reported, with SSA accounting for 95% of all global malaria cases [2]. Ghana accounts for ~ 2.5% of all global malaria cases and ~ 1.9% of global malaria deaths, and is among the 15 countries with the highest malaria burden globally [2]. Vector control relies primarily on insecticide-based interventions, particularly insecticide-treated nets (ITNs) and indoor residual spraying (IRS), which have driven major reductions in malaria over the past two decades [2].

In Ghana, ITNs were introduced in the early 1990s and scaled up through nationwide mass campaigns every three years, alongside continuous distribution via antenatal and child health services, while IRS was introduced in 2008 and implemented annually in selected high-transmission districts [3, 4]. However, the effectiveness of these interventions is increasingly threatened by widespread insecticide resistance [5–7]. Malaria vectors in SSA, including in Ghana, have been identified as resistant to all four main classes of insecticides used in public health interventions: pyrethroids, organochlorines, carbamates and organophosphates [7–9]. Resistance to newer insecticide classes prequalified by the WHO and introduced for vector control, specifically Pyrroles (Chlorfenapyr) and neonicotinoids (Clothianidin) in malaria vectors, is rapidly emerging, with some *Anopheles* (*An.*) populations showing reduced susceptibility to these insecticides [10, 11].

Target-site resistance and metabolic resistance are the most well-known mechanisms of resistance evolution in malaria vectors [7]. Non-synonymous mutations in the insecticide target sites have been associated with insecticide resistance phenotypes in many African malaria vectors [12, 13]. The target site for pyrethroids and organochlorine insecticides is the voltage-gated sodium channel (*vgsc*) while carbamates and organophosphates target the acetylcholinesterase enzyme (*ace1*) [14]. Several non-synonymous mutations in the *vgsc* gene have been implicated in pyrethroid resistance in *An. gambiae* s.l. [12, 13, 15]. The first genetic variants identified in the *vgsc* gene were the knockdown resistance (*kdr*) L995F/S mutations [16]. The L995F mutation is predominant in West and Central Africa [17], and has been found in higher frequencies in local *An. gambiae* s.l. mosquitoes from Ghana and other countries across West Africa [8, 17–20]. The L995S mutation is common in Central and East Africa [21, 22]. However, recently the L995S has also been detected in West African countries such as Côte d'Ivoire, Ghana, and Burkina Faso [8, 23, 24]. Additionally, the N1570Y mutation, which enhances resistance when combined with L995F, has been reported in vectors from Guinea, Ghana, Burkina Faso, and Cameroon [15, 17]. The G280S mutation in the *ace1* gene, and *ace1* gene duplications, have been linked to organophosphate and carbamate resistance [25–27], while mutations in the GABA receptor (*rdl*), including A296G/S, have been associated with resistance to organochlorines, particularly dieldrin [28]. Mutations in the metabolic gene, glutathione-S-transferase *epsilon* (*gste2*) such as L119V and I114T have been linked to pyrethroid and

organophosphate resistance [29]. Other insecticide resistance mechanisms including metabolic and cuticular resistance have also been reported in local *An. gambiae* s.l. populations in Ghana [30].

Whole genome sequencing (WGS) has facilitated the identification of additional target site and metabolic resistance loci [17, 20]. Recently, thirteen novel *vgsc* non-synonymous mutations were found occurring almost exclusively with the L995F *kdr* haplotype [17]. A novel mutation, I1527T, adjacent to a predicted pyrethroid-binding site, was found in tight linkage with the V402L mutation [17]. Studies have shown temporal increases in insecticide resistance alleles in response to insecticide exposure from vector interventions [31]. Therefore, enhanced genomic surveillance is required to identify new, reliable genomic markers and determine the spread of resistance haplotypes in vector populations, with the aim of informing insecticide resistance management.

In addition to conventional WGS platforms, Oxford Nanopore Technologies (ONT) sequencing offers important operational advantages for surveillance: its portability, rapid real-time data generation, reusable flow cells, and relatively low cost allow resistance monitoring to be carried out in field laboratories, enabling faster detection of emerging resistance variants [32]. These features make nanopore-based approaches particularly valuable for strengthening genomic monitoring in resource-limited settings where vector-control decisions often need to be made quickly.

In Ghana, *An. gambiae* s.l. and *An. funestus* mosquitoes are the primary vectors of malaria. These vectors vary in their distribution, ecology and behaviour across Ghana's ecological zones [33, 34], impacting malaria transmission dynamics and the efficacy of vector control interventions [19, 35, 36]. Accurate identification of species within *An. gambiae* s.l. is crucial for malaria control, but morphological methods are inadequate due to overlapping traits, requiring molecular tools for precise species identification [37, 38]. Genetic markers including internal transcribed spacers 1 and 2 (*ITS1*, *ITS2*), the *sine200* insertion and the mitochondrial genes *cox1* and *nd4* are effective for species identification and genetic and phylogenetic analysis [39–41]. These analyses provide insights into evolutionary history, genetic diversity and gene flow, guiding vector control strategies. Across Africa, *An. gambiae* s.l. shows high genetic diversity and differentiation, especially at insecticide-resistance loci [20, 29, 42, 43], shaped by ecological and chemical-based intervention pressures [31, 44]. However, major geographical gaps persist in Ghana, where data on *An. gambiae* s.l. phylogeny and genetic diversity remain limited across different ecological zones and intervention settings.

In this study, we used high-throughput multiplex amplicon PCRs and nanopore sequencing to investigate insecticide resistance alleles, species identification markers, and genetic diversity metrics to characterise *An. gambiae* s.l. populations across different ecological and vector intervention settings in Ghana [45]. This high-throughput method allowed for the screening of a large number of mosquito samples across multiple genetic loci simultaneously, and still discriminated between individual mosquitoes based on unique primer barcodes, hereby optimizing throughput, scalability, and cost-efficiency for population genomic surveillance.

Methodology

Population and Mosquito sampling

A total of 330 *Anopheles gambiae* s.l. mosquitoes were used for this study. The samples were collected as part of ongoing entomological surveillance across Ghana by the Centre for Vector-Borne Disease Research (CVBDR) based at the University of Ghana Medical School. Mosquito samples from 18 study sites spanning the three ecological zones of Ghana were used (**Fig. 1**). Mosquitoes were collected from sites in the coastal savannah zone: Ada, Takoradi, Tuba, Dodowa, Apam, Antsaadze, Tema and Pediatorkope. Forest zone sites included Elubo, Twifo Praso, Dawurampong, Obuasi, Konongo, Begoro and Kade. Sites within the Guinea savannah zone included Kpalsogu, Tolon and Kulaa. The list of the number of mosquitoes per site, sampling method and year of collection is available (**Additional File 1: Table S1**). For all sites excluding Apam, Antsaadze, Dawurampong, Twifo Praso, Begoro, Kade, Pediatorkope and Tuba, mosquitoes were collected as larvae, raised to adults and phenotyped using WHO tube insecticide susceptibility tests. These mosquitoes were exposed to papers impregnated with pyrethroids {type I permethrin (0.75%) and type II deltamethrin (0.05%)} and organophosphate {pirimiphos-methyl (0.25%)}

Mortality was assessed after a 24-hour recovery period, and resistance status was determined according to WHO criteria [46] to determine phenotypic resistance. Mosquitoes from Begoro, Kade, Pediatorkope, Apam, Antsaadze, Dawurampong, Twifo Praso and Tuba were collected as adults using Prokopack aspirators. IRS is deployed in two of the sites, Obuasi and Kpalsogu while ITNs (standard, PBO and chlorfenapyr nets) are deployed in the other sites.

Figure 1: Entomological surveillance sites and their associated ecological zones within Ghana, including recent vector control interventions.

DNA Extraction

DNA was extracted from individual mosquitoes using Qiagen DNeasy Blood and Tissue Kits according to the manufacturer's protocol, with minor adjustment to the elution volume. The DNA samples were then quantified using the Qubit 2.0 fluorimeter HS DNA kit (ThermoFisher, Waltham, MA, USA) and stored at -20°C.

Multiplex PCR Reactions

Gene specific multiplex PCR assays were used to amplify genes associated with insecticide resistance, speciation and phylogeny using established protocols [45]. Primers targeting 7 genomic regions containing 17 SNPs associated with insecticide resistance were used. This included 4 domains of the voltage-gated sodium channel (*vgsc*), partial sequences of the genes acetylcholinesterase 1 (*ace1*), glutathione S- transferase 2 (*gste2*) and gamma-aminobutyric acid receptor (*rdl*). Primers targeting seven genes were used for speciation and phylogenetic analysis: nuclear ribosomal internal transcribed spacers 1 and 2 (*ITS1*, *ITS2*), cytochrome c oxidase I (*cox1*), mitochondrially encoded NADH dehydrogenases 4 (*nd4*), 28S ribosomal RNA and rRNA intergenic spacer region (*IGS*), and short-interspersed elements *sine200* were also used. Forward and reverse primers were tagged with unique 5' barcodes (8bp long, a total of 6 different barcodes were used) to permit pooling of samples before sequencing, as described previously [45, 47]. The multiplex PCR reactions were performed consisting of 5 µl of 5X Q5 Reaction Buffer, 0.25 µl of Q5 high fidelity DNA polymerase (NEB), 1 µl of each primer (10uM), 0.5 µl of dNTPs (10mM), 2 µl of DNA and nuclease free water to a final reaction volume of 25 µl. Reactions were performed in four multiplexes for gene-specific primers: multiplex 1 (*ITS1*, *ace1*, *vgsc-DIIS6*, *cox1*), multiplex 2 (*ITS2*, *nd4*, *rdl* and *vgsc-DIIS6*), multiplex 3 (*IGS*, *gste2*, *vgsc-DIVS5*) and multiplex 4 (*vgsc-DIS6*, *SINE200*). PCR assays were performed using the following reaction conditions: heat start activation at 98°C for 30 seconds, followed by 35 cycles of denaturation at 98°C for 10 seconds, annealing at 57°C for 60s and elongation at 72°C for 60 seconds, followed by a final elongation step at 72°C for 2 min.

Amplicon purification and nanopore sequencing

PCR products were run on a 1% agarose gel to confirm the presence and sizes, before pooling amplicons for sequencing. Pools of PCR products of 6 different mosquitoes containing different unique barcode combinations were created. The pools were then purified using KAPA pure beads (Roche Diagnostics, Indianapolis, IN, USA) according to manufacturer's protocol, using a bead-to-DNA ratio of 0.8:1. The purified pools were quantified using the Qubit dsDNA HS Kit (Thermo Fisher). Purified DNA pools were adjusted to a final concentration of 200 fmol, after which the DNA ends were prepped and nanopore native barcodes were ligated onto the DNA ends using the Native Barcoding Kit 24 V14 (Oxford Nanopore Technologies, Oxford, UK). Sequencing adapters were ligated to the DNA ends, loaded on the MinION flow cell r10.4.1 and sequenced on the MinION Mk1B device (Oxford Nanopore Technologies, Oxford, UK). A minimum of 50,000 reads were obtained for each pool of amplicons.

Bioinformatics analysis

Sequenced reads were basecalled using Oxford Nanopore Technologies basecaller, Dorado v0.9.1 super high accuracy model, generating BAM files from the raw pod5 signal data [48]. Basecalled reads were then converted to FASTQ format and demultiplexed to separate individual mosquito sequences by identifying the inline sample-specific barcodes. The demultiplexed reads were then processed using a custom python script from the London School of Hygiene and Tropical Medicine (LSHTM)

pathogen sequencing lab (<https://github.com/LSHTMPathogenSeqLab/amplicon-seq>). Sequences were trimmed and the quality was assessed using FastQC (v 0.11.5) and aligned to the *Anopheles gambiae* reference genome (assembly accession: GCA_000005575.1; *Anopheles gambiae*.AgamP4.dna.toplevel. fa) using the BWA-MEM algorithm (v0.7.17, default parameters) [49]. SNPs and small indels were called using freebayes (v1.3.5, -haplotype-length - 1) [50] and GATK HaplotypeCaller (v4.1.4.1, default parameters) software [51]. All variant calls were combined and SNPs were filtered using BCFtools (version 1.16) for an allele depth of at least 20, minimum read depth of 30 and phred score of > 30 per base [52]. Variants were annotated using the SnpEff software (version 5.1d) [53]. Variants were then genotyped based on the percentage of alternative allele reads within the total depth coverage: (i) homozygous reference (< 25% alternate allele calls); (ii) heterozygous (25–75% alternate allele calls); and (iii) homozygous alternate (> 75% alternate allele reads) [45]. Species identification of individual mosquito samples was assigned using the *SINE200* repeat region, for which a 230 bp insertion exists in *An. coluzzii* which does not exist in *An. gambiae*. Specimens with $\leq 30\%$ of *SINE200* reads containing this *An. coluzzii* insertion were classified as *An. gambiae*, and those with $\geq 70\%$ of *SINE200* reads containing this insertion were classified as *An. coluzzii*. Those with a mixture of *SINE200* reads containing the insertion (between 30 and 70%), were classified as *An. gambiae/coluzzii* hybrids.

Phylogenetic, Nucleotide and Haplotype diversity analysis

Consensus FASTA sequences for *ITS2* and *cox1* amplicons were generated using an in-house pipeline (<https://github.com/LSHTMPathogenSeqLab/fastq2matrix>). Before consensus calling, low-quality regions were masked, and variant calls were converted to consensus sequences using bcftools consensus. The resulting consensus sequences were aligned using MAFFT [54], after which the alignments were inspected and manually trimmed in AliView [55]. Publicly available *Anopheles* sequences from other African studies were downloaded from GenBank and included in the alignments [56]. A full list of accession numbers used is provided in Supplementary File 2. Maximum likelihood trees were generated using the software RAxML using a bootstrap value of 1000 [57], and visualized in iTOL [58]. For haplotype network construction, *cox-1* and *mtnd4* FASTA sequences from all specimens were aligned, and subsequent analyses were conducted using the R packages Pegas and ggplot2 [59, 60]. The Pegas package was employed to compute nucleotide diversity, haplotype diversity and Tajima's D statistic [59].

Results

Anopheles gambiae s.l. molecular species distribution across the study sites

Species identification was performed based on species-specific nucleotide sequences for *IGS* and an insertion sequence in the *SINE200* amplicon, which is fixed in *An. coluzzii* but absent in *An. gambiae* s.s mosquitoes [61]. Out of a total of 323 *An. gambiae* s.l, 239 (74.0%) were *An. coluzzii*, 64 (19.8%) were *An. gambiae* s.s. and 20 (6.2%) were *An. coluzzii/An. gambiae* s.s. hybrids. *An. coluzzii* was the predominant species in the Coastal savannah zone (90/116, 77.6%) and Forest zone (128/148, 86.5%) while *An. gambiae* s.s was the most prevalent in the Guinea savannah zone (30/59, 50.0%). However, site-based differences were observed (Fisher exact, $P < 0.001$). *An. gambiae* s.s was most predominant in only three sites, Dodowa (75.0%), Begoro (70.0%) and Tolon (75.0%) whereas *An. coluzzii* dominated in the remaining sites (Table 1). Seven samples were not speciated because of low read coverage for these speciation markers and hence were excluded from the analysis.

Table 1
Species identification of collected mosquitoes from across all entomological surveillance sites.

Eco- zones	Variables	Total N (%)	<i>An. gambiae</i> s.l N (%)		
			<i>An. coluzzii</i>	<i>An. gambiae</i> s.s	Hybrids
Coastal Savannah Zone	Ada	18 (100.0)	18 (100.0)	0	0
	Dodowa	20 (100.0)	4 (20.0)	15 (75.0)	1 (5.0)
	Tema	19 (100.0)	18 (94.7)	0	1 (5.3)
	Pediatorkope	10 (100.0)	6 (60.0)	1 (10.0)	3 (30.0)
	Takoradi	20 (100.0)	20 (100.0)	0	0
	Tuba	10 (100.0)	6 (60.0)	2 (20.0)	2 (20.0)
	Antsaadze	13 (100.0)	12 (92.3)	1 (7.7)	0
	Apam	6 (100.0)	6 (100.0)	0	0
	Total	116 (100.0)	90 (77.6)	19 (16.4)	7 (6.0)
Forest Zone	Begoro	10 (100.0)	3 (30.0)	7 (70.0)	0
	Elubo	18 (100.0)	18 (100)	0	0
	Twifo Praso	62 (100.0)	58 (93.5)	4 (6.5)	0
	Dawurampong	9 (100.0)	5 (55.6)	3 (33.3)	1 (11.1)
	Kade	10 (100.0)	7 (70.0)	1 (10.0)	2 (20.0)
	Konongo	19 (100.0)	19 (100.0)	0	0
	Obuasi	20 (100.0)	18 (90.0)	2 (10.0)	0
	Total	148 (100.0)	128 (86.5)	17 (11.5)	3 (2.0)
Guinea Savannah Zone	Kpalsogu	19 (100.0)	8 (42.1)	7 (36.8)	4 (21.1)
	Kulaa	20 (100.0)	6 (30.0)	8 (40.0)	6 (30.0)
	Tolon	20 (100.0)	2 (10.0)	15 (75.0)	3 (15.0)
	Total	59 (100.0)	16 (26.7)	30 (50.0)	14 (23.3)
N= number of samples, %= Percentage, Hybrids = <i>An. coluzzii</i> / <i>An. gambiae</i> s.s hybrid					

Phylogenetic and Genetic Variation of *An. gambiae* s.l Populations

A maximum likelihood tree was generated using ribosomal *ITS2* sequences of *Anopheles gambiae* s.l from Ghana combined with publicly available *ITS2* sequences of *An. gambiae* complex across Africa. The phylogenetic analysis revealed no distinct clustering of isolates according to their geographic origin or species, suggesting gene flow across populations (**Fig. 2**). Similarly, there was low genetic and nucleotide diversity of mitochondrial *cox1* ($\pi = 0.0047$), *mtnd4* ($\pi = 0.0054$) and *ITS* ($\pi = 0.0013$) sequences. However, haplotype diversity for these sequences was high, driven largely by the high proportion of singleton haplotypes (**Additional File 1: Table S2**).

Figure 2: Maximum-likelihood tree constructed using *its2* gene sequences of *An. gambiae* s.l generated in this study (Coastal Savannah, Forest and Guinea Savannah zones of Ghana). **A:** Including publicly available *Anopheles ITS2* sequences from other African countries. **B.** *ITS2* gene sequences of *An. coluzzii*, *An. gambiae/coluzzii* hybrids, and *An. gambiae* s.s and across Ghana's ecological zones (Coastal Savannah, Forest and Guinea Savannah zones).

Amplicon sequencing coverage and single nucleotide polymorphisms (SNPs) variations.

The presence of single nucleotide polymorphisms (SNPs) was assessed in all seven amplicons. The average coverage for each amplicon was high (mean: 1906.27bp), ranging from 610.40 to 3381.40 bp, allowing for proper identification of SNPs present. In total, 202 SNPs were detected across the amplicons targeting the *vgsc*, *rdl*, *gste2* and *ace1* loci. This included 73 synonymous SNPs, 31 non-synonymous SNPs, 79 SNPs in intron regions, 6 SNPs in splice regions and 13 upstream gene variants (Table 2).

Table 2
Distribution of single-nucleotide polymorphisms (SNPs) detected in each resistance associated gene.

Gene/ amplicon	Average coverage of amplicon (bp)	Synonymous (n)	Non- synonymous (n)	Intron (n)	Splice region (n)	Upstream gene variant (n)	Total (n)
<i>vgsc</i> /DIS6	3361.40	0	1	14	0	0	15
<i>vgsc</i> /DIIS6	1400.26	3	2	4	0	0	9
<i>vgsc</i> /DIIS6	814.92	5	4	5	0	0	14
<i>vgsc</i> /DIVS5	610.40	1	2	4	1	0	8
<i>rdl</i>	1158.94	3	2	15	4	0	24
<i>gste2</i>	3381.22	24	10	0	1	13	48
<i>ace1</i>	2616.78	37	10	37	0	0	84
Total		73	31	79	6	13	202

bp = Base pairs

Non-synonymous SNPs detected in genes associated with insecticide resistance

Overall, 31 non-synonymous mutations were detected in the genes associated with insecticide resistance. In the *vgsc* gene, a total of 9 SNPs were detected across four domains (*vgsc*-DIS6, *vgsc*-DIIS6, *vgsc*-DIIS6, *vgsc*-DIVS5). In *vgsc*-DIS6, one non-synonymous SNP, V402L, which has been previously associated with insecticide resistance, was detected in the *An. gambiae* s.l population at an allelic frequency of 21%. In *vgsc*-DIIS6, two known insecticide resistance SNPs were detected, the L995F allele at high frequency (75%) and L995S at low frequency (6%). In *vgsc*-DIIS6, 4 SNPs were detected, including three known insecticide resistance mutations: N1570Y at 1% allelic frequency, I1527T at 19% allelic frequency, and F1529C at 4% allelic frequency. A novel SNP, A1553T was detected at a low frequency in the *An. gambiae* s.l population (3%). Two SNPs were detected in the *vgsc*-DIVS5, one known insecticide resistance mutation, A1746S (11%) and one novel SNP, L1680F (3%). Within the *rdl* amplicon, two known insecticide resistance SNPs were detected: A296G (51%) and A296S (4%). In the *ace1* gene, 10 non-synonymous SNPs were detected, including the known resistance mutation G280S (22%). Nine (9) novel SNPs (V218L, D181E, V190A, P203A, A212T, A212V, E213D and T221A) were detected in *ace1* at frequencies of 4% to 12%. In *gste2*, 10 non-synonymous SNPs were detected, including 3 known resistance mutations (I114T, F120L and L119V), one recently detected SNP (T154S) and 6 novel SNPs (A185T, P174L, G171N, N148E, I126F and D148E) (Table 3). A total of 19 indels were also detected within three insecticide resistance loci, *vgsc*, *rdl* and *gste2* (**Additional File 3. Table S1**).

Table 3
Frequencies of non-synonymous SNPs in *An. gambiae* s.l mosquitoes from Ghana.

Chromosome	Amplicon	Position	N ^a	Mutation	Genotype frequencies, N			Alternate allele frequency (%)
					Homozygous (reference)	Heterozygous	Homozygous alternate	
2L	vgsc-I	2391228	233	V402L	150	68	15	21
	vgsc-II	2422652	283	L995F	22	97	164	75
	vgsc-II	2422651	212	L995S	190	17	5	6
	vgsc-III	2429745	194	N1570Y	192	2	0	1
	vgsc-III	2429617	251	I1527T	170	66	15	19
	vgsc-III	2429623	252	F1529C	234	16	2	4
	vgsc-III	2429694	194	A1553T	184	8	2	3
	vgsc-IV	2430424	163	A1746S	136	19	8	11
	vgsc-IV	2430226	149	L1680F	146	2	1	1
2L	<i>rdl</i>	25429236	249	A296G	73	97	79	51
		25429235	193	A296S	182	8	3	4
2R	<i>ace1</i>	3492074	293	G280S	180	99	14	22
		3491888	290	V218L	288	2	0	0.3
		3491779	87	D181E	86	0	1	1.1
		3491805	87	V190A	86	0	1	1.1
		3491843	87	P203A	86	0	1	1.1
		3491870	87	A212T	86	0	1	1.1
		3491871	87	A212V	86	0	1	1.1
		3491875	97	E213D	86	0	1	1
		3491897	87	T221A	86	1	0	0.6
		3492189	87	F318Y	86	0	1	1.1
3R	<i>gste2</i>	28598166	283	I114T	159	96	28	27
		28598057	283	F120L	107	114	62	42
		28598062	285	L119V	230	34	21	13
		28597956	284	T154S	181	67	36	24
		28597864	196	A185T	193	3	0	0.8
		28597896	195	P174L	194	0	1	1
		28597905	196	G171N	195	1	0	0.3
		28597973	195	N148E	194	1	0	0.3
		28598041	197	I126F	195	2	0	1
		28597973	85	D148E	84	0	1	1.2

Bold SNPs are novel SNPs.

Linkage disequilibrium of insecticide resistance SNPs

Linkage disequilibrium (LD) analysis of *vgsc* mutations showed strong linkage between the *vgsc* mutations V402L and I1527T ($r^2 = 0.90$), indicating that these substitutions frequently occur on the same haplotype background. Moderate LD was also observed between L995F-I1527T ($r^2 = 0.51$) and V402L and L995F ($r^2 = 0.49$). Most other mutations, including L995S, F1529C, N1570Y, and A1746S, exhibited weak LD ($r^2 < 0.2$), indicating largely independent segregation. For the *gste2* gene amplicon, modest LD within *gste2* was detected between L119V-T154S ($r^2 = 0.50$) and F120L-T154S ($r^2 = 0.25$). R^2 values and linkage disequilibrium figures are shown in **Additional File 2 (Tables S2, S3 and Fig S1, S2)**.

Location-based differences of non-synonymous SNPs observed in *An. gambiae* s.l

Location based differences in the frequencies of non-synonymous SNPs were observed. Moderate to high allele frequencies (53% to 100%) of the *vgsc* L995F mutation were observed across the entomological surveillance sites. The *vgsc* L995S mutation was only observed in *An. gambiae* s.l from the Coastal savannah site, Ada (38%) and the GUINEA savannah sites, Kpalsogu (5%), Kulaa (47%) and Tolon (6%). The *vgsc* N1570Y mutation was only detected at very low allele frequencies in Dodowa (3%) and Begoro (6%). The *vgsc* V402L and I1527T mutations were found in frequencies ranging from 0 to 43%. The *rdl* mutation, A296G was detected in *An. gambiae* s.l. across almost all the sites, with the highest frequencies being observed in Coastal savannah sites, Dodowa (94%) and Tema (92%). The *ace1* G280S mutation was found in low to moderate frequencies in *An. gambiae* s.l mosquitoes (0 to 56%). Four known non-synonymous SNPs were identified in the *gste2* loci, three insecticide-resistant linked SNPs (I114T, F120L and L119V) and a recently detected SNP (T154S) were detected in varying frequencies across the study sites (**Fig. 3**).

Figure 3: Distribution of known non-synonymous SNPs detected in *An. gambiae* s.l from 18 sites across Ghana.

Additionally, 17 novel SNPs were identified across the *vgsc*, *ace1* and *gste2* loci. These mutations ranged from 1% to 22% allelic frequencies across the study sites. The novel SNP, A1553T detected in the *vgsc*-DIIS6 was found in only five sites with *An. gambiae* s.l from Tuba having the highest frequency (22%). In addition, the novel SNPs L1680F (*vgsc*-DIVS5) and V218L (*ace1*) were only found in *An. gambiae* s.l populations from the forest zone sites Konongo (13%) and Kade (7%) respectively. Eight out of nine novel SNPs detected in the *ace1* loci were found in one mosquito sample (**Fig. 4**).

Figure 4: Distribution of novel non-synonymous SNPs detected in *An. gambiae* s.l from 18 sites across Ghana

We also investigated the number of mosquitoes with multiple non-synonymous SNPs across the insecticide resistance loci. For the *vgsc* gene, most of the mosquitoes across the three ecological zones of Ghana have one or multiple insecticide resistance SNPs. For the *rdl* gene, over 60% of the mosquitoes had one insecticide resistance SNP, with 23.04% of *An. gambiae* s.l mosquitoes from the Guinea savannah zone having two insecticide resistant SNPs. For the *gste2* gene, the majority of the mosquitoes have one or more insecticide-resistant linked SNP (**Fig. 5**). Only one mosquito from the forest zone was found to have 8 SNPs in the *ace1* loci (**Fig. 5**).

Figure 5

Distribution of SNPs in each insecticide resistance loci (*vgsc*, *rdl*, *ace1* and *gste2*) across the different ecological zones of Ghana. Map of Ghana showing sampling locations across three major ecological zones (Guinea Savannah, Forest, and Coastal Savannah zones). Pie charts represent for each insecticide resistance gene (*vgsc*, *rdl*, *Ace1*, *gste2*), the number of SNPs identified per mosquito population (0–≥4, colour-coded) and the proportion of mosquitoes in each ecological zone carrying that SNP count.

Distribution of non-synonymous SNPs per mosquito species and phenotype

Higher frequencies of the *vgsc* L995F mutation were observed in *An. gambiae* s.s (88%) compared to *An. coluzzii* (73%) and *An. coluzzii/An.gambiae* hybrids (67%) ($\chi^2 = 25.46$, $df = 3$, $P < 0.001$). However, low allele frequencies of *vgsc* L995S were observed across all *An. gambiae* s.l species observed (4% to 12%). Significantly higher allele frequencies of *vgsc* V402L and I1527T SNPs were detected in *An. coluzzii* mosquitoes (Fisher exact, $P < 0.05$), while the *vgsc* N1570Y mutation was only detected in *An. gambiae* s.s at a very low frequency (2%). For the *rdl* gene, the highest allelic frequency of the A296G mutation was detected in *An. gambiae* s.s (84%), and the A296S mutation was identified at low frequency across all of the species (2% to 11%). All the novel SNPs detected in the *ace1* loci were found only in *An. coluzzii* mosquitoes at very low frequencies ($\leq 1.3\%$). The *gste-2* SNP, T154S, had the highest allele frequency in *An. gambiae* s.s (66%), followed by *An. gambiae* s.s/*An. coluzzii* hybrids (23%) and *An. coluzzii* (12%) ($P < 0.001$) (Table 4).

Table 4
Distribution of non-synonymous SNPs among the *Anopheles gambiae* s.l species identified.

Amplicons	Non-synonymous SNPs	Allele frequencies (%)		
		<i>An. coluzzii</i> N = 216	<i>An. gambiae</i> s.s N = 59	Hybrids N = 18
vgsc-I	V402L*	26	5	27
vgsc-II	L995F*	72	90	53
	L995S	6	1	28
vgsc-III	N1570Y	0	2	0
	I1527T*	24	3	12
	F1529C	5	0	0
	A1553T	3	2	10
vgsc-IV	A1746S*	0	42	0
	L1680F	2	0	0
rdl	A296G	46	69	46
	A296S	3	2	18
ace1	G280S	18	36	20
	V218L	0.4	0	0
	D181E	1.3	0	0
	V190A	1.3	0	0
	P203A	1.3	0	0
	A212T	1.3	0	0
	A212V	1.3	0	0
	E213D	1.3	0	0
	T221A	1.3	0	0
	F318Y	1.3	0	0
gste2	I114T*	33	6	8
	F120L	49	20	27
	L119V*	2	54	8
	A185T	0	0	2
	P174L	0	2	0
	G171N	0	1	0
	T154S*	12	66	50
	N148E	0.4	0	0
	I126F	0.4	0	0.00
	D148E	0.4	0	0

Hybrids = *An. gambiae* s.s./*An. coluzzii* hybrids, N = sample number, * = SNPs with significant differences across the species identified (*An. gambiae* s.s., *An. coluzzii* and Hybrids) ($P < 0.05$).

Overall, there were no significant associations between the allelic frequency of these non-synonymous SNPs and permethrin and deltamethrin resistance phenotypes ($P > 0.05$) (**Additional File 1: Table S3**). However, comparing associations across the genotypes of insecticide resistance SNPs, only two mutations were found to be significantly associated with resistance. The heterozygous genotype of the *vgsc* L995S and I1527T mutations was found to be significantly associated with deltamethrin resistance ($P = 0.032$) and permethrin resistance ($P = 0.034$), respectively (**Additional File 1: Table S4 and S5**).

Discussion

Genomic surveillance is an important tool to detect and track the emergence and geographical distribution of known and new genomic variants associated with insecticide resistance, as well as patterns of population structure and diversity in malaria vectors [12]. This study provides comprehensive data on the phylogenetic relationships and genetic variants in insecticide-resistance genes in *An. gambiae* s.l populations from sites across different ecological zones of Ghana. Phylogenetic and genetic diversity analysis showed limited genetic divergence, suggesting high gene flow across *An. gambiae* s.l populations across ecological zones in Ghana. Consistent with previous studies, *Anopheles coluzzii* was the most predominant species found in most of the study sites with *An. gambiae* s.s dominating in only a few locations. This aligns with other studies done in Ghana [19, 62, 63]. Phylogenetic analysis also revealed no clear variations of *An. gambiae* s.l. populations from Ghana to other *An. gambiae* s.l mosquitoes from West and East Africa. This genetic structuring suggests gene flow between regional populations and points to local adaptation in *An. gambiae* s.l mosquitoes, similar to other studies [64]. Low genetic diversity of the mitochondrial genes *cox1* and *mtnd4* were observed in Ghanaian *An. gambiae* s.l mosquitoes, similarly to findings in Nigeria [40, 65].

Using multiplex amplicon sequencing, we identified extensive genetic variation, with 202 SNPs across resistance associated loci (*vgsc*, *rdl*, *ace1*, and *gste2*), out of which 31 were non-synonymous SNPs. We detected thirteen SNPs previously associated with insecticide resistance in *An. gambiae* s.l, which includes 7 *vgsc*, 2 *rdl*, 1 *ace1* and 3 *gste2* (mutations. For the *vgsc* gene, the known *kdr* mutation, L995F was found at moderate to high frequencies (53% to 100%) across the study sites. This is consistent with other studies on *An. gambiae* s.l mosquitoes in Ghana and West Africa [8, 19, 66–68]. The widespread distribution of L995F is consistent with the intense pyrethroid selection pressure from pyrethroid-based vector control tools such as ITNs, agricultural pesticides and environmental volatiles [9, 67, 69]. The *vgsc* L995S mutation was absent in *An. gambiae* s.l from most of the study sites except in the Guinea savannah zone sites (Kpalsogu, Kulaa and Tolon) and coastal savannah site, Ada, where frequencies between 0.05 to 0.47 were observed. This suggests that the *vgsc* L995S mutation is spreading in West African *An. gambiae* s.l populations, as observed in other studies [8, 23, 24]. Although the *vgsc* L995S mutation has historically been present in East African *Anopheles* populations, in recent years, several studies have reported this *kdr* mutation in Ghana and other countries in West Africa [8, 18, 66].

Other *vgsc* resistance-associated SNPs including the N1570Y, V402L, I1527T, F1529C and A1746S, were detected at low frequencies in this study, similarly to previous studies across West and East Africa [8, 17, 20, 70]. The N1570Y mutation, first identified in 2012, has been found to amplify the effect of the L995F mutation in pyrethroid resistance [15], and was found at allele frequencies ranging from 5% to 35% in *Anopheles gambiae* s.l mosquitoes from Ghana, Burkina Faso, Benin and Cameroon. The *vgsc* V402L and I1527T mutations were observed at similar frequencies in the *An. gambiae* s.l population. These two mutations were found in tight linkage ($r^2 = 0.90$) from our LD analysis, similar to previous studies [17, 25]. Moderate to strong LD observed among *vgsc* mutations, particularly V402L, L995F and I1527T supports earlier evidence that pyrethroid target-site resistance often arises through combinations of mutations carried on specific haplotypes that have undergone positive selection and rapid geographic spread [17, 71]. Two novel mutations, A1553T and L1680F, were detected in the *vgsc* gene at low frequencies (3% and 1% respectively). However, further functional studies are needed to investigate their potential impact on insecticide resistance. The presence of other *kdr* variants, including these novel SNPs, highlights their persistence within *An. gambiae* s.l. populations and indicates that these SNPs could be enhancing tolerance to pyrethroid insecticides, warranting further investigation.

Mutations in other resistance loci were also detected, including *rdl* A296G/S, *ace1* G280S, and *gste2* variants (I114T, F120L, L119V), consistent with previous reports in Ghana and across Africa [28]. The *rdl*-A296G mutation was widespread across all the entomological surveillance sites while the *rdl*-A296S mutation was only present in *An. gambiae* s.l. populations from the Guinea savannah sites and at lower frequencies. Other studies in Ghana and Africa have also reported high frequencies and site-based variations of the A296G/S mutations [28, 72, 73]. Although dieldrin is no longer used for malaria vector control and has been banned for agricultural use in Ghana, the persistence of the *rdl* A296G mutation is likely maintained by their low fitness costs, compensatory mutations, and introgression between *Anopheles* species and chromosomal backgrounds, including the 2La inversion, which facilitates their persistence in natural populations [28, 72]. The *ace1* G280S mutation was also present at low to moderate frequencies (0 to 56%) across the study sites, consistent with other studies across Ghana [8, 18, 62].

The *ace1* G280S mutation is strongly associated with resistance to pirimiphos-methyl, an organophosphate insecticide currently used for indoor residual spraying (IRS) in Ghana [27, 74]. Hence, reports of pirimiphos-methyl resistance and the persistence of the *ace1* G280S mutation are concerning for current control efforts in Ghana and need more attention. Nine novel SNPs were also detected in the *ace-1* loci, with eight of them appearing in a single *An. coluzzii* mosquito from Dawurampong (in the forest zone), suggesting that these SNPs are not yet selected in the wider population.

In this study, we identified ten non-synonymous SNPs in the *gste2* gene, including three known insecticide resistance markers, I114T, F120L and L119V. Mutations in the *gste2* gene have been associated with organophosphate and organochlorine resistance [75, 76]. However, recent studies have also implicated the *gste-2* gene in pyrethroid resistance [77]. The *gste2* I114T and L119V mutations have previously been associated with DDT and pyrethroid resistance in African malaria vectors, while the F120L mutation has been associated with DDT resistance [77, 78]. Another SNP, T154S was detected at a frequency of 24% in the *An. gambiae* s.l. population. This novel SNP was recently detected in other *An. gambiae* s.l. populations from Guinea-Bissau (19% allelic frequency) and at high frequency in the Democratic Republic of Congo (78%) [47, 66]. T154S lies within the C-terminal domain of *gste2*, a region important for binding hydrophobic substrates such as insecticides. Hence, mutations in this region can alter substrate binding, potentially modifying detoxification efficiency and resistance. Six other novel SNPs were detected at very low frequencies ($\leq 1\%$). However, additional surveillance is needed to assess their spread in other *An. gambiae* s.l. populations in Ghana and any functional significance in conferring phenotypic resistance.

Species-based variations were observed in the frequencies of the non-synonymous SNPs identified. The *vgsc* L995F was found at higher frequencies in *An. gambiae* s.s. (90%) compared to *An. coluzzii* (72%). Also, significant species variations were observed for seven SNPs (V402L, I1527T, L995S, A1746S, I114T, L119V and T154S). The V402L and I1527T SNPs were significantly higher in *An. coluzzii* and hybrids compared to *An. gambiae* s.s., in line with studies on *An. gambiae* s.l. populations from Ghana and other West African countries [17, 20]. However, *vgsc* A1746S was only present in *An. gambiae* s.s. mosquitoes at a frequency of 39%. Similar studies found the A1746S mutation only in *An. gambiae* s.s. populations from Ghana, Togo, Benin and Cote d'Ivoire with frequencies $\leq 52\%$ [20]. The *vgsc* T154S SNP was detected at a significantly higher frequency in *An. gambiae* s.s. (66%) compared to *An. gambiae* s.s. (12%). Understanding these species-specific variations is crucial for designing and implementing effective insecticide resistance management strategies and predicting how resistance genes will spread within mosquito populations [64].

Although most SNPs were not significantly associated with resistance phenotypes, several known resistance-linked variants (L995F, I114T, F120L, A1746S, L119V) occurred at higher frequencies in resistant *An. gambiae* s.l., suggesting a possible contribution to resistance despite the limited sample size. This aligns with previous reports linking such SNPs to pyrethroid resistance (11,19,26,76,78), and suggests that metabolic mechanisms may play a prominent role in shaping insecticide resistance in Ghanaian populations. Larger genomic studies, coupled with functional validation, will be required to identify metabolic determiners of resistance and also to understand the contribution of the novel *vgsc* variants T154S and A1553T, detected in this study at frequencies above 1%.

Conclusion

This study demonstrates the utility of multiplex amplicon nanopore sequencing as a scalable and cost-effective approach for genomic surveillance of malaria vectors. Extensive genetic variation, including both known and novel resistance-associated variants, was identified, alongside evidence of gene flow across *An. gambiae* s.l. populations in Ghana. These findings highlight the dynamic nature of insecticide resistance and underscore the need for continued genomic monitoring to inform targeted and adaptive vector control strategies.

Abbreviations

WHO
World Health Organization
VGSC
voltage-gated sodium channel
kdr
knockdown resistance
SNP
single nucleotide polymorphism
WGS
Whole Genome sequencing
Ace1
Acetylcholinesterase 1
LD
Linkage Disequilibrium
bp
base pair
ITS
Internal Transcribed Spacers
COX1
Cytochrome C Oxidase I
Mtnd4
mitochondrially encoded NADH dehydrogenases 4
IGS
Intergenic Spacer Region
SINE
Short-Interspersed Elements
gst
glutathione s transferase
IRS
Indoor Residual Spraying
LLINs
Long lasting insecticide-treated nets.

Declarations

Ethical Considerations /Consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Author Contribution

AA, SM, SC, TGC, MK, FAA-A, PFA-K and YAA were responsible for the study design, supervised the project and contributed to the writing of the manuscript. AA, KB-T, CMO-A, YA-B, ARMS and IS performed the sample collection and processing. AA, SM, EC, KBT and PVDL undertook the laboratory work and sequencing. AA and SM performed the bioinformatic analysis and statistical analysis under the supervision of SC, TGC. AA drafted the manuscript. All the authors read and approved the final manuscript.

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Data Availability

All datasets generated and/or analysed during this study are included in the manuscript. The raw sequence data generated during the current study are available in the European Nucleotide Archive (Project ID: PRJEB108525, Accession Numbers: ERS29166292– ERS29166616).

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Figures

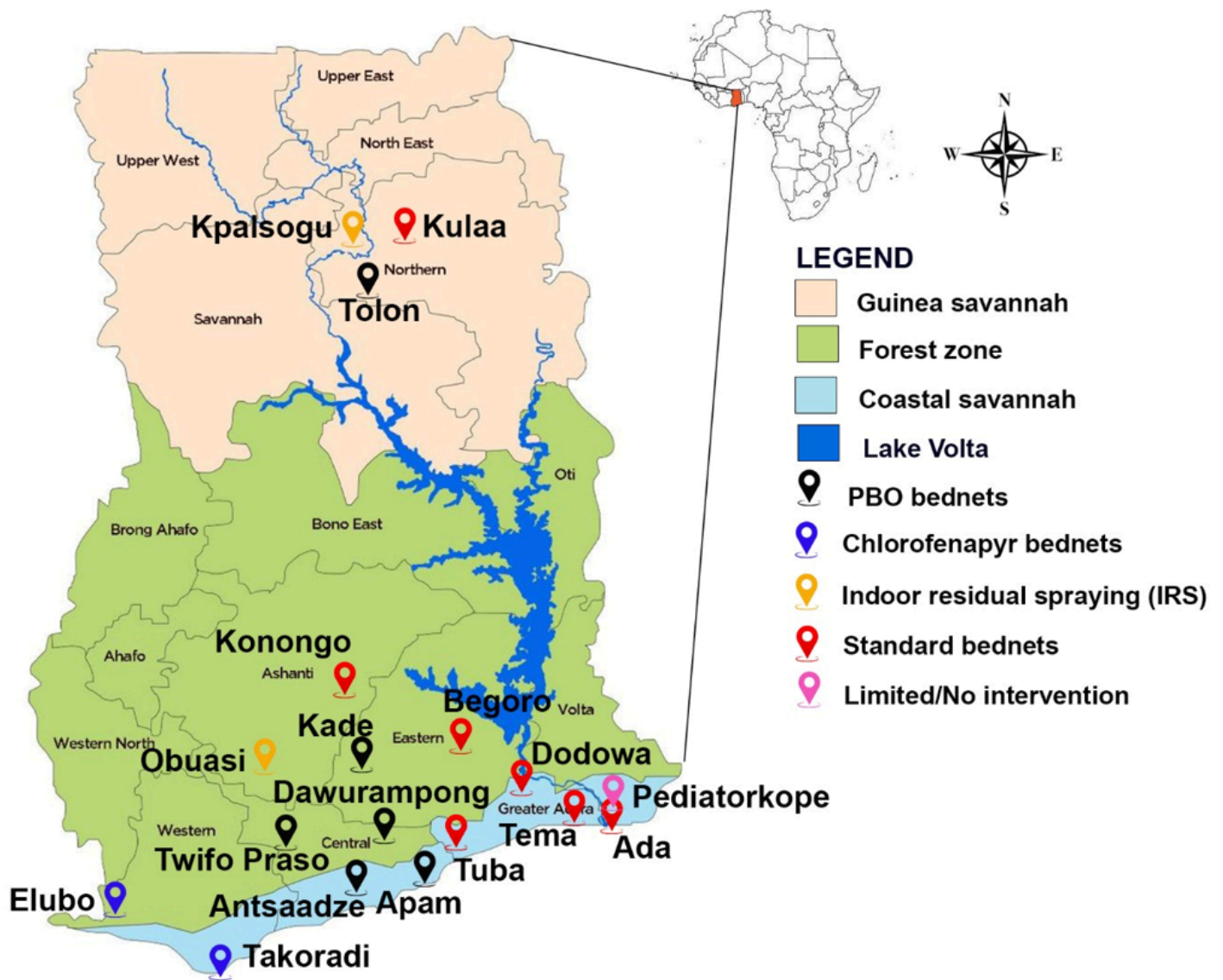


Figure 1

Entomological surveillance sites and their associated ecological zones within Ghana, including recent vector control interventions.

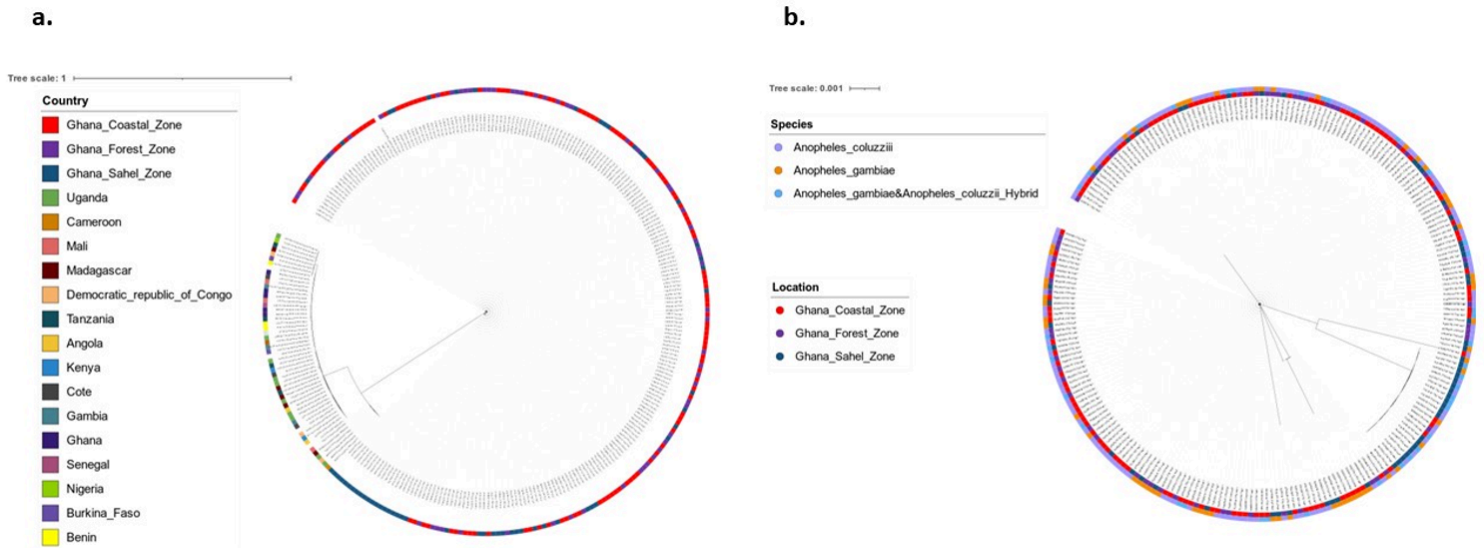


Figure 2

Maximum-likelihood tree constructed using *its2* gene sequences of *An. gambiae* s.l generated in this study (Coastal Savannah, Forest and Guinea Savannah zones of Ghana). **A.** Including publicly available *Anopheles ITS2* sequences from other African countries. **B.** *ITS2* gene sequences of *An. coluzzii*, *An. gambiae/coluzzii* hybrids, and *An. gambiae* s.s and across Ghana's ecological zones (Coastal Savannah, Forest and Guinea Savannah zones).

Ecozones	Sites	vgsc-I	vgsc-II			vgsc-III			vgsc-IV	rdl		ace-I	gste2				
		V402L	L995F	L995S	N1570Y	I1527T	F1529C	A1746S	A296G	A296S	G280S	I114T	F120L	L119V	T154S		
Coastal Zone	ADA (N= 16)	38%	63%	38%	0	38%	0	0	13%	0	56%	44%	56%	0	25%	100%	
	Dodowa (N= 20)	11%	55%	0	3%	11%	0	39%	94%	0	29%	12%	24%	32%	53%		
	Tema (N= 18)	25%	78%	0	0	22%	0	0	92%	0	44%	27%	75%	3%	11%		90%
	Pediatorkope (N= 9)	43%	67%	0	0	17%	0	0	30%	0	36%	17%	67%	6%	11%		
	Tuba (N= 10)	0	65%	0	0	33%	0	0	85%	0	30%	20%	65%	0	5%		80%
	Antsaadze (N= 11)	32%	68%	0	0	31%	0	0	59%	0	8%	15%	38%	4%	27%		
	Apam (N= 6)	0	100%	0	0	0	0	0	50%	0	17%	20%	60%	0	0		70%
	Takoradi (N= 17)	13%	61%	0	0	32%	25%	0	56%	0	17%	33%	50%	0	0		
Forest Zone	Begoro (N= 10)	17%	87%	0	6%	11%	0	28%	65%	0	40%	15%	10%	6%	65%	50%	
	Elubo (N= 17)	17%	88%	0	0	12%	12%	0	41%	0	0	38%	47%	0	30%		40%
	Twifo Praso (N= 62)	18%	85%	0%	0%	16%	8%		42%	0%	4%	28%	45%	6%	12%	30%	
	Dawurampong (N=8)	30%	70%	0	0	30%	0	0	50%	0	31%	25%	19%	38%	56%		20%
	Kade ((N= 8)	0	87%	0	0	0	0	0	50%	0	14%	33%	30%	3%	30%	10%	
	Konongo (N= 18)	34%	67%	0	0	33%	0	0	66%	0	55%	67%	75%	0	6%		0%
	Obuasi (N= 20)	18%	82%	0	0	18%	3%	8%	26%	0	6%	5%	55%	8%	13%		
	Guinea Savannah Zone	Kpalsogu (N= 19)	31%	71%	5%	0	34%	0.04	11%	32%	9%	22%	29%	11%	26%		34%
Kulaa (N= 18)		5%	53%	47%	0	0	0	15%	38%	38%	14%	8%	4%	21%	71%		
Tolon (N= 17)		0	85%	6%	0	0	0	35%	44%	3%	24%	4%	10%	54%	79%		

Figure 3

Distribution of known non-synonymous SNPs detected in *An. gambiae* s.l from 18 sites across Ghana.

Ecozones	Sites	vgsc-III	vgsc-IV	ace-1									gste2					
		A1553T	L1680F	V218L	D181E	V190A	P203A	A212T	A212V	E213D	T221A	F318Y	A185T	P174L	G171N	N148E	I126F	D148E
Coastal Zone	ADA (N= 16)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3%	0	0
	Dodowa (N= 20)	3%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Tema (N= 18)	3%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Pediatorkope (N= 9)	0	0	0	0	0	0	0	0	0	0	0	0	10%	5%	0	0	0
	Tuba (N= 10)	22%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Antsaadze (N= 11)	0	0	4%	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Apam (N= 6)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Takoradi (N= 17)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Forest Zone	Begoro (N= 10)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Elubo (N= 17)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Twifo Praso (N= 62)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.01
	Dawurampong (N=8)	0	0	0	13%	13%	13%	13%	13%	13%	13%	13%	0	0	0	0	0	1%
	Kade ((N= 8)	0	0	7%	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Konongo (N= 18)	0	13%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Obuasi (N= 20)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Kpalsogu (N= 19)	3%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3%	0
Guinea Savannah Zone	Kulaa (N= 18)	14%	0	0	0	0	0	0	0	0	0	0	9%	0	0	0	0	0
	Tolon (N= 17)	0	0	0	0	0	0	0	0	0	0	0	4%	0	0	0	4%	0

Figure 4

Distribution of novel non-synonymous SNPs detected in *An. gambiae* s.l from 18 sites across Ghana

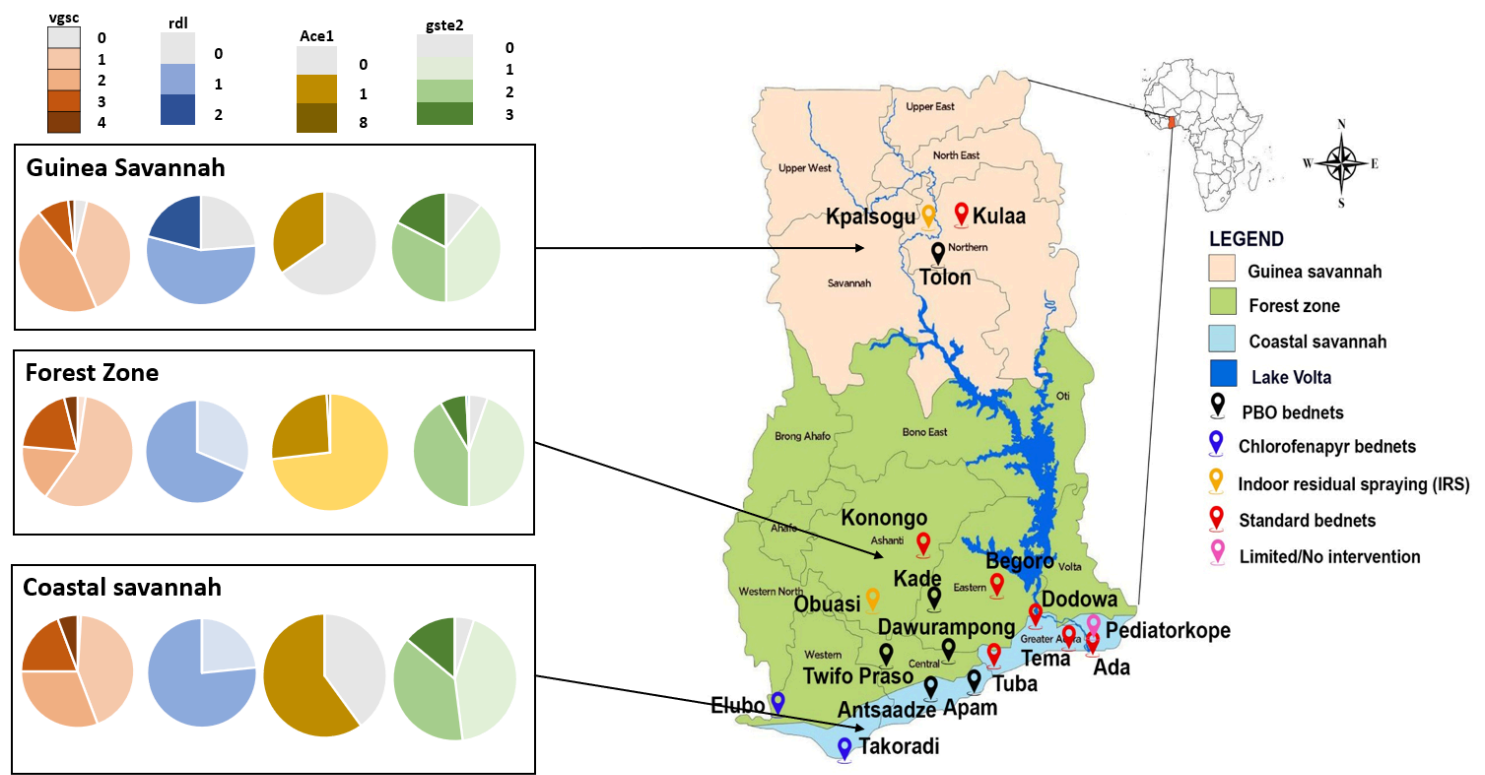


Figure 5

Distribution of SNPs in each insecticide resistance loci (*vgsc*, *rdl*, *ace1* and *gste2*) across the different ecological zones of Ghana. Map of Ghana showing sampling locations across three major ecological zones (Guinea Savannah, Forest, and Coastal Savannah zones). Pie charts represent for each insecticide resistance gene (*vgsc*, *rdl*, *Ace1*, *gste2*), the number of SNPs identified per mosquito population (0–≥4, colour-coded) and the proportion of mosquitoes in each ecological zone carrying that SNP count.

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