

Supplementary file Ngannang et al

Supplementary Figures

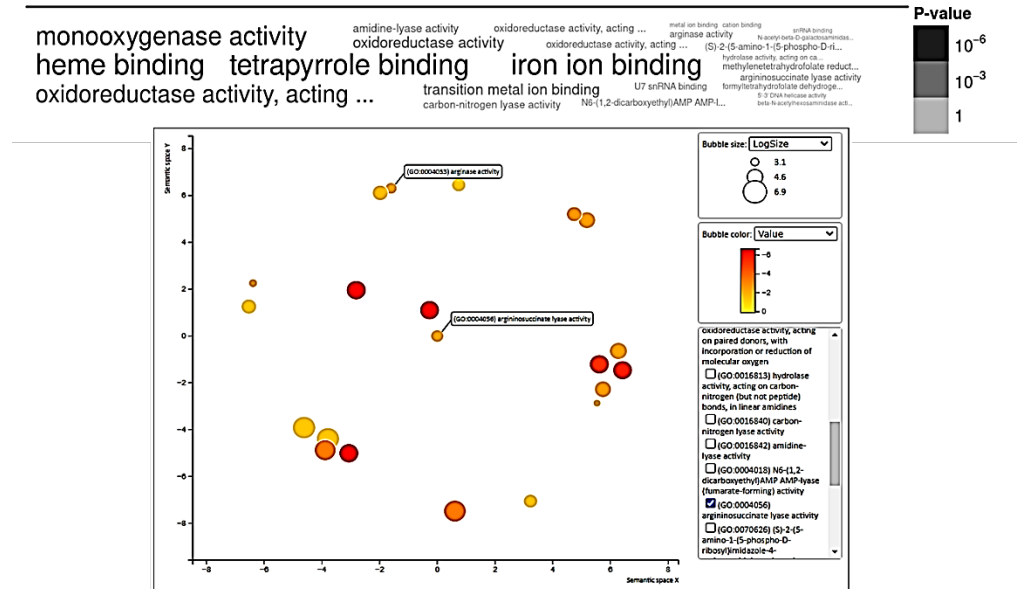


Fig. S1: Gene Ontology (GO) terms enriched in transcripts up-regulated in the same comparison as *ASL*.

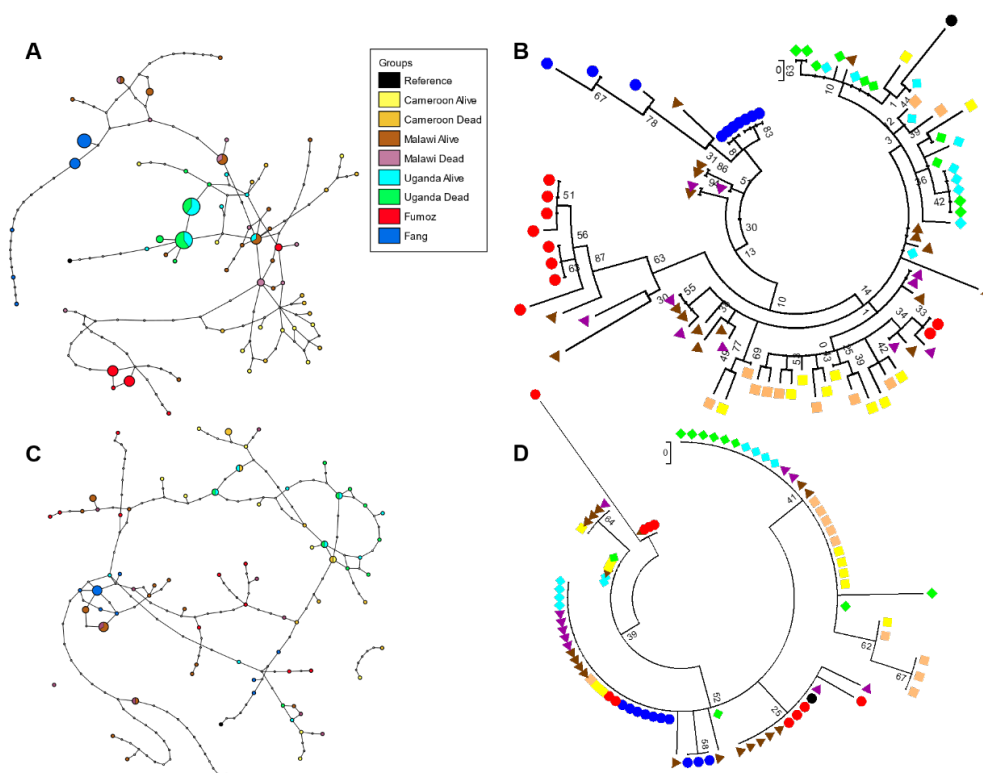
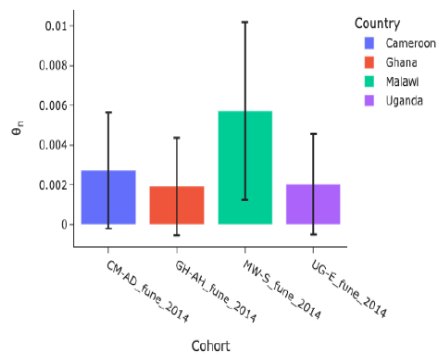
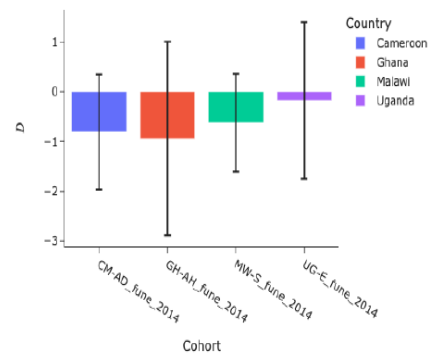


Fig. S2: Polymorphism analysis of *ASL* using retrieved sequences from Sure Select data. Analysis of the coding part **(A)** haplotype network and **(B)** Phylogenetic tree of *ASL*. Analysis of 1kb putative promoter, **(C)** haplotype network and **(D)** Phylogenetic tree of *ASL*.

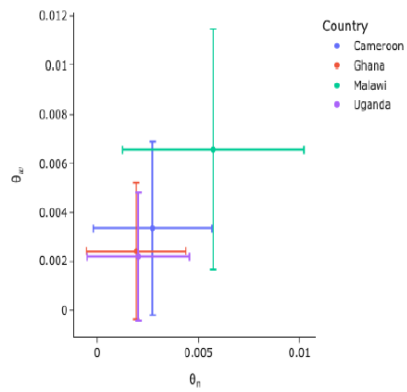
A Nucleotide diversity



B Tajima's D



C Diversity estimators



D Watterson's estimator

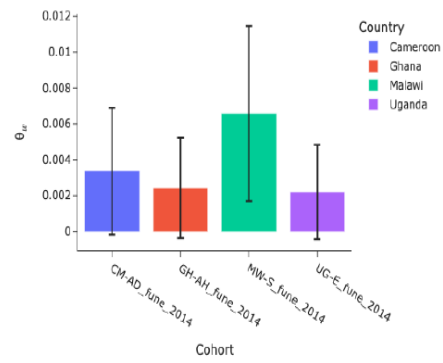


Fig. S3: Genetic diversity of *ASL* gene in *An. funestus* across Africa.

sample sets: 1231-VO-MULTI-WONDJI-VMF00043
sample query: country in ["Cameroon", "Ghana", "Malawi", "Uganda"]
genomic region: 2RL:89596355-89598161 (160 SNPs)

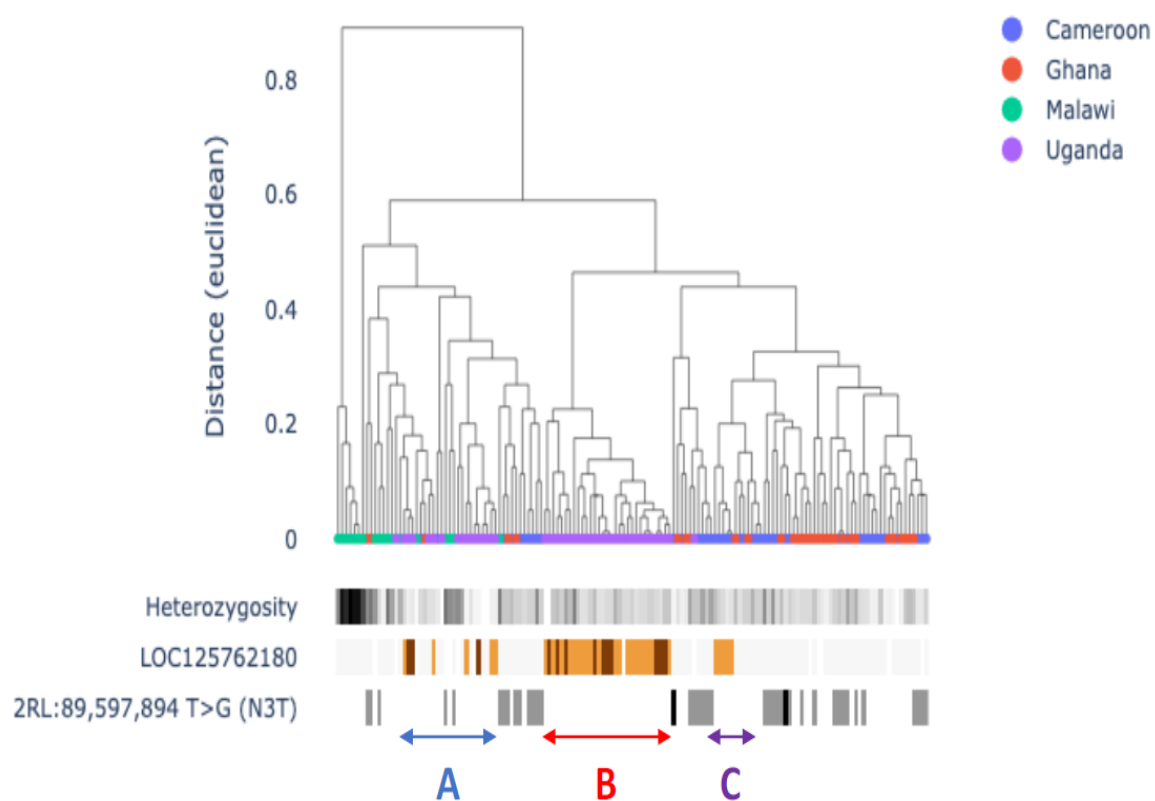


Fig. S4: DiploTYPE clustering at *ASL* gene (*LOC125762180*).

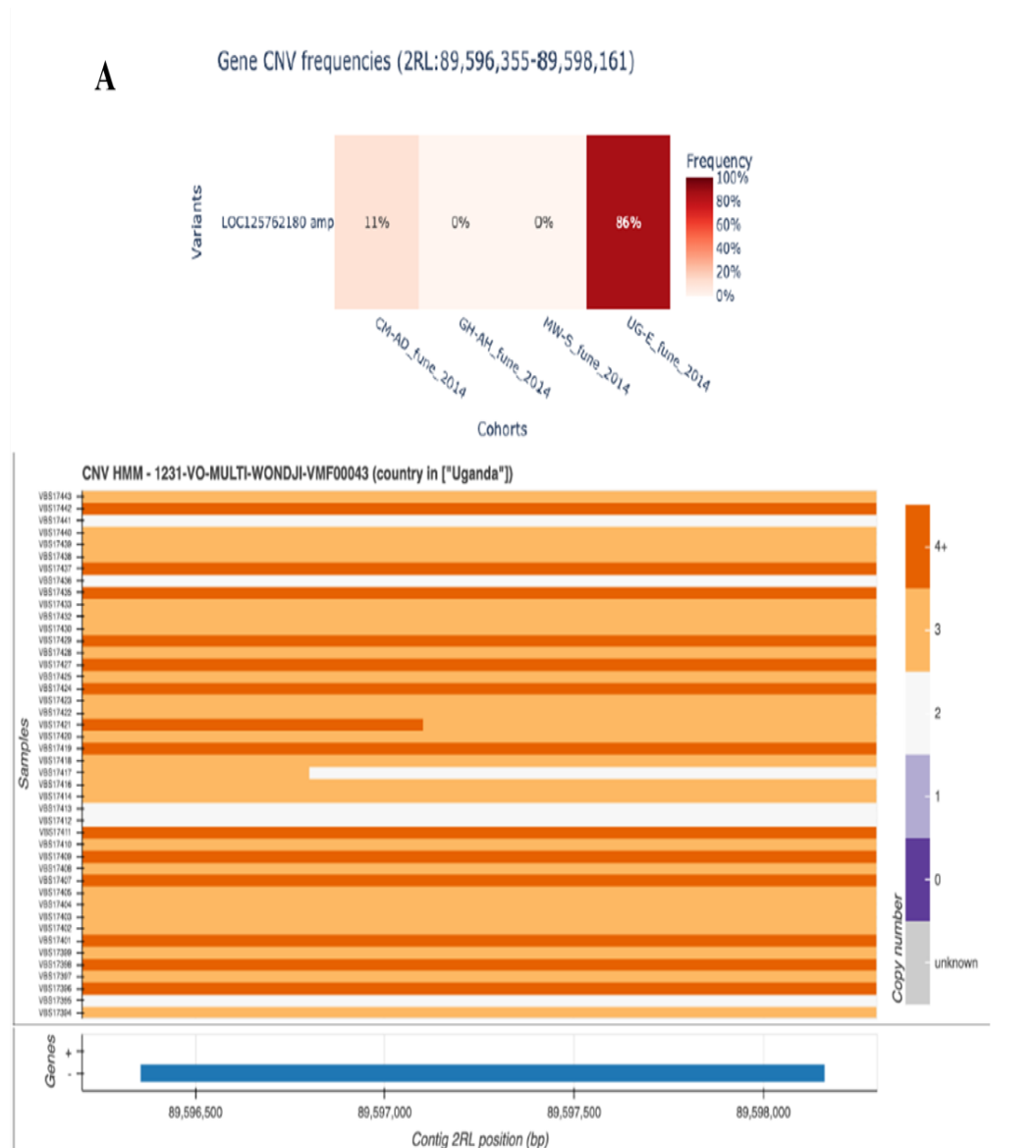


Fig. S5: Evidence of CNV in *An. funestus* *ASL* gene from Uganda. **(A)** shows the copy number allele frequency heatmap across our four samples and **(B)** indicates the CNV frequency heatmap in Uganda samples.

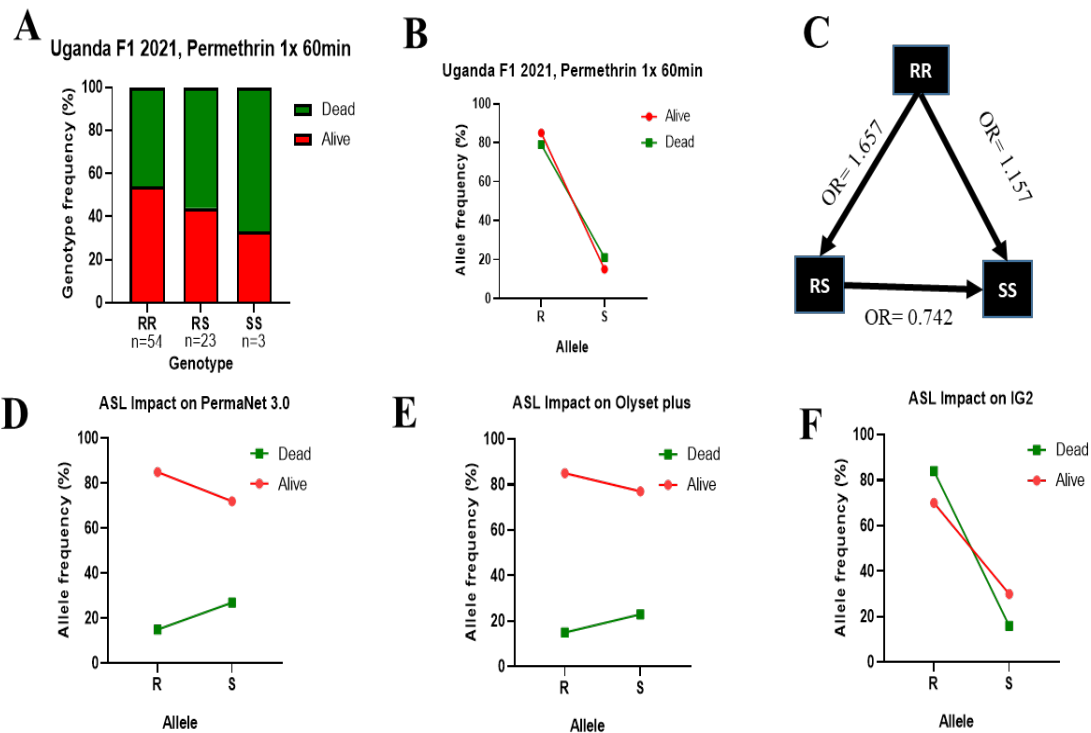


Fig. S6: Distribution of *ASL* marker. (A), Distribution of T-277A-*ASL* resistance marker among Uganda field population F1 *An. funestus* mosquitoes exposed to Permethrin for 60 mins. (B) Percentage allele frequency distribution Uganda F₁ permethylrin alive and dead female mosquitoes. (C) Estimation of odds ratio (OR) and associated significance between different *ASL* genotypes in permethylrin alive and dead Uganda F₁ female mosquitoes. (D) Percentage allele frequency distribution 2023 F1 field population in PermaNet 3.0 in experimental huts. (E) Percentage allele frequency distribution 2023 F1 field population in Olyset Plus in experimental huts. (F) Percentage allele frequency distribution 2023 F1 field population in IG2 nets in experimental huts.

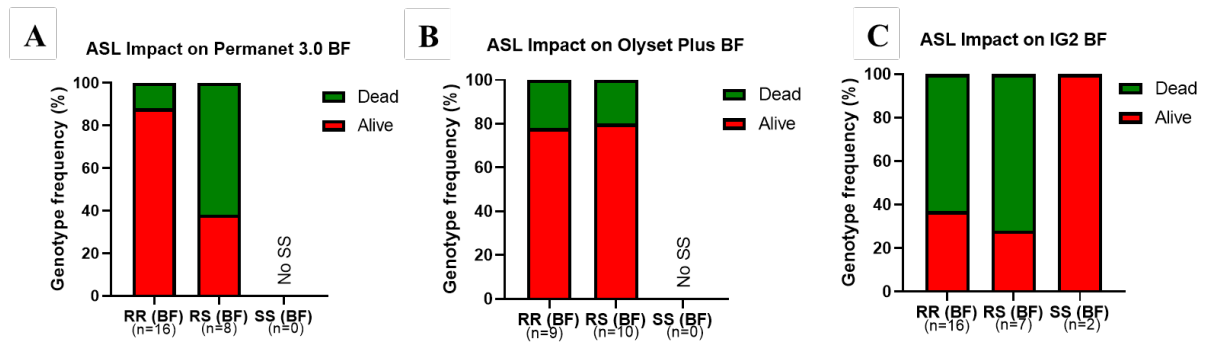


Fig. S7: Distribution of the *ASL* genotypes between alive and dead blood feed mosquitoes.
(A) Expose to PermaNet 3.0. **(B)** Expose to Olyset Plus. **(C)** Expose to IG2.

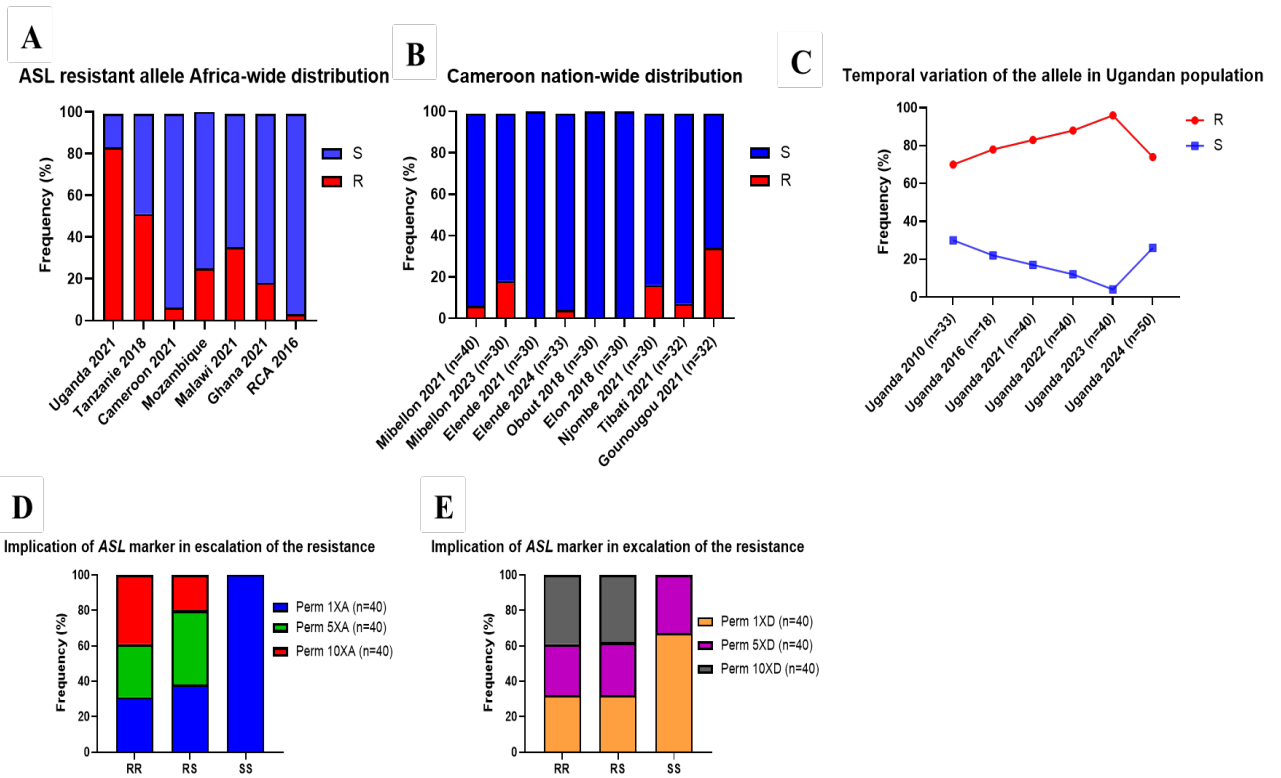


Fig. S8: Allele distribution of the marker. (A) Across Africa. **(B)** Nationwide Cameroon. **(D)** ASL marker frequency in the alive mosquitoes 1X, 5X and 10X. **(E)** ASL marker frequency in the dead mosquitoes 1X, 5X and 10X.

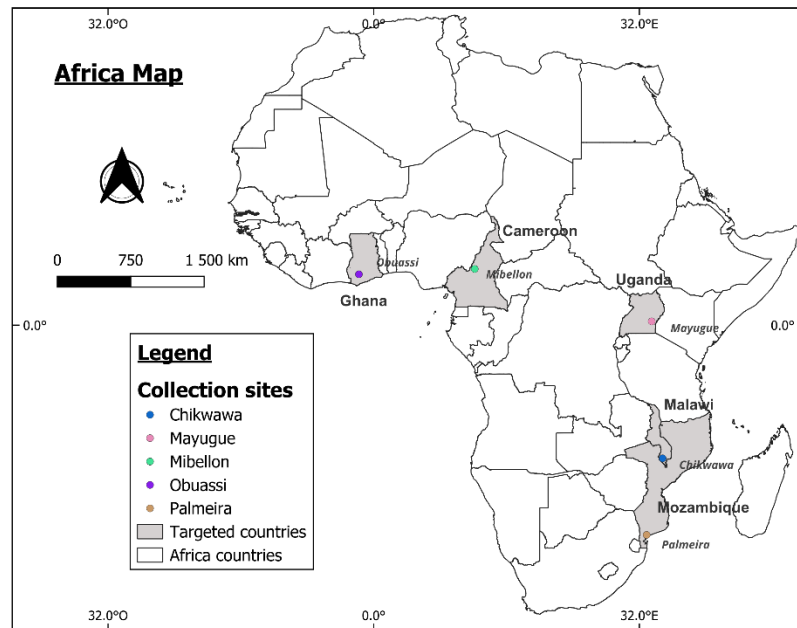


Fig. S9: Map of the different collection areas across the continent.

Supplementary Tables

Table S1: RNAseq-based expression profile of *ASL* gene in *An. funestus* across Africa

geneID	Chr	log2FC	FDR	Name	FC	Comparison
AFUN004002	AfunF3_3	0.79	0.0014***	<i>ASL</i>	1.72	C.Cam.Unx_vs_S.Fang
AFUN004002	AfunF3_3	0.74	0.0028***	<i>ASL</i>	1.67	C.Gha.Unx_vs_S.Fang
AFUN004002	AfunF3_3	0.06	0.8068ns	<i>ASL</i>	1.04	C.Mal.Unx_vs_S.Fang
AFUN004002	AfunF3_3	1.17	0.0000***	<i>ASL</i>	2.25 up	C.Uga.Unx_vs_S.Fang
AFUN004002	AfunF3_3	0.79	0.0011***	<i>ASL</i>	1.73	R.Cam.Al_vs_S.Fang
AFUN004002	AfunF3_3	0.36	0.1178ns	<i>ASL</i>	1.29	R.Gha.Al_vs_S.Fang
AFUN004002	AfunF3_3	1.51	0.0000***	<i>ASL</i>	2.85 up	R.Mal.Al_vs_S.Fang
AFUN004002	AfunF3_3	0.06	0.7976ns	<i>ASL</i>	1.04	R.Uga_Al_vs_S.Fang
AFUN004002	AfunF3_3	1.29	0.00008***	<i>ASL</i>	2.62 up	R.Mal.Al_vs_C.Mal_Unx
AFUN004002	AfunF3_3	-1.04	0.0009***	<i>ASL</i>	2.05	R.Uga.Al_vs_C.Uga_Unx
AFUN004002	AfunF3_3	-2.60	0.9999ns	<i>ASL</i>	1.00	R.Cam.Al_vs_C.Cam.Unx
AFUN004002	AfunF3_3	-0.05	0.5497ns	<i>ASL</i>	1.04	R.Gha.Al_vs_C.Gha.Unx
AFUN004002	AfunF3_3	1.04	0.0003***	<i>ASL</i>	2.05 up	C.Uga.Unx_vs_C.Mal.Unx
AFUN004002	AfunF3_3	1.39	0.00004***	<i>ASL</i>	2.62 up	R.Mal.Al_vs_R.Uga_Al

ASL refers to argininosuccinate lyase. In comparisons such as R.Uga_Al_vs_C.Uga_Unx and C.Uga.Unx_vs_S.Fang, R denotes resistant mosquitoes' post-exposure to permethrin, S represents the susceptible Fang strain, and C refers to the control group of unexposed mosquitoes. Statistical significance levels are indicated as follows: ns (not significant), * p<0.05 (significant), ** p<0.01 (very significant), and *** p<0.001 (highly significant).

Table S2: Gene Ontology (GO) terms enriched in transcripts up-regulated

TermID	Name	Value	LogSize	Frequency	Uniqueness	Dispensability	Representative
GO:0004053	arginase activity	-2.111070390454287	3.8402315949581087	0.01510673824043235	0.9160665287169059	0.03019435	null
GO:0004497	monooxygenase activity	-5.945931765556388	5.7829360542600465	1.324150025517313	0.820297237524953	0	null
GO:0004563	beta-N-acetylhexosaminidase activity	- 1.3412137379433005	4.313614159578132	0.044936052616064266	0.9423354191558742	0.14242767	null
GO:0005506	iron ion binding	-6.573509932144194	5.825053334454326	1.4589974726717228	0.8927818001116844	0.05262948	null
GO:0016155	formyltetrahydrofolate dehydrogenase activity	- 1.8117033543297874	3.0870712059065353	0.0026651246050524344	0.8079571486970744	0.6775977	null
GO:0016491	oxidoreductase activity	- 3.5063275759592094	6.705334884838153	11.074819433442528	0.9436299411908284	0.0607237	null
GO:0016645	oxidoreductase activity, acting on the CH-NH group of donors	- 2.2949276122711098	5.21037203402529	0.35430004976863727	0.8358213885411911	0.39264768	null
GO:0016646	oxidoreductase activity, acting on the CH-NH group of donors, NAD or NADP as acceptor	-2.430071610047555	5.027626815293221	0.23260797197921604	0.7657022163091626	0.37674254	null
GO:0004489	methylentetrahydrofolate reductase (NAD(P)H) activity	-2.111070390454287	4.139627248480638	0.030102156779916567	0.7846567317987181	0.79351239	16646
GO:0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	-5.459624424933764	5.842845042414387	1.5200093997482385	0.8186085141071984	0.45983527	null
GO:0016813	hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in linear amides	-1.637273035421649	4.725258066359961	0.11594492538556941	0.9098460624375921	0.5031034	null
GO:0016840	carbon-nitrogen lyase activity	-2.430071610047555	5.123570728140037	0.2901144198454826	0.81425659643575	0.03897058	null
GO:0016842	amidine-lyase activity	-2.592044618821726	4.6347391785612535	0.0941306294782033	0.7458461213298219	0.50398704	null
GO:0004018	N6-(1,2-dicarboxyethyl)AMP AMP-lyase (fumarate-forming) activity	-2.111070390454287	4.101059354908116	0.02754398639734371	0.7392197778772516	0.84831743	16842
GO:0004056	argininosuccinate lyase activity	-2.111070390454287	4.092966137594365	0.02703540815575713	0.7394374876642501	0.84432637	16842
GO:0070626	(S)-2-(5-amino-1-(5-phospho-D-ribosyl)imidazole-4-carboxamido) succinate lyase (fumarate-forming) activity	-2.111070390454287	4.067368470915269	0.02548784603865461	0.7401248826167197	0.76437877	16842
GO:0017069	snRNA binding	- 1.3412137379433005	4.6344268850476285	0.09406296456194072	0.9398156878215569	0.30901014	null
GO:0020037	heme binding	-6.626653471902241	5.829499322166181	1.4740103531250812	0.9496100823461787	0	null
GO:0043139	5'-3' DNA helicase activity	-1.418740105540949	4.337299491406797	0.04745493369242013	0.9637235543460836	0.03307989	null
GO:0043169	cation binding	-1.442069124027476	6.935587114255789	18.818669658416372	0.8962965940473413	0.24087335	null
GO:0046872	metal ion binding	- 1.4591903543178895	6.929372070476243	18.551279736738948	0.8551526303084247	0.60761832	null
GO:0046906	tetrapyrrole binding	-6.599983076521069	5.874642401587303	1.6354741225022669	0.9491128963549412	0.1375018	null
GO:0046914	transition metal ion binding	-3.432535886840426	6.454241915208814	6.212174084018391	0.8716417561018456	0.39169143	null
GO:0071209	U7 snRNA binding	-2.111070390454287	3.28397928423848	0.004195224808280737	0.9475684332841829	0.08012276	null

Table S3: Polymorphism analysis of *ASL* gene in *An. funestus* across Africa using SureSelect data.

1.4 kb coding region <i>ASL</i> gene (Sure-Select)									
Sample	N	S	h	Hd	Syn	Nsyn	π	D	D*
FUMOS	10	16	5	0.84	15	1	0.004	-0.191ns	0.556ns
FANG	10	15	5	0.80	15	0	0.041	0.606ns	0.746ns
Cameroon									
Alive	10	21	10	1	16	05	0.004	-1.115ns	-0.899ns
Dead	10	22	10	1	23	06	0.004	-1.228ns	-0.972ns
All	20	28	10	1	26	07	0.004	-1.138ns	-0.533ns
Uganda									
Alive	10	05	06	0.90	03	01	0.001	-0.279ns	-0.024ns
Dead	10	08	07	0.91	07	01	0.001	-0.618ns	-0.834ns
All	20	11	11	0.88	08	3	0.001	-1.228ns	-1.645ns
Malawi									
Alive	18	37	15	0.98	na	na	0.005	-1.251ns	-1.406ns
Dead	10	19	09	0.97	15	04	0.004	-0.670ns	-0.735ns
All	28	39	22	0.98	na	na	0.004	-1.209ns	-1.634ns
All samples from the three countries									
All alive	38	55	31	0.98	na	na	0.004	-1.783ns	-2.499ns
All dead	30	39	25	0.98	na	na	0.004	-1.584ns	-1.719ns
Total all	68	64	52	0.98	na	na	0.004	-1.843s	-2.683s

n, number of sequences; S, number of polymorphic sites; Syn, Synonymous mutations; Nsyn, Non-synonymous mutations; π , nucleotide diversity; D and D* Tajima's and Fu and Li's statistics; ns, not significant; s, significant; n.a, not applicable.

Table S4: Polymorphism analysis of *ASL* 1kb promoter region in *An. funestus* across Africa using SureSelect data.

1kb putative promoter region (Sure Select)								
Samples	N	S	h	Hd	Syn	π	D	D*
FANG	10	02	04	0.73	02	0.000	1.033ns	1.026ns
FUM0Z	10	10	07	0.93	10	0.003	-0.671ns	-0.768ns
Cameroon								
Alive	10	04	05	0.82	04	0.001	0.022ns	-0.338ns
Dead	10	04	06	0.84	04	0.002	0.686ns	0.450ns
All	20	05	06	0.77	05	0.001	0.139ns	0.387ns
Malawi								
Alive	18	08	09	0.89	08	0.002	-0.340ns	-0.294ns
Dead	10	07	07	0.91	07	0.002	0.025ns	0.377ns
All	28	08	11	0.89	08	0.002	-0.143ns	0.082ns
Uganda								
Alive	10	07	06	0.87	07	0.002	-1.002ns	-1.13149ns
Dead	10	04	08	0.96	04	0.002	1.109ns	1.23914ns
All	20	07	10	0.89	07	0.002	-0.139ns	-0.57766ns
All samples from the three comparisons								
All Alive	38	07	09	0.83	07	0.001	-0.371ns	-0.94838ns
All Dead	30	11	14	0.91	11	0.002	-0.698ns	-0.56511ns
Total all	88	16	15	0.82	16	0.0015	-1.393ns	-2.92569ns

n, number of sequences; S, number of polymorphic sites; Syn, Synonymous mutations; Nsyn, Non-synonymous mutations; π , nucleotide diversity; D and D* Tajima's and Fu and Li's statistics; ns, not significant; s, significant; n.a, not applicable.

Table S5: Genetic diversity of *ASL* from coding sequences.

Coding sequenced region (1437bp)									
Samples	N	S	h	Hd	Syn	Nsyn	π	D	D*
FUMOZ	10	21	06	0.867	16	05	0.00317	-1.82139s	-2.22572s
FANG	14	18	05	0.593	18	01	0.00457	0.40826ns	0.82563ns
Cameroon	11	11	07	0.909	10	01	0.00187	-1.21985ns	-1.01417ns
Uganda	17	08	09	0.900	04	04	0.00119	-0.98712ns	-1.33662ns
Malawi	12	45	11	0.985	n.a	n.a	0.00804	-1.02541ns	-1.47878ns
Ghana	10	13	08	0.933	12	01	0.00306	-0.19341ns	-0.26257ns
All samples from the four countries									
Total all	50	71	33	0.977	na	na	0.00572	-1.69153ns	-3.15443s

n, number of sequences; S, number of polymorphic sites; Syn, Synonymous mutations; Nsyn, Non-synonymous mutations; π , nucleotide diversity; D and D* Tajima's and Fu and Li's statistics; ns, not significant; s, significant; n.a, not applicable.

Table S6: Genetic parameters of 1kb putative promoter of *ASL* upstream of ATG region between permethrin Alive and Dead samples.

1kb putative promoter region (1080bp)								
Samples	N	S	h	Hd	Syn	π	D	D*
FANG	11	5	6	0.727	5	0.00084	-1.79107s	-2.12517s
Uganda								
Alive	13	10	7	0.830	10	0.00376	1.05928ns	1.42657s
Dead	12	30	8	0.920	30	0.00331	-0.47107ns	-0.61349ns
All	25	30	15	0.940	30	0.00768	-0.30127ns	0.16866ns
Crossing Fang/Uganda								
Alive	14	27	10	0.923	27	0.00870	0.43830ns	0.49861ns
Dead	12	31	10	0.955	32	0.00841	-0.65405ns	-0.89321ns
All	26	43	19	0.932	44	0.00858	-0.076677ns	-2.36205ns
Crossing Fang/Cameroon								
Alive	15	30	8	0.733	30	0.01548	-1.24577ns	1.16260ns
Dead	15	67	12	0.943	67	0.01986	0.17353ns	0.00590ns
All	30	85	20	0.922	86	0.00153	0.65236ns	-0.05951ns
All samples from the three comparisons								
All Alive	42	57	26	0.947	57	0.02287	-0.14500ns	-1.64435ns
All Dead	39	94	30	0.981	99	0.17609	0.17609ns	-1.08505ns
Total all	81	123	55	0.978	128	0.01905	0.76939ns	-3.08675ns

n, number of sequences; S, number of polymorphic sites; Syn, Synonymous mutations; Nsyn, Non-synonymous mutations; π , nucleotide diversity; D and D* Tajima's and Fu and Li's statistics; ns, not significant; s, significant; n.a, not applicable.

Table S7: Correlation between the T-277A-*ASL* genotypes and the ability to survive pyrethroid insecticide exposure using WHO tube assay

ASL-T-277A	Genotypes	Odds ratio (OR)	95% Confidence interval (CI)	P value
Uganda F1 Permethrin 1x	RR vs RS	1.657	0.913 to 2.945	P=0.1308
	RR vs SS	1.1569	0.022 to 60.423	P=0.9424
	RS vs SS	0.7419	0.014 to 40.247	P=0.8835
	R vs S	1.441	0.694 to 2.949	P=0.4464
Fang/Uganda F3 Permethrin 1x	RR vs RS	1.847	0.7646 to 4.439	P=0.1980
	RR vs SS	5.70	2.072 to 15.37	P=0.0007
	RS vs SS	3.089	1.523 to 6.183	P=0.0016
	R vs S	1.9329	1.0954 to 3.4110	P=0.0229
Experimental Huts				
PermaNet 3.0	RR vs RS	2.13	1.116 to 3.594	P=0.0182
	RR vs SS	0	0.6521 to 235.27	P=0.015
	RS vs SS	6.23	0.323 to 119.89	P=0.151
	R vs S	2.204	1.05 to 4.300	P=0.036
Olyset Plus	RR vs RS	1.909	1.041 to 3.345	P=0.359
	RR vs SS			
	RS vs SS			
	R vs S	1.598	0.77 to 3.299	P=0.2047
Interceptor G2 ®	RR vs RS	0.616	0.341 to 1.109	P=0.1064
	RR vs SS	0	0.002 to 0.622	P=0.0004
	RS vs SS	0	0.0033 to 1.019	P=0.0051
	R vs S	0.478	0.243 to 0.938	P=0.0283
Resistance escalation				
Perm 1X vs Perm 10X	RR vs RS	0.398	0.187 to 0.847	P=0.016
	RR vs SS	0.166	0.0078 to 3.515	P=0.249
	RS vs SS	0.408	0.0182 to 9.156	P=0.572
	R vs S	0.362	0.134 to 0.9746	P=0.044
Perm 5X vs Perm 10X	RR vs RS	0.334	0.159 to 0.701	P=0.003
	RR vs SS	0.1294	0.0065 to 2.5587	P=0.179
	RS vs SS	0.837	0.4719 to 1.4842	0.542
	R vs S	0.1543	0.0077 to 3.0876	0.222

Table S8: List of Primers used for this study.

List of Primers used		
	qPCR Primers	Product size
Forward	AAGAGTGGCATGTTCGGAGT	150bp
Reverse	GTAACCGAGCGAGCTGAGAC	
	PCR Primers	
Forward	ATGAGCAATCGTAATAATCGC	1437bp
Reverse	CTATTGTACATGCCCATCGAC	
	Primers for Screening colonie	
Forward	CCGAAAAGTGCCACCTGAACGTCTAA	2162bp
Reverse	TCCTGTCTCAGTTTCCTGAAGCTTGCTC	
	PCR Primers with restriction enzyme for Flies work	
Forward	GAATTCATGAGCAATCGTAATAATCGCATCACG	1437bp
Reverse	CATATGCTATTGTACATGCCCATCGAC	
	qPCR flies Primers	
Forward	AAGAGTGGCATGTTCGGAGT	114bp
Reverse	GTAACCGAGCGAGCTGAGAC	
	Double strand Primers	
Forward	TAATACGACTCACTATAGGGTGAACAACTCGCTTACGGTG	450bp
Reverse	TAATACGACTCACTATAGGGTCTTCGAACCGGTCAAAATC	
	1kb Promoter region Primers	
Forward	ATCTCTACCAACCGAATAGA	1080bp
Reverse	GGTGGCTTTGGAGTAACGG	
	DNA-based assay primers	
ASL AS OF	ATCTCTACCAACCGAATAGA	654bp
ASL AS OR	GGTAGCGGAAACTGGAAGGT	
ASL AS IF	TGCCAAACTCTACCGGCCCGGT	381bp
ASL AS IR	GTTACGTCGCAAAAATAGACCGCTAT	272bp
	LNAssay Probe-Based	
Forward	GACGGAATGAGGTTTTGCTTTATTT	109bp
Reverse	CGTCCACTTCTTTATCGCCTAG	
WT- Probe-HEX	ACC+GCC+A+ A +CTGGG	
MUT-Probe-FAM	ACCGCC+A+ T +CTGGG	

Table S9: Design of T-277A DNA-based assay for LNA. Sequence and LNA Probe-based TaqMan Assay design for the T-277A mutation. The probes were designed through IDT and they were able to delineate the -277A (resistant) allele from the susceptible -277T allele. The mutation is indicated in blue and bold, with primer binding sites in green and bold in the sequence.

Species:	<i>Anopheles funestus</i>
Assay target(s):	> <i>ASL</i> gene (T-277A in Promoter)
Sequence:	ACTTATATCTCTACCAACCGAATAGATGTGTGTATACCTATGTAGGTGTATAATACA ACATCATCCCGCTAAACATTTCATACACACATACGTACAAACATTATAATCCAGCCT ACTTTTCTAATTATGAAACGAAGCTATCGCGTACCGCTCTCCACGCTGATTAAAGC TATATGTACATTTTTAATATGACGTCCACAGGCAATCAAAACACCGTGACGGAAT GAGGTTTGGCTTTATTCTAGATTGGCTTCTGCCAAACTCTACCGGCCCAGT ATGGCGGTCTATTTTTCGCGACGTAACCCTAGGCCGATAAAGAAGTGGACGTTTG AGATTTTGGGGGGTCGGAAATAGTTCTAAGCTTGAAGTTTTTCGGGTTCAAACGT GTCAAGATGGTTGTTTGGCTTCTTTTCGAAAACAATCGAGGACAACGAACCTGGG TAACGATACGTAAGGGATGTAACAAGAACGGCGCAATATTAAATTCTGCTTTAGT CCAGTTTTAAAGTGTCATCCTGCATAATCCAAGTGGGCATGGGGTTTGCCATCAG TGACTTCCATTTCCGGTTCGTTCAAGTTCAAAGTCGCCAATTCTTGGAATCGTTTC GGAACGACGTCGCCAGCTGTTACGGGTACATAACCTTCCAGTTTCCGCTACCCGA TCGTACAAACTGCGTTCTGCGAAGCAATCTGATAACCTTTCTGCCAGGTTGACCT GCAGCACATCATCGTAATCGCTTATCGCACTGGCTGGGATGGGTATTTAAGTGCGC TTCCAAGCTCACCAACGTTTTTAGAAGCTCAGTGCCTCCAGCTGTTTCGGGGCCGTT GTAGTGCATAGACACAGAGAAAGCGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGC GAGAAAAAATAGTAACTCTAAACGGTTGTTAGCGAGTGAGAATTACACCAGTCT AGAAGATCTAGTGGCACCAAGTAGACTAGAAGTTCCAGTGGCACCGGGAAATCCT GATTTGGCAGCAGAGATGAGCAATCGTAATAATCGCATCACGTCCGCAACCGAGC AGGTGCAGACCCCGTTCAAGCTGTGGGGTGGCCGTTACTCCAAAGCCACCGATC ACGTCCT
Detection method:	<i>LNA Probe-based</i> <i>We want to design a probe to detect a SNP mutation.</i>
Amplicon size:	<i>90-150 bp (optional)</i>
Exon target:	<i>Assay to span all exons</i>