

Investigating Molecular Mechanisms of Insecticide Resistance in the Eastern Democratic Republic of the Congo

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Research

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Abstract

Background

Malaria vector control in the Democratic Republic of the Congo is plagued by several major challenges, including inadequate infrastructure, lack of access to health care systems and preventative measures, and more recently the widespread emergence of insecticide resistance among *Anopheles* mosquitoes. Across 26 provinces, insecticide resistance has been reported from multiple sentinel sites. However, to date, investigation of molecular resistance mechanisms among *Anopheles* vector populations in DRC has been more limited.

Methods

Adult *Anopheles gambiae* s.l. and *Anopheles funestus* s.l. were collected from two sites in Sud-Kivu province and one site in Haut-Uélé province and PCR-screened for the presence of 11 resistance mutations, to provide additional information on frequency of resistance mechanisms in the eastern DRC, and to critically evaluate the utility of these markers for prospective country-wide resistance monitoring.

Results

L1014F-*kdr* and L1014S-*kdr* were present in 75.9% and 56.7% of *An. gambiae* s.l. screened, respectively, with some individuals harbouring both resistant alleles. Across the three study sites, L43F-CYP4J5 allele frequency ranged from 0.42-0.52, with evidence for ongoing selection. G119S-*ace1* was also identified in all sites but at lower levels. A triple mutant haplotype (comprising the point mutation CYP6P4-I236M, the insertion of a partial Zanzibar-like transposable element and duplication of CYP6AA1) was present at high frequencies. In *An. funestus* s.l. *cis*-regulatory polymorphisms in CYP6P9a and CYP6P9b were detected, with allele frequencies ranging from 0.82-0.98 and 0.65-0.83, respectively.

Conclusions

This study screened the most up-to-date panel of DNA-based resistance markers in *An. gambiae* s.l. and *An. funestus* s.l. from the eastern DRC, where resistance data is lacking. We identified several new candidate markers (CYP4J5, G119S-*ace1*, the triple mutant, CYP6P9a and CYP6P9b), which are diagnostic of resistance to major insecticide classes, and warrant future, larger-scale monitoring in the DRC to inform vector control decisions by the National Malaria Control Programme.

Background

Malaria is a significant public health concern in the Democratic Republic of the Congo (DRC), where it is currently responsible for 12% and 11% of global malaria cases and deaths, respectively [1]. In the DRC, approximately 60% of the population reside in zones with an average *Plasmodium (P) falciparum* prevalence above 50% [2]; malaria is the leading cause of medical consultations, hospitalizations and death [3], accounting for 44% of all outpatient visits and 22% of deaths in children under 5 years old [4]. Malaria vector control in the DRC relies on universal coverage of insecticide-treated nets (ITNs), via mass campaigns, community-based top-ups to maintain high coverage, and continuous distributions through routine antenatal care (ANC) and child immunization services by the national expanded programme of immunization (EPI) [5]. Small-scale indoor residual spraying (IRS) is also undertaken by private enterprises (usually mining operations) in focal areas [5]. Between 2011 and 2018, an estimated 134.8 million pyrethroid-treated ITNs were distributed nationwide through such mechanisms [6]. However, recent estimates of net access and use across the country remain low, with the proportion of households with at least one ITN for every two people declining from 47% in 2013/14 to 44% in 2017/18, and proportion of children under five years old reported sleeping under an ITN the previous night also decreasing from 56% in 2013/14 to 51% in 2017/18 [7].

Malaria control in the DRC is plagued by several major challenges, including poor transportation and communication infrastructure, a majority rural population, high poverty, political and socio-economic instability, lack of access to health care systems and preventative measures for at-risk populations, and more recently the widespread emergence of insecticide resistance among *Anopheles (An.)* vector populations. Across 26 provinces in the DRC, insecticide resistance in *An. gambiae* s.l. and *An. funestus* s.l. has been reported from multiple sentinel sites [8], including reduced susceptibility to DDT, deltamethrin and permethrin in Kwilu province [9], Nord-Ubangi [10] and Sud-Kivu provinces [11], and to deltamethrin and DDT in Haut-Uélé province [12]. In Kinshasa, high intensity pyrethroid and organochlorine resistance has been observed, with *An. gambiae* populations displaying low mortality after six hours of exposure to DDT and permethrin [13], and significant proportions of vectors are capable of surviving exposure to five and ten times the diagnostic doses of alpha-cypermethrin, deltamethrin and permethrin [14]. Importantly, nationwide Demographic and Health surveys (DHS) in the DRC indicate higher levels of protection with ITNs containing deltamethrin, compared to permethrin, suggesting a partial influence of insecticide resistance on vector control intervention efficacy [15]. By comparison to pyrethroids, resistance profiles to other insecticide classes (e.g. organophosphates and carbamates) have been less clearly established, restricting the ability of the national malaria control programme (NMCP) to make evidence-based decisions for resistance management.

To date, the investigation of insecticide resistance mechanisms among *Anopheles* vector populations in DRC has been limited (summarised in Table 1). L1014F- and L1014S-*kdr* mutations are found at variable frequencies in *An. gambiae* across the country, with the former predominating in western and central provinces and the latter to the east (Table 1); a proportion of individuals have also been documented harbouring both L1014F and L1014S alleles. N1575Y is present at very low prevalence in Nord-Ubangi province [10], while other commonly described mutations, such as G119S-*ace1*, have not been detected in the DRC (Table 1). Among local *An. funestus* populations, overexpression of key detoxification enzymes (including CYP6P9a, CYP6P9b, CYP6M7, CYP6P4a, CYP6P4b and GSTE2) has been implicated in pyrethroid resistance [12, 13] (Table 1). Furthermore, increased mortality following pre-exposure of resistant *An. gambiae* populations to the synergist piperonyl butoxide (PBO) before pyrethroid bioassays, also indicates a role for metabolic resistance mechanisms in this species complex in the DRC [8, 10], supported by reports of overexpression of GSTS1-2, GSTE2, TPX2, CYP6M2 and CYP6P1 [12].

While recent next-generation sequencing initiatives have characterised substantial genetic diversity within natural *Anopheles* populations, concerns have been raised for the rapid evolution and spread of novel insecticide resistance mechanisms [16, 17]. However, real-time tracking of resistance mechanisms in the field, especially the identification of diagnostic markers that are predictive of vector control intervention failure [18, 19], is still lacking. As an intermediate step for future insecticide resistance monitoring efforts in the DRC, this study assessed *P. falciparum* infection prevalence and the frequency of 11 published insecticide resistance mutations among populations of *An. gambiae* s.l. and *An. funestus* s.l., collected from an area of pyrethroid resistance in the eastern DRC.

Table 1
Summary of all published insecticide resistance mechanism data from the DRC.

Study Year	Study Province	Study Site	Vector Species	Resistance Mechanism		References
				Target Site Mutations (R Frequency)	Detoxification Enzymes	
2009	Kinshasa Province	Kingasani	<i>An. gambiae</i> s.l.	L1014F- <i>kdr</i> (0.68) G119S- <i>ace1</i> (0.0)	-	[20]
	Equateur Province	Bolenge	<i>An. gambiae</i> s.l.	L1014F- <i>kdr</i> (0.78) G119S- <i>ace1</i> (0.0)	-	
	Kongo Central Province	Kimpese	<i>An. gambiae</i> s.l.	L1014F- <i>kdr</i> (0.95) G119S- <i>ace1</i> (0.0)	-	
	Sud-Kivu Province	Katana	<i>An. gambiae</i> s.l.	L1014F- <i>kdr</i> (0.13) G119S- <i>ace1</i> (0.0)	-	
2010	Kinshasa Province	Kindele, Kinshasa City	<i>An. gambiae</i> s.s.	L1014F- <i>kdr</i> (0.38)	-	[21]
		Kimbangu, Kinshasa City	<i>An. gambiae</i> s.s.	L1014F- <i>kdr</i> (0.33)	-	
2011-2012	Haut-Uélé Province	Kibali Gold Mine	<i>An. gambiae</i> s.s.	L1014F- <i>kdr</i> (0.1) L1014S- <i>kdr</i> (0.61) L1014F/S- <i>kdr</i> (0.26) A296G- <i>rdI</i> (0.02) G119S- <i>ace1</i> (0.0)	Overexpression of GSTS1-2, GSTE2, TPX2, CYP6M2 and CYP6P1	[12]
			<i>An. funestus</i> s.s.	-	Overexpression of CYP6M7, CYP6P9a, CYP6P9b, CYP6P4a, CYP6P4b, GSTE2 and GSTS1	
2013-2016	Sankuru Province	Lodja	<i>An. gambiae</i> s.l.	L1014F- <i>kdr</i> (0.06)	-	[8]
	Tshopo Province	Kabondo	<i>An. gambiae</i> s.l.	L1014F- <i>kdr</i> (0.99)	-	
	Lulua Province	Tshikaji	<i>An. gambiae</i> s.l.	L1014F- <i>kdr</i> (0.81)	-	
		Mikalayi	<i>An. gambiae</i> s.l.	L1014F- <i>kdr</i> (0.82)	-	
	Haut-Katanga Province	Kapolowe	<i>An. gambiae</i> s.l.	L1014F- <i>kdr</i> (0.22)	-	
	Kinshasa Province	Kingasani	<i>An. gambiae</i> s.l.	L1014F- <i>kdr</i> (0.73)	-	
2015	Kinshasa Province	Ndjili-Brasserie, Kinshasa City	<i>An. gambiae</i> s.l.	L1014F- <i>kdr</i> (0.87) L1014S- <i>kdr</i> (0.10) L1014F/S- <i>kdr</i> (0.16)	-	[13]
			<i>An. funestus</i> s.s.	A296S- <i>rdI</i> (0.10) 119F-GSTe2 (0.67)	Overexpression of CYP6P9a, CYP6P9b, CYP6M7 and GSTE2	
2015-2016	Kwilu Province	Bagata	<i>An. gambiae</i> s.s.	L1014F- <i>kdr</i> (0.61)	-	[9]
			<i>An. coluzzii</i>	L1014F- <i>kdr</i> (0.87)		
		Vanga	<i>An.</i>	L1014F- <i>kdr</i> (0.63)	-	

			<i>gambiae</i> s.s.			
			<i>An. coluzzii</i>	L1014F- <i>kdr</i> (0.77)		
		Bandundu-City	<i>An. gambiae</i> s.s.	L1014F- <i>kdr</i> (0.76)	-	
			<i>An. coluzzii</i>	L1014F- <i>kdr</i> (0.90)		
2016	Nord-Ubangi	Pambwa	<i>An. gambiae</i> s.s.	L1014F- <i>kdr</i> (0.82) L1014S- <i>kdr</i> (0.01) L1014F/S- <i>kdr</i> (0.17) N1575Y (0.01)	-	[10]
		Fiwa	<i>An. gambiae</i> s.s.	L1014F- <i>kdr</i> (0.64) L1014S- <i>kdr</i> (0.07) L1014F/S- <i>kdr</i> (0.29) N1575Y (0.01) L1014F- <i>kdr</i> (1.0)	-	
			<i>An. coluzzii</i>	L1014S- <i>kdr</i> (0.0) L1014F/S- <i>kdr</i> (0.0) N1575Y (0.13)		
		Bassa	<i>An. gambiae</i> s.s.	L1014F- <i>kdr</i> (0.79) L1014S- <i>kdr</i> (0.01) L1014F/S- <i>kdr</i> (0.20) N1575Y (0.0)	-	
2016	Nord-Ubangi	Pambwa/ Fiwa/Bassa	<i>An. gambiae</i> s.s.	L1014F- <i>kdr</i> (0.86) L1014S- <i>kdr</i> (0.14) A296G- <i>rdl</i> (0.05) G119S- <i>ace1</i> (0.0) I114T-GSTe2 (0.0) L119V-GSTe2 (0.35) N1575Y (0.01)	-	[22]
			<i>An. coluzzii</i>	L1014F- <i>kdr</i> (0.75) A296S- <i>rdl</i> (0.25) I114T-GSTe2 (0.50) G119S- <i>ace1</i> (0.0) N1575Y (0.0)		
2016	Nord-Ubangi	Pambwa	<i>An. gambiae</i> s.s.	ZZB-TE-CYP6P4-236M- CYP6AA1-Duplication (0.40)	-	[23]
		Fiwa	<i>An. gambiae</i> s.s.	ZZB-TE-CYP6P4-236M- CYP6AA1-Duplication (0.61)	-	
		Bassa	<i>An. gambiae</i> s.s.	ZZB-TE-CYP6P4-236M- CYP6AA1-Duplication (0.73)	-	
2018	Sud-Kivu Province	Tchonka	<i>An. gambiae</i> s.s.	L1014F- <i>kdr</i> (0.98) G119S- <i>ace1</i> (0.0)	-	[11]
2016- 2018	Kinshasa Province	Bu	<i>An. gambiae</i>	L1014F- <i>kdr</i> (0.78)	-	[14]

	s.s.	L1014S- <i>kdr</i> (0.0)	
		L1014F/S- <i>kdr</i> (0.22)	
Kasangulu	<i>An. gambiae</i> s.s.	L1014F- <i>kdr</i> (0.70)	-
		L1014S- <i>kdr</i> (0.08)	
		L1014F/S- <i>kdr</i> (0.23)	
Kimpoko	<i>An. gambiae</i> s.s.	L1014F- <i>kdr</i> (0.86)	-
		L1014S- <i>kdr</i> (0.0)	
		L1014F/S- <i>kdr</i> (0.14)	
	<i>An. coluzzii</i>	L1014F- <i>kdr</i> (0.33)	
		L1014S- <i>kdr</i> (0.67)	
		L1014F/S- <i>kdr</i> (0.0)	
Kingasani	<i>An. gambiae</i> s.s.	L1014F- <i>kdr</i> (0.91)	-
		L1014S- <i>kdr</i> (0.02)	
		L1014F/S- <i>kdr</i> (0.07)	
Kinkole	<i>An. gambiae</i> s.s.	L1014F- <i>kdr</i> (0.91)	-
		L1014S- <i>kdr</i> (0.02)	
		L1014F/S- <i>kdr</i> (0.07)	
	<i>An. coluzzii</i>	L1014F- <i>kdr</i> (0.0)	
		L1014S- <i>kdr</i> (1.0)	
		L1014F/S- <i>kdr</i> (0.0)	

Methods

Mosquito collections and species identification

Adult *Anopheles* mosquitoes were collected from households in two sites in Sud-Kivu province (Tchonka; 2°19'18"S 27°32'24"E and Tushunguti; 1°48'19"S 28°45'00.5"E) and one site in Haut-Uélé province (Kibali; 3°6'59"N, 29°35' 8"E) using Centers for Disease Control (CDC) light traps during the rainy season in Tchonka (100 houses: April-June 2018), Tushunguti (50 houses: December 2017-February 2018) and Kibali (25 houses: June 2019) (Figure 1). Mosquitoes were identified morphologically as members of the *An. gambiae* s.l. or *An. funestus* s.l. complexes [24]. A subset of mosquitoes, morphologically classified as *An. gambiae* s.l. (n=24) or *An. funestus* s.l. (n=16), were further identified to species-level by PCR [25, 26]. A total of 163 *An. funestus* s.l. from Tchonka (n=133) and Tushunguti (n=30) and 192 *An. gambiae* s.l. from Tchonka (n=131), Tushunguti (n=32) and Kibali (n=29) were used for insecticide resistance mutation analyses.

Plasmodium falciparum screening

Individual mosquitoes (n=355) were screened for the presence of *P. falciparum* using a SYBR green real-time assay targeting the parasite cytochrome c oxidase subunit 1 (*cox1*) mitochondrial gene (present in all stages of the *P. falciparum* life cycle) [27, 28].

An. gambiae s.l. target site mutation screening

Mosquito individuals morphologically identified as *An. gambiae* s.l. (n=192) were screened for eight mutations: L1014S-*kdr*, L1014F-*kdr*, G119S-*ace1*, N1575Y, L43F-CYP4J5, CYP6P4 (I236M), Zanzibar-like transposable element (TE) and CYP6AA1 duplication.

For L1014F-*kdr* and L1014S-*kdr* detection, the following primers were used: IPCF (5'-GATAATGTGGATAGATTCCCGACCATG-3'), AltRev (5'-TGCCGTTGGTGACAGACAAGGATG-3'), WT (5'-GGTCCATGTTAATTTGCATTACTTACGAATA-3'), West (5'-CTTGGCCACTGTAGTGATAGGAAATGTT-3') and East (5'-CTTGGCCACTGTAGTGATAGGAAAATC-3') [29]. For L1014F-*kdr*, amplifications were performed in 25 µL reactions containing 2 µL template DNA, 1 µL IPCF (2.5 pmol/µL), 1 µL AltRev (2.5 pmol/µL), 1 µL WT (25.0 pmol/µL), 3 µL West (8.0 pmol/µL), 4.5 µL H₂O, and 12.5 µL 2X Hot Start Taq PCR Master Mix (New England Biolabs). For L1014S-*kdr*, amplifications were performed in 25 µL reactions containing 2 µL template DNA, 2 µL IPCF (2.5 pmol/µL), 2 µL AltRev (2.5 pmol/µL), 2 µL WT (5 pmol/µL), 2 µL East (2.5 pmol/µL), 2.5 µL H₂O, and 12.5 µL 2X Hot Start Taq PCR Master Mix (New England Biolabs). Samples were run on a Bio-Rad T100™ Thermal Cycler, according to the following conditions: for 5 minutes at 95°C, followed by 35 cycles of 30 seconds at 95°C, 30 seconds at 59°C (L1014F) or 57°C (L1014S), and 30 seconds at 72°C, and a final extension step of 5 minutes at 72°C. PCR products were separated in 2% E-Gel™ agarose gels with SYBR Safe (Invitrogen, UK). A control band at 314 base pairs (bp) indicated a successful reaction, a band at 214 bp indicated the susceptible wild type allele, and a band at 156 bp indicated the resistant allele. No template controls (NTCs) were run in parallel for all assays.

For N1575Y, PCR reactions were prepared with 10 µL of 2X QuantiTect™ Probe PCR master mix (Qiagen, UK), a final concentration of 1 µM of forward (5'-TGGATCGCTAGAAATGTTTCATGACA-3') and reverse (5'-CGAGGAATTGCCTTTAGAGGTTTCT-3') primers, 0.5 µM of N probe (5'-HEX-ATTTTTTTCATTGCATTATAGTAC-3') and Y probe (5'-6-FAM-TTTTTTCATTGCATAATAGTAC-3'), and 2 µL template DNA for a final reaction volume of 20 µL [30]. Reaction plates were run on the Agilent Technologies Stratagene Mx3005P qPCR system, according to the following conditions: 15 minutes at 95°C followed by 40 cycles of 15 seconds at 94°C and 1 minute at 60°C. Positive controls from gDNA extraction from known *An. gambiae* s.s. with and without the N1575Y mutation were included in each run, alongside NTCs.

For G119S-*ace1*, PCR reactions were prepared with 5 µL 2X PrimeTime® Gene Expression Master Mix (Integrated DNA Technologies, UK), a final concentration of 0.8 µM of forward (5'-GGCCGTTCATGCTGTGGAT-3') and reverse (5'-GCGGTGCCGAGTAGA-3') primers, 0.2 µM of G probe (5'-HEX-TTCGGCGGCGGCT-3') and S probe (5'-6-FAM-TTCGGCGGCAGCT-3'), and 2 µL template DNA for a final reaction volume of 10 µL [29]. Reaction plates were run on the Agilent Technologies Stratagene Mx3005P qPCR system, according to the following conditions: 10 minutes at 95°C followed by 40 cycles of 10 seconds at 95°C and 35 seconds at 60°C. Positive controls from gDNA extraction from known *An. gambiae* s.s. with and without the G119S mutation were included in each run, alongside NTCs.

For L43F-CYP4J5, PCR reactions were prepared with 10 µL of 2X QuantiTect™ Probe PCR master mix (Qiagen, UK), a final concentration of 1 µM of forward (5'-AGCCTGCGCGTGTGATA-3') and reverse (5'-CTTCTTCTCTGTGGTTGTTG-3') primers, 0.5 µM of SNP-specific probes (5'-6-FAM-TTGCCGGAAGGCAGT-3') and (5'-HEX-TTGCCGAGGGCAGT-3'), and 2 µL template DNA for a final reaction volume of 20 µL [31]. Reaction plates were run on the Agilent Technologies Stratagene Mx3005P qPCR system, according to the following conditions: 10 minutes at 95°C followed by 40 cycles of 15 seconds at 92°C and 1 minute at 60°C.

Three recently designed, independent, locked-nucleic acid (LNA) probe-based PCR assays were used to genotype the point mutation CYP6P4-I236M, the insertion of the partial Zanzibar-like TE and the duplication of CYP6AA1 [23]. For CYP6P4-I236M, PCR reactions were prepared with 5 µL 2X PrimeTime® Gene Expression Master Mix (Integrated DNA Technologies, UK), 0.35 µM of each primer, CYP6P4_I236M_Forward (5'-AGTTTATGTTTGCACCACGTT-3') and CYP6P4_I236M_Reverse (5'-TCCACCGTCTCGGCACAAAC-3'), 0.2 µM of each probe, CYP6P4_I-Wild (5'-6-FAM-TTC+ATGC+C+G+ATGC-3') and CYP6P4_M-Mutant (5'-HEX-TTC+ATGC+C+C+ATGC-3'), and 2 µL template DNA for a final reaction volume of 10 µL. Reaction plates were run on the Agilent Technologies Stratagene Mx3005P qPCR system, according to the following conditions: 3 minutes at 95°C followed by 20 cycles of 15 seconds at 95°C and 30 seconds at 66°C, followed by 23 cycles of 10 seconds at 95°C, 20 seconds at 58°C and 11 seconds at 72°C. For the Zanzibar-like TE, PCR reactions were prepared with 5 µL 2X PrimeTime® Gene Expression Master Mix (Integrated DNA Technologies, UK), 0.35 µM of each primer, ZZB_Flank_A (5'-CAAAATCAATGKACACRGAGC-3'), ZZB_Flank_B (5'-CGCTACAATGAAGGAAAGTC-3'), ZZB_Int_A (5'-CATTACATGGCAGCCGTACCT-3'), 0.2 µM of each probe, ZZB_Mutant (5'-6-FAM-A+C+ATTA+CA+CTTTGT+CA+GTA-3') and ZZB_WT (5'-HEX-GATG+TT+CTTTK+T+CA+G+TATT-3'), and 2 µL template DNA for a final reaction volume of 10 µL. Reaction conditions were 3 minutes at 95°C followed by 40 cycles of 10 seconds at 95°C, 20 seconds at 58°C and 11 seconds at 72°C. For the CYP6AA1 duplication, PCR reactions were prepared with 5 µL 2X PrimeTime® Gene Expression Master Mix (Integrated DNA Technologies, UK), 0.35 µM of each primer, AA1_Dup1_ins1 (5'-CAGTGGGTACGCTCGTTAA-3'), AA1_Dup1_outs (5'-GGATCGGTTTACAGCGGACG-3'), AA1_Dup1_ins2 (5'-CATCACCTGTGCTCGCAARTT-3'), 0.2 µM of each probe, AA1_Dup1_outs (5'-Cy5-CCAT+CA+C+CGAA+CG-3'), AA1_Dup1_ins (5'-HEX-AGAA+CCTGCA+C+CAA-3') and AA1_Dup1_junct (5'-6-FAM-A+CA+AT+TAATT+G+CAT+CGG-3'), and 2 µL template DNA for a final reaction volume of 10 µL. Reaction conditions were 3 minutes at 95°C followed by 40 cycles of 10 seconds at 95°C, 20 seconds at 57°C and 15 seconds at 72°C.

An. funestus s.l. target site mutation screening

Mosquito individuals morphologically identified as *An. funestus* s.l. (n=160) were screened for three target site mutations: L119F-GSTe2, CYP6P9a and CYP6P9b.

For L119F-GSTe2, PCR reactions were prepared with 10 µL of 2X QuantiTect™ Probe PCR master mix (Qiagen, UK), a final concentration of 1 µM of forward (5'-AACAATTTTTATTCTTATTCTATTAC-3') and reverse (5'-CGACTCGATCTTCGGGAATGTC-3') primers, 0.5 µM of probes L119 (5'-5-HEX-AGGAGCGTATTCTTTCTAC-3') and 119F (5'-6-FAM-AGGAGCGTATTTTTTTCTA-3'), and 2 µL template DNA for a final reaction volume of 20 µL [32]. Reaction plates were run on the Agilent Technologies Stratagene Mx3005P qPCR system, according to the following conditions: 10 minutes at 95°C followed by 40 cycles of 15 seconds at 92°C and 1 minute at 60°C.

For CYP6P9a, PCR reactions were performed in a final volume of 25 µL, containing 2X Hot Start Taq PCR Master Mix (New England Biolabs), 1 µM of forward (5'-TCCCGAAATACAGCCTTTTCTAG-3') and reverse (5'-ATTGGTGCCATCGCTAGAAG-3') primers, and 2 µL template DNA [18]. Samples were run on a Bio-Rad T100™ Thermal Cycler, according to the following conditions: 95°C for 3 minutes, 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute; followed by 72°C for 10 minutes. Ten microlitres of each PCR product was digested by adding 1 µL 10X buffer *TaqI*, 0.2 µL *TaqI* restriction enzyme (Thermo Scientific, UK) and 3.8 µL of H₂O. Digests were incubated at 65°C for 4 hours. Digested products were separated in 2% E-Gel™ agarose gels with SYBR Safe (Invitrogen, UK). A band at 450 bp indicated the susceptible wild type allele, a band at 350 bp and 100 bp indicated the resistant allele.

For CYP6P9b, PCR reactions were performed in a final volume of 25 µL, containing 2X Hot Start Taq PCR Master Mix (New England Biolabs), 1 µM of forward (5'-CCCCACAGGTGGTAACATCTGAA-3') and reverse (5'-TTATCCGTAACCAATAGCGATG-3') primers, and 2µL template DNA [19]. Samples were run on a Bio-Rad T100™ Thermal Cycler, according to the following conditions: 95°C for 3 minutes, 40 cycles of 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 1 minute; followed by 72°C for 10 minutes. Ten microlitres of each PCR product was digested by adding 1 µL CutSmart buffer, 0.2 µL *Tsp45I* restriction enzyme (New England Biolabs, UK) and 3.8 µL of H₂O. Digests were incubated at 65°C for 4 hours. Digested products were separate in 2% E-Gel™ agarose gels with SYBR Safe (Invitrogen, UK). A band at 550bp indicated the resistant allele and two bands at 400bp and 150bp indicated the susceptible wild type allele.

For both CYP6P9a (n=8) and CYP6P9b (n=8), PCR-RFLP results for a subset of resistant and susceptible individuals were confirmed by sequencing as previously described [33]. In brief, PCR products used in the enzyme digests were submitted to Source BioScience (Source BioScience Plc, Nottingham, UK) for PCR reaction clean-up, followed by chain termination sequencing. Sequencing analysis was conducted in Geneious Prime®2021.1.1.

Data analysis

Stratagene MxPro qPCR software (Agilent Technologies) was used to produce standard curves for genotypic analysis. All statistical analyses were conducted in Stata/SE 17.0, including Pearson's Chi squared test to investigate deviations from Hardy-Weinberg equilibrium and associations between study site, presence/absence of resistance mutation and *P. falciparum* infection prevalence. Analysis of the triple LNA PCR assay to detect the point mutation CYP6P4-I236M, the insertion of the partial Zanzibar-like TE and the duplication of CYP6AA1, was conducted according to [23]. Heterozygotes and homozygotes for the CYP6AA1 duplication were differentiated by analysis of the ratio of the HEX, FAM and Cy5 Ct values: $2^{\text{Ct(Cy5)-(FAM+HEX)}}$; ratio values were then arranged in ascending order, plotted on a line graph and heterozygotes and homozygotes differentiated by a change in the line gradient.

Results

Mosquito species identification and *P. falciparum* screening

A subset of eight individual *An. gambiae* s.l. each from Tchonka, Tushunguti and Kibali and *An. funestus* s.l. from Tchonka and Tushunguti were identified to species-level by PCR; all were determined to be *An. gambiae* s.s. (24/24) and *An. funestus* s.s. (16/16), respectively. *P. falciparum* infection rate (indicative of any parasite lifecycle stage) was 11.04% (18/163) and 9.4% (21/192) among *An. funestus* s.l. and *An. gambiae* s.l., respectively. By study site, *P. falciparum* infection rate for *An. funestus* s.l. was not significantly different between Tchonka (12.03%; 16/133) and Tushunguti (6.7%; 2/30) ($\chi^2=0.717$, $p=0.397$), nor for *An. gambiae* s.l. (13.0%; 17/131 in Tchonka, 6.25%; 2/32 in Tushunguti and 6.9%; 2/29 in Kibali; $\chi^2=1.77$, $p=0.413$).

An. gambiae s.l. target site mutation screening

Mosquito individuals morphologically identified as *An. gambiae* s.l. were screened for the presence of eight mutations (Table 2 and 3). L1014F-*kdr* was present in 75.9% (142/187) of *An. gambiae* s.l. screened; 50% (71/142) were homozygous L1014F/L1014F and 50% were heterozygous L1014F/L1014S (Table 2). L1014S-*kdr* was present in 56.7% (106/187) of *An. gambiae* s.l. tested; 33.0% (35/106) were homozygous L1014S/L1014S and 67.0% (71/106) were heterozygous L1014F/L1014S (Table 2). No individuals were heterozygous for L1014F or L1014S in isolation. By study site there was a clear predominance of L1014F/L1014F (52.4%; 66/126) and L1014F/L1014S (44.4%; 56/126) in Tchonka, and L1014S/L1014S in Kibali (75.9%; 22/29) (Table 2). In Kibali, there was evidence for significant deviations from Hardy-Weinberg equilibrium for L1014S ($\chi^2=22.0$ and 27.0 ; $p<0.0001$), respectively. N1575Y was not detected in any individual tested (190/190).

L43F-CYP4J5 was identified in 83.9% (156/186) of *An. gambiae* s.l. tested; 3.8% (6/156) were homozygous and 96.2% (150/156) were heterozygous (Table 3). Across the three study sites, L43F-CYP4J5 allele frequency ranged from 0.42-0.52, with evidence for significant deviations from Hardy-Weinberg equilibrium ($\chi^2=38.26$, 28.24 and 15.75 for Tchonka, Tushunguti and Kibali, respectively; $p<0.0001$ for all). G119S-*ace1* was detected in 30.9% (59/191) of *An. gambiae* s.l. tested; 3.4% (2/59) were homozygous and 96.6% (57/59) were heterozygous (Table 3). There was no evidence for ongoing selection for G119S-*ace1* in any study site, with the resistant allele frequency ranging from 0.11-0.19 (Table 3).

For *An. gambiae* s.l., there was no significant association with presence of any resistant allele and *P. falciparum* infection for L1014F-*kdr* ($\chi^2=1.15$, $p=0.283$), L1014S-*kdr* ($\chi^2=1.75$, $p=0.186$), L43F-CYP4J5 ($\chi^2=1.30$, $p=0.254$) or G119S-*ace1* ($\chi^2=3.05$, $p=0.081$).

The triple LNA PCR assay, used to genotype the point mutation CYP6P4-I236M, the insertion of the partial Zanzibar-like TE and the duplication of CYP6AA1, identified high frequencies of the triple homozygote mutant in all study sites (Table 4). Furthermore, the double mutant CYP6P4-I236M-ZZB-TE was detected in nine *An. gambiae* s.l. from Tchonka.

Table 2
L1014F and L1014S allele frequencies among *An. gambiae* s.l. three study sites in East DRC.

Study Site (Province)	# Mosquitoes Tested*	Homozygote L1014F/L1014F	Homozygous L1014S/L1014S	Heterozygous L1014F/L1014S	Homozygous Wild Type	Allele Frequency			χ^2 Test	
						L1014F	L1014S	L1014	L1014F	L1014S
Tchonka (Sud-Kivu)	126	66	4	56	0	0.75	0.25	-	-	-
Tushunguti (Sud-Kivu)	32	4	9	14	5	0.34	0.5	0.16	1.11	0.01
Kibali (Haut-Uélé)	29	1	22	1	5	0.05	0.78	0.17	2.32	22.0

* Sample numbers adjusted to reflect non-amplifiers per assay.

Table 3
An. gambiae s.l. target site allele frequencies from three study sites in East DRC.

Resistance Mutation	Study Site (Province)	# Mosquitoes Tested*	Homozygote Mutation (RR)	Heterozygote Mutation (RS)	Homozygote Wild Type (SS)	Allele Frequency		χ^2 Test	p-value
						R	S		
N1575Y	Tchonka (Sud-Kivu)	129	0	0	129	0	1.0	-	-
	Tushunguti (Sud-Kivu)	32	0	0	32	0	1.0	-	-
	Kibali (Haut-Uélé)	29	0	0	29	0	1.0	-	-
L43F-CYP4J5	Tchonka (Sud-Kivu)	126	5	95	26	0.42	0.58	38.26	<0.0001
	Tushunguti (Sud-Kivu)	32	1	31	0	0.52	0.48	28.24	<0.0001
	Kibali (Haut-Uélé)	28	0	24	4	0.43	0.57	15.75	<0.0001
G119S- <i>ace1</i>	Tchonka (Sud-Kivu)	131	1	41	89	0.16	0.84	2.59	0.11
	Tushunguti (Sud-Kivu)	32	0	12	20	0.19	0.81	1.70	0.19
	Kibali (Haut-Uélé)	28	1	4	23	0.11	0.89	1.80	0.18

* Sample numbers adjusted to reflect non-amplifiers per assay.

Table 4
An. gambiae s.l. triple mutant genotype (CYP6P4-I236M-Zanzibar-like TE-CYP6AA1 duplication) frequencies from three study sites in East DRC.

Study Site (Province)	Homozygote CYP6P4-I236M-ZZB-TE Wild Type	Double CYP6P4-I236M-ZZB-TE Mutant	Heterozygote CYP6P4-I236M-ZZB-TE-CYPAA1 Duplication (Triple Mutant)	Homozygote CYP6P4-I236M-ZZB-TE-CYPAA1 Duplication (Triple Mutant)	CYP6P4-I236M-ZZB-TE-CYPAA1 Duplication (Triple Mutant) Frequency
Tchonka (Sud-Kivu)	0	9	4	33	0.76
Tushunguti (Sud-Kivu)	1	0	0	1	0.5
Kibali (Haut-Uélé)	0	0	1	14	0.97

An. funestus s.l. target site mutation screening

Mosquito individuals morphologically identified as *An. funestus* s.l. were screened for three mutations in metabolic genes (Table 5). L119F-GSTe2 was not detected in any individual tested (Table 5). CYP6P9a was present in 100% of *An. funestus* s.l. tested (152/152); 67.8% (103/152) were homozygous and 32.2% (49/152) were heterozygous (Table 5). In Tchonka CYP6P9a allele frequency was 0.82, with significant deviations from Hardy-Weinberg equilibrium ($\chi^2=6.59$; $p=0.01$); no evidence for ongoing selection was observed in Tushunguti (Table 5). CYP6P9b was present in 94.9% of *An. funestus* s.l. screened (131/138); 70.2% (92/131) were homozygous and 29.8% (39/131) were heterozygous (Table 5). In Tushunguti CYP6P9b allele frequency was 0.65, with significant deviations from Hardy-Weinberg equilibrium ($\chi^2=4.0$; $p=0.05$); no evidence for ongoing selection was observed in Tchonka (Table 5). Presence of the CYP6P9b resistant allele was significantly associated with *P. falciparum* infection ($\chi^2=7.03$, $p=0.008$), while presence of the CYP6P9a resistant allele was not ($\chi^2=1.39$, $p=0.238$).

Table 5
An. funestus s.l. target site allele frequencies from two study sites in East DRC.

Resistance Mutation	Study Site	# Mosquitoes Tested*	Homozygote Mutation (RR)	Heterozygote Mutation (RS)	Homozygote Wild Type (SS)	Allele Frequency		χ^2 Test	p-value
						R	S		
L119F-GSTe2	Tchonka	126	0	0	126	0	1.0	-	-
	Tshunguti	27	0	0	27	0	1.0	-	-
CYP6P9a	Tchonka	131	83	48	0	0.82	0.18	6.59	0.01
	Tshunguti	21	20	1	0	0.98	0.02	0.01	0.91
CYP6P9b	Tchonka	121	83	35	3	0.83	0.17	0.09	0.76
	Tshunguti	17	9	4	4	0.65	0.35	4.0	0.05

* Sample numbers adjusted to reflect non-amplifiers per assay.

Discussion

By comparison to neighbouring malaria-endemic countries, there is a considerable paucity of available molecular insecticide resistance data in the DRC. This study assessed the frequency of 11 resistance mutations among field populations of *An. gambiae* s.l. and *An. funestus* s.l., to provide additional information on resistance mechanisms in the eastern DRC, and to critically evaluate the utility of these markers for prospective country-wide resistance monitoring.

Because the NMCP vector control strategy relies almost exclusively on universal coverage of ITNs, high levels of pyrethroid resistance and cross-resistance to DDT are widespread, with some evidence for increasing pyrethroid resistance intensity following distribution of ITNs in parts of Kinshasa province [14]. Unsurprisingly, the pyrethroid-associated L1014F-*kdr* and L1014S-*kdr* mutations in the voltage-gated sodium channel (VGSC) are present in *An. gambiae* s.l. across the country and can be found in high frequencies in some locales [11, 14]. Prior to this study, molecular resistance monitoring had been undertaken in Kibali in 2011-2012, reporting a moderate prevalence of L1014S-*kdr* (0.61) and lower levels of L1014F-*kdr* (0.1) and L1014F/S-*kdr* (0.26) [12] (Table 1). Our dataset from 2017-19 supports these previous surveys, with L1014S-*kdr* present in 75.9% of *An. gambiae* s.l. and evidence for ongoing selection of this allele. One additional study had also been performed in Tchonka from April-November, 2018 [11], indicating almost complete fixation of L1014F-*kdr* (0.98), which was also consistent with our observations of slightly lower frequencies of this mutation (0.75), which may have risen over time under selection. The predictive association between L1014F-*kdr* and L1014S-*kdr* and resistance phenotype (i.e. survival or death following an insecticide bioassay or exposure to a vector control intervention) is not absolute [34], but both mutations are suspected to play a larger contributing role in resistance to type I (permethrin) versus type II (alpha-cypermethrin and deltamethrin) pyrethroids [35], which broadly aligns with observations of lower permethrin susceptibility, compared to deltamethrin or alpha-cypermethrin, in some local vector populations [10, 11, 14, 36, 37]. This study also demonstrated a proportion of *An. gambiae* s.l. individuals with both L1014F-*kdr* and L1014S-*kdr* mutations co-occurring in Sud-Kivu province. This phenomenon has also been observed in Kinshasa [14] and Nord-Ubangi provinces [10], as well as in other areas of East and West Africa [38, 39]. While the biological implications of harbouring both resistant alleles are unclear, it adds an additional complication to routine genotypic surveillance and supports the use of newly-developed single detection method assays [10]. A second mutation in the VGSC, N1575Y, located downstream of L1014F-*kdr*, can have a synergistic effect on pyrethroid and DDT resistance [30, 40] but was not observed in any *An. gambiae* s.l. screened. It has only been reported once previously in the DRC, at very low frequencies in *An. gambiae* s.s. and *An. coluzzii* from Nord-Ubangi [10]. The recent characterization of 20 additional non-synonymous nucleotide substitutions in the VGSC has revealed that the molecular basis of target-site pyrethroid resistance in malaria vectors may be more complex than previously thought [41]. This is of particular relevance in the DRC where high genetic diversity in *kdr* haplotypes has been described, suggesting that these resistance alleles may have either originated in central Africa and spread across the continent or converged in the DRC and persisted without replacement [22].

In all three study sites, a non-synonymous substitution in CYP4J5 was identified at moderate levels (0.42-0.52), for the first time in the DRC. The point mutation used in this assay is in tight linkage disequilibrium (LD) with the L43F-CYP4J5 variant, which has previously been associated with resistance to lambda-cyhalothrin in *An. gambiae* s.s. from Uganda and to deltamethrin in Uganda and Kenya [31]. Furthermore, this mutation has been shown to be highly diagnostic of extreme pyrethroid resistance, with survivors of two hour deltamethrin exposure significantly more likely to have L43F-CYP4J5, compared to those killed within one hour [31]. Given the significant deviations from Hardy-Weinberg expectations observed for L43F-CYP4J5 in our dataset, this marker may warrant further monitoring as a potential predictor of extreme pyrethroid resistance in the DRC. We also present the first report of G119S-*ace1* from all study sites at low frequencies (0.11-0.19), which did not appear to be under local selection at the time of sample collection. Duplication of the G119S-*ace1* mutation can enhance resistance to organophosphates and carbamates [42], including pirimiphos-methyl which is widely used in IRS campaigns [43], by reducing sensitivity to the neurotransmitter acetylcholinesterase [44]. In our study sites, limited insecticide spraying operations have been conducted, except in Kibali in the gold mining region. It is difficult to infer the emergence of G119S-*ace1* in direct response to public health insecticides; rather this may have been selected under the pressure of widespread, unregulated agricultural pesticide use [45, 46]. Finally in *An. gambiae* s.l. the presence of a triple mutant haplotype (a non-synonymous SNP in CYP6P4, an upstream insertion of a partial Zanzibar-like TE and duplication of the CYP6AA1 gene), associated with high levels of deltamethrin resistance [23], was identified at high frequencies across our study sites and represents an additional promising diagnostic marker for future surveillance of pyrethroid resistance.

Previous studies in the DRC have identified overexpression of CYP6P9a and CYP6P9b in *An. funestus* s.l. as mechanisms of pyrethroid and DDT resistance in Kinshasa and Haut-Uélé provinces [12, 13]. The presence of *cis*-regulatory polymorphisms in CYP6P9a and CYP6P9b, which drive overexpression, have been correlated with reduced efficacy of deltamethrin ITNs [18, 19]. Using two DNA-based assays, we identified moderate to high frequencies of both resistant

alleles, with evidence for ongoing selection of CYP6P9a in Tchonka and CYP6P9b in Tushunguti. For both *An. gambiae* s.l. and *An. funestus* s.l., the impact of some of these mutations on intervention effectiveness, coupled with recent data demonstrating partial restoration of pyrethroid susceptibility following PBO pre-exposure in bioassays [8, 10], improved killing of populations containing triple mutants with PBO-ITNs [23] and high mortality to the putative diagnostic doses of chlorfenapyr [47], strongly support targeted deployment of next-generation dual-active ingredient ITNs to control pyrethroid-resistant vector populations in the DRC.

Consistent with previous reports from the DRC, we detected very high *P. falciparum* infection rates, which were comparable between both *An. gambiae* s.l. and *An. funestus* s.l. across study sites (9.4-11.04%); *P. falciparum* infection prevalence was twice as high for both species in Tchonka compared to Tushunguti. Similarly high sporozoite rates in *An. gambiae* s.l. have been described from nearby Kashuga, Nord-Kivu (13.9%) [37]. While malaria transmission is known to be highly heterogeneous across the country, these observations of high *Plasmodium* infection levels in pyrethroid-resistant vector populations, which may be less responsive to standard ITNs, are of considerable concern.

Conclusion

Real-time tracking of insecticide resistance is currently limited by the lack of diagnostic markers of intervention efficacy and difficulties dissecting the relative contributions of different mechanisms to resistance phenotype, particularly those involved in metabolic resistance. This study screened the most up-to-date panel of DNA-based resistance markers for target site and metabolic resistance in *An. gambiae* s.l. and *An. funestus* s.l. populations from the eastern DRC, where resistance data is lacking. We identified several new candidate markers (CYP4J5, G119S-*ace1*, the triple mutant: CYP6P4-I236M-Zanzibar-like TE-CYP6AA1 duplication, CYP6P9a and CYP6P9b), which are diagnostic of resistance to major insecticide classes used for malaria vector control and/or reduced pyrethroid ITN efficacy, and warrant future, larger-scale monitoring in the DRC to inform vector control decisions by the NMCP.

Abbreviations

Ace1
acetylcholinesterase
ANC
Antenatal care
Cox-1
cytochrome c oxidase subunit 1
CDC
Centers for Disease Control and Prevention
CNV
copy number variant
CYP450
cytochrome-dependent monooxygenase 450
DDT
dichlorodiphenyltrichloroethane
DHS
Demographic and Health surveys
DRC
Democratic Republic of the Congo
EPI
expanded programme on immunization
GST
glutathione-s-transferase
IRB
institutional review board
IRS
indoor residual spraying
ITN
insecticide-treated net
Kdr
knock-down resistance
LD
linkage disequilibrium
LNA
locked-nucleic acid
LSHTM
London School of Hygiene and Tropical Medicine
NMCP
national malaria control programme

OR

odds ratio

PBO

piperonyl butoxide

rdl

resistance to dieldrin

SNP

single-nucleotide polymorphism

TE

transposable element

VGSC

voltage-gated sodium channel

Declarations

Ethics approval and consent to participate

The study protocol in Tshunguti and in Tchonka was reviewed and approved by the

National Ethics Committee of Health in the Democratic Republic of the Congo (approval number CNES001/DPSK/111PM/2017). For Kibali the protocol was reviewed and approved by the scientific ethics committee of the Centre de Recherche en Sciences Naturelles and of the Université Officielle de Bukavu.

Consent for publication

Not applicable

Availability of data and material

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

JB, TW and LAM designed the overall study and were responsible for data analysis and interpretation. JB, SND, CB, VB, MJ, MG, BZ, AD and JP contributed to study conception, led the entomology field activities and participated in data collection. CML, under the supervision of LAM generated the molecular data and produced Figure 1. DRC Map using QGIS Software. LAM drafted the manuscript, which was revised by co-authors. All authors read and approved the final manuscript.

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Figures

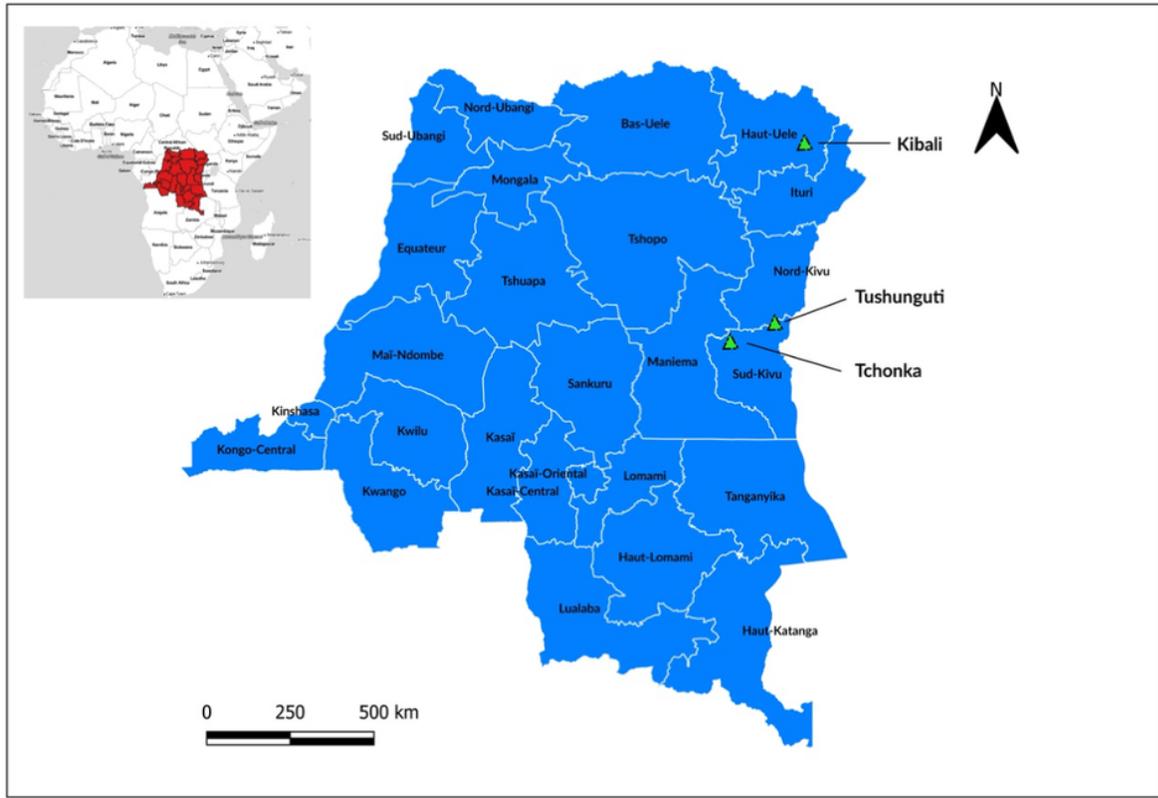


Figure 1

Map of study sites in Sud-Kivu (Tchonka, and Tshunguti) and Haut-Uélé (Kibali) provinces, in the Democratic Republic of the Congo.