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Mapping *Plasmodium falciparum* sequence variants associated with antimalarial drug resistance across populations of the Democratic Republic of Congo using 'MOSAIC' a high throughput methodology.

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Abstract

Background

Geographical differences in antimalarial drug efficacy are known to exist in the Democratic Republic Congo (DRC), and so accurate mapping of resistance is required to inform and optimise the sub-national tailoring of treatment and chemoprevention. Therapeutic efficacy studies (TES) are the gold standard for monitoring clinical and parasitological response to treatment but to perform them with adequate coverage and regularity is costly. A further limitation of efficacy studies is that resistance to one drug in a combination treatment is masked by the antiparasitic effects of its partner. Genetic surveillance for the sequence determinants of parasite resistance is therefore a useful addition to efficacy studies, enabling an affordable expansion of coverage and actionable warnings of resistance before it becomes apparent in the clinic.

Methods

We used a sampling framework based upon national sentinel surveillance sites, sampling between 100-200 patients from a single clinic location in 16 of the 26 provinces in DRC. We employed a high-throughput low-cost genotyping methodology pipeline to characterize molecular determinants of resistance and generate maps of the distribution of artemisinin and partner drug resistance across the Country. There were few observations of PfK13 validated or candidate markers of artemisinin resistance in any part of the DRC but genotype profiles for resistance to companion drugs were regionally distinct.

Results

In Eastern provinces bordering the Great Lakes Region, we found high prevalence of *Pfcrt* and Pfmdr1 markers linked to 4-aminoquinoline resistance; these are associated with a significant risk of ASAQ treatment failure. By contrast, parasite populations in the North and South of DRC predominantly carried *Pfmdr1* and *Pfcrt* haplotypes associated with risk of ALU treatment failure.

Conclusion

We extended the coverage of national drug resistance surveillance by TES using supplementary sampling and then used high throughput genotyping to describe drug resistance profiles of parasites in 16 of the 26 Provinces. We demonstrate the utility of this approach to inform subnational tailoring of malaria treatment and chemoprevention to administrative level 1.

Keywords.

Plasmodium falciparum, drug resistance, *Pfkelch13*, *Pfcrt*, *Pfmdr1*, Democratic Republic of Congo, Illumina, Sequencing.

Background

According to the World Health Organization (WHO) 94% of global malaria cases and 95% of malaria deaths occurred in Africa in 2024 (WMR2025). The Democratic Republic of Congo (DRC) remains one of the most heavily affected countries, accounting for 13.3% of cases and 11.7% of deaths in Africa (WMR 2025). As one of ten countries to adopt the High Burden to High Impact (HBHI) strategy, DRC prioritizes sub-national tailoring of malaria control interventions to accelerate the reduction in disease burden. However, with wide genetic differentiation of parasites across the country, there is a need for scalable surveillance for drug resistance that can inform treatment strategies at administrative level in near real-time. This is especially important in the context of emerging resistance to artemisinin derivatives which threatens the efficacy of artemisinin-based combinations therapies (ACTs) used in malaria treatment across Africa. Importantly, surveillance must also include markers for resistance to the partner drugs that are combined with artemisinin derivatives in ACTs, to ensure that protection from direct selection is maintained. In 2024 the recommended first line malaria treatment in DRC changed from artesunate-amodiaquine (ASAQ) and artemether-lumefantrine (ALU) to a Multi First Line Therapy (MFT) strategy. The MFT includes ASAQ and ALU, and in addition, Artesunate-Pyronaridine (AP) and Dihydroartemisinin-Piperaquine (DHAPQ) used in epidemic outbreaks, and Quinine Clindamycin in cases of intolerance and failure of ACTs. (Programme national de lutte contre le paludisme.).

Single nucleotide polymorphisms (SNPs) in the *Pfkelch13*-propeller region were first identified as the molecular basis for artemisinin resistance when they emerged in Southeast Asia in 2014 (Ariey *et al.* 2014). In Africa, early surveys found that *Pfk13*-propeller SNPs were rare, with synonymous and non-synonymous changes occurring at comparably low frequency (Menard

111 *et al.* 2016). In 2014, just 3% of samples in Africa had a non-reference *Pfk13*-propeller
112 sequence and this baseline was a fairly uniform across Africa suggesting that *Pfk13*-propeller
113 was not subject to strong directional selection in Africa at the time (Menard *et al.* 2016).

114 Since that time, novel non-synonymous SNP mutations have emerged in Africa, particularly
115 within the Great Lakes Region. Some SNPs are experimentally confirmed to be associated with
116 delayed parasite clearance and validated by WHO as markers of artemisinin resistance,
117 others are classified only as candidate or potential markers of resistance (WHO compendium,
118 WHO artemisinin information). Notable examples of validated SNP mutations emerging in the
119 Great Lakes Region include one coding for the R561H substitution. This was reported at
120 prevalences of 13% and 22% in Rwanda in 2018 (Uwimana A. *et al*. 2021, Straimer *et al.* 2022),
121 22% in Northwest Tanzania in 2022 (Ishengoma *et al.* 2024), and 23% in Southwest Uganda in
122 2022 (Conrad *et al.* 2023). Further the validated A675V was recorded at 9-30% prevalence in
123 Eastern Uganda in 2024 (Olupot Olupot *et al.* 2025) and 5% (range 0%–35.5%) in Rwanda in
124 2022-2023 (Wernsman Young *et al.* 2025). The validated C469Y reached 35% prevalence in
125 northern Uganda in 2016-2017 (Ogwang 2024), while candidate marker C469F at the same
126 codon position was reported at 40% prevalence in Southwestern Uganda in 2021-2022
127 (Conrad *et al.* 2023). Another candidate, the P441L, is also increasing, reaching prevalences
128 of 12% -23% in three districts in western Uganda in 2022 (Conrad *et al.* 2023) and 1.4% in
129 Zambia in 2023 (Aranda-Diaz *et al.* 2025).
130
131 In DRC the first observation of a validated *Pfk13* mutation was of R561H, found in a single
132 sample from Kabondo in the Tshopo Province collected in 2021 (Mesia *et al.* 2024), the
133 second, a single C469Y detected in Bukavu, South Kivu in 2022 (Van Loon *et al.* 2024). Further,

a single observation of the *candidate* resistance mutation P441L was found in 2020 in Rutshuru in North Kivu (Mesia *et al.* in 2024). All three observations were in Eastern provinces of DRC bordering the Great Lakes Region where these SNPs are already well established. The characterisation of *Pfk13* mutant lineages has been used to demonstrate the dispersal of resistance in this region (Uwimana *et al*., 2021, Bergmann C. *et al*., 2021, Straimer *et al*., 2022, Tumwebaze PK. *et al*., 2022, and Conrad MD. *et al*., 2023) and to document the dispersal of resistance across borders (Tukwasibwe *et al*. 2024).

A separate geographical hotspot for emerging resistance has appeared in Northeast Africa, where the validated SNP R622I has been detected in 22% of isolates from South-eastern Sudan in 2017 (L'Episcopia *et al.* 2025) and in 15.7% of isolates from Eritrea in 2019–2023 (Brhane *et al.* 2025). The establishment and dispersal of resistance in multiple locations bordering DRC further emphasises the need for an effective molecular surveillance framework at this time.

Molecular monitoring of partner drug resistance can provide an early warning of emerging resistance before loss of clinical efficacy is apparent (Roper *et al.* 2014). Mutations in the chloroquine resistance transporter (*Pfcrt*) gene, specifically those coding for substitutions in amino acids 72–76 have been demonstrated to confer resistance to chloroquine as well as modifying the parasites susceptibility to many other antimalarial drugs (Yuan *et al.* 2011). Mutations in the *Pfmdr1* gene first described in the context of emerging resistance to chloroquine, were subsequently associated with modified susceptibility to a range of other antimalarial drugs (Veiga *et al.* 2016).

Addressing the need for scalable resistance surveillance within modest resource availability, we combined parasite sampling within the National TES framework with supplementary sampling. The aim was to map resistance as far as possible to the level of the first administrative unit (in the DRC this unit is Province). We developed a high-throughput amplicon sequencing pipeline which is specifically designed for rapid genotyping of multiple resistance-associated loci in a high volume of samples. The *Multi-locus high through-put Oligo-based Sequencing of targeted Amplicons on Illumina Miseq for high sample capacity* (MOSAIC) described here is a genotyping pipeline established as part of the Plus Project (Home - PSI). Using the MOSAIC system, we were able to analyse a larger set of genes without a major impact on costs.

To demonstrate the utility of this approach to surveillance mapping, we here provide a detailed description of the laboratory and analytical workflow, illustrating its utility by mapping resistance to artemisinin and partner drugs in the DRC. We describe here an analysis of *Pfcrt, Pfmdr1* and *Pfk13* (both propeller and non-propeller regions) in samples collected over a five-to six-year period in sentinel sites in 16 of 26 provinces of the DRC.

Methodology

The genotyping approach described here was developed by the Plus Project, to provide National Maps of antimalarial drug resistance to inform sub-national tailoring of chemoprevention and treatment. For this we aimed to collect 100-200 dried blood spot (DBS) samples per administrative level 1 (province), using samples collected as part of the National TES sentinel surveillance framework where feasible.

This protocol describes a high-throughput genotyping pipeline processing sampled dried blood spots (DBS) through DNA extraction, diagnostic qPCR analysis, targeted amplicon sequencing, and bioinformatic analysis. The workflow enables simultaneous analysis of multiple gene fragments from large sample sets in a single sequencing run, while maintaining traceability of individual samples through fragment barcoding ("indexing"). A summary of the pipeline is shown in Figure 1. As outlined in Figure 1, the workflow spans the full analytical pipeline from sample processing through sequencing and downstream data analysis. While the core amplicon genotyping strategy follows the principles established by Nag *et al.* 017 & 2019, the laboratory and analytical steps have been adapted to support an expanded range of resistance markers, and an updated bioinformatic analysis pipeline for variant detection and quality analysis has been implemented. The genetic analysis pipeline is designed with flexibility, allowing selection of genes of interest through targeted amplification of gene fragments using outer and nested PCR. Nested PCR products originating from the same DNA plate were subsequently pooled to generate sequencing libraries. The current protocol supports analysis of fragments from *P. falciparum dhfr, dhps, crt, mdr1, exo, aat, ubp1, coronin, csp, cpmp, k13*, and mitochondrial loci, with additional targets readily incorporated as needed. A complete overview of the genes and fragments is shown in Figure 2.

While the platform enables broad multi-gene surveillance, the present study focuses primarily on *Pfmdr1*, *Pfcrt*, and *Pfk13* in the context of the DRC.

Sampling Framework.

We sought to genotype recent *Plasmodium falciparum* parasite samples from as many of the 26 DRC provinces as possible. Coverage of 16 of the 26 provinces was achieved with sample collections in each case donated by malaria patients attending a single sentinel health facility in each province. Where possible, samples collected as part of routine therapeutic efficacy studies that were conducted by the Ministry of Health. Table 1 shows the coverage of 16 of the 26 Provinces of the DRC and specifies the place of sampling, year of sampling and the study of origin. All samples were collected between 2019 and 2024.

Sample Collections.

Samples from Kalima in Maniema n=179, Vanga in Kwilu n=177 were collected by the National Malaria Control Program (NMCP) during 2023 as part of a study testing the therapeutic efficacy and safety of the artesunate-amodiaquine (ASAQ) and artemether-lumefantrine (ALU) for the treatment of uncomplicated *P. falciparum* malaria. Malaria patients aged 6-59 months with parasitaemia confirmed by microscopy, were recruited and finger-prick blood samples collected prior to treatment. *Ethical Approval was obtained in DRC (reference number ESP/CE/77C/2023), and in UK (LSHTM reference number 31393)*.

Samples from Katana in South Kivu n=69, from Kamina in Haut-Lomami n=92, and from Fungurume in Lualaba n=68, were collected from febrile malaria patients of all ages, with parasitaemia confirmed with either rapid diagnostic test (RDT) or microscopy during 2019. The samples were part of larger cross-sectional survey of molecular markers of resistance in 10 NMCP sentinel sites described in Yobi *et al.* 2022. *Ethical Approval was obtained in DRC (ESP/CE/071/2019) and UK (LSHTM reference number 31398).*

Samples from six sites were collected as part of a therapeutic efficacy study of AL and ASAQ conducted by the NMCP; Kimpese in Kongo Central (n=200) and Rutshuru in North Kivu (n=200) during 2020; Kapolowe in Haut Katanga (n=180), Kabondo in Tshopo (n=176), Mikalayi in Kasai Central (n=180) and Boende in Tshuapa (n=179) during 2021. In all sites patients aged 6-59 months with uncomplicated *P. falciparum* malaria confirmed by microscopy were recruited. *Finger prick blood* samples were collected prior to treatment. The study was previously described in Mesia *et al.* 2024. *DRC Ethical Approval (ESP/CE/69/2020).*

Samples from Inongo in Mai Ndombe n=150, Bunia in Ituri n=150, Lisala in Mongala n=150, Gemena in Sud-Ubangi n=150 and Kalemie in Tanganyika n=150 were collected in 2024 from febrile malaria patients of all ages, with parasitaemia confirmed with rapid diagnostic tests (RDTs). *Ethical Approval was obtained in DRC (reference; 496/CNES/BN/PMMF/2023), UK (LSHTM reference number 29281) and from the WHO (ERC reference number 0003949).*

MOSAIC: High-throughput sequencing pipeline.

DNA extraction and qPCR

DNA was extracted from DBS at London School of Hygiene and Tropical Medicine (LSHTM) using a high throughput robotic DNA extraction technology (QIAsymphony, Qiagen) (Robinson *et al*, .2019). DNA was stored at -20°C, at LSHTM until shipment.

An in-house developed multiplex qPCR Assay (Beshir *et al.* 2010) was used to detect *P. falciparum* DNA, with a human housekeeping gene reference (*Cytß* gene). Samples positive for the presence of *P. falciparum* DNA were shipped to the University of Copenhagen for next-

generation sequencing. Parasite genotyping for drug resistance mutations was performed at the University of Copenhagen using a high-throughput Illumina amplicon deep-sequencing workflow with custom dual-indexed primers.

PCR amplification and library preparation

In this study target regions of *Pfk13, Pfmdr1* and *Pfcrt* were amplified using an outer and nested PCR protocol optimized for Illumina MiSeq library preparation. All primer mixes were prepared at a final concentration of 2 µM by combining 960 µl nuclease-free water with 20 µl forward primer (100 µM stock) and 20 µl reverse primer (100 µM stock).

The gene-specific primers for the outer PCR have no modifications, whereas the gene-specific nested primers carry uniform overhangs which do not anneal to any genomic sequences. All primer sequences and gene references for *Pfk13, Pfmdr1* and *Pfcrt* and as well, for *Pfdhfr, Pfdhps, Pfexo, Pfaat, Pfubp1, Pfcoronin, Pfcsp, Pfcpmp* and mitochondrial loci of *P. falciparum* are listed in Supplemental table 1.

The indexing is performed, adding a unique combination of a forward and a reverse primer-sequence to a PCR reaction using the nested PCR product as templates. Multiple nested PCR products from a specific DNA sample can be pooled together before indexing. The overhangs incorporated in the nested PCR serve as annealing sites for the indexing primers. The indexing primers contain individual indices for flow cell annealing as well.

Outer PCR

Outer PCR reactions were set up in a total volume of 14 µl, consisting of 2.4 µl forward/revers primer mix (2 µM; final primer concentration 0.32 µM), 3 µl 5× AHPol HS - Ready to Load 7.5mM, 5 x Master Mix (AH diagnostics, Denmark), and 8.6 µl nuclease-free water. Finally,

one µl of template DNA was added to each reaction. Thermocycling was performed as follows: initial denaturation at 95 °C for 15 min; 40 cycles of 95 °C for 20 s, 51 °C for 2 min, and 72 °C for 2 min; followed by a final extension at 72 °C for 10 min.

Each PCR plate included the following controls: a *P. falciparum* positive control strain, a malaria naive whole-blood control, and a no-template control (master mix only). Plate ID and date were recorded for each run.

Nested PCR

Nested PCR reactions were prepared in a total volume of 19 µl, containing 1.3 µl forward/revers primer mix (2 µM; final concentration 0.13 µM), 4 µl AHPol HS - Ready to Load 7,5mM, 5 x Master Mix (AH diagnostics, Denmark), and 13.7 µl nuclease-free water. One µl of the outer PCR product was used as template. Thermocycling conditions were: 95 °C for 15 min; 30 cycles of 95 °C for 20 s, 52 °C for 2 min, and 72 °C for 2 min; and a final extension at 72 °C for 10 min.

Gel electrophoresis

A subset of each plate of nested PCR amplicons was verified by agarose gel electrophoresis on a 1.5% agarose gel containing ethidium bromide. Five µl of each nested PCR product was loaded onto the gel alongside a 100 bp Plus DNA ladder. DNA fragments were visualized using a Bio-Rad Gel Doc system with UV illumination.

Index PCR

Before index PCR, all nested PCR fragments from each sample are pooled into one or two pools. All fragments originating from the same sample are assigned the same index

combination, enabling simultaneous sequencing of multiple fragments across a large number of samples. The indexing library consists of 50 forward and 50 reverse primers, allowing for 2,450 unique index combinations.

Index PCR reactions were prepared in a total volume of 11 µl, containing 1.5 µl of each unique index primer (2 µM; final concentration 0.07 µM), 2 µl AHPol HS Ready-to-Load 7.5 mM 5× Master Mix (AH Diagnostics, Denmark), and 5 µl nuclease-free water. One µl of pooled nested PCR product was used as template. Thermocycling conditions were: 95 °C for 15 min; 20 cycles of 95 °C for 20 s, 60 °C for 1 min, and 72 °C for 2 min; followed by a final extension at 72 °C for 10 min.

Following index PCR, all reactions from a single plate were pooled into one tube, generating one combined library per plate.

Bead-based purification of indexed PCR products

Pooled index PCR products were purified using a magnetic bead–based clean-up with a bead-to-sample ratio of 0.7:1. Purification was performed in 1.5 ml low-binding tubes labelled according to each pooled library. The beads (SPRI select beads, Fischer Scientific, Denmark) were mixed thoroughly before use.

For each pool, 175 µl magnetic beads were added to 250 µl pooled PCR product (0.7× ratio). Tubes were gently inverted several times to mix and incubated on a magnetic rack for 5–10 min until the beads had fully separated from the supernatant. After magnetic separation, the supernatant was carefully removed and discarded. Beads were washed twice with 500 µl 80% ethanol, freshly made, while kept on the magnet, allowing 30 s incubation for each wash. Care

was taken to remove all residual polymerase-containing solutions. Following the second wash, tubes were opened and air-dried on the magnet for 5–10 min, or until all visible liquid had evaporated. Beads were then removed from the magnet and resuspended in 25 µl EB buffer. After 2 min at room temperature, tubes were returned to the magnet for 5 min to allow separation. A total of 20 µl clarified eluate was transferred to a new labelled low-binding tube; eluates displaying any residual green coloration were discarded due to insufficient purification.

To confirm successful amplification and absence of primer dimers, 5 µl from each pool was verified by agarose gel electrophoresis. Pools were subsequently diluted 1:10 or 1:20 depending on the number of samples and expected fragment complexity. DNA concentrations were quantified using a Qubit fluorometer with the 1× dsDNA High Sensitivity (HS) assay (Termo Fischer Scientific, Denmark) and measurements converted to nM using the formula:

$$\mathrm{nM} = \left(\frac{\mathrm{ng/\mu l}}{660}\right) \times 750 \times 10^6$$

Each pool was diluted to 4 nM using the calculated dilution factors. Finally, all 4 nM pools were mixed into a single tube to a total volume of 100 µl at equimolar concentrations, accounting for the number of samples and amplicons in each pool when combining. The concentration of the final library pool (4 nM) was verified by Qubit.

Sequencing

Pooled indexed libraries were sequenced on an Illumina MiSeq instrument using paired-end 2×300 bp chemistry. A 5% PhiX spike-in control was included to improve base-calling accuracy in low-diversity amplicon libraries. Sequencing was carried out according to Illumina standard protocols.

Analysis workflow.

Quality control and trimming

After sequencing, the data is sorted into folders corresponding to the indexing based on the original DNA plates using “Drop it” before uploading the data to online tools on the Galaxy platform. ??????) and trimming??) at a Phred score of 20.

Alignment and data processing

The Python Assimpler program (Leekitcharoenphon *et al.* 2014), developed by Ole Lund at the Technical University, Denmark (Nag *et al*. 2017), was used to align all sequences to *P. falciparum 3*D7 reference sequences (Supplemental table 1). For each sample, all information across all targeted genes was compiled into a single output file, ensuring that all sequence and alignment results for that sample were consolidated in one place.

The consolidated files were then run through R scripts developed by Max Sauerland and Helle Hansson at University of Copenhagen resulting in 3 outputs: 1) Reads for predefined known positions for fast determination of known haplotypes, 2) Reads for all positions in any of the

fragments differing from the references sequences, 3) Reads to determine fully sequenced sequences. The analysis was finalized in Excel. A minimum read depth of 50 reads per position was required for variant calling. Samples were classified as polyclonal (mixed) infections when two nucleotide variants each accounted for more than 20% of the total reads at a given position.

Dataanalyses of CSP-and CPMP markers

Following the MiSeq run, sequencing data for the genes CSP and CPMP were analyzed using SeekDeep (v3.0.1) (Hathaway *et al.* 2018). All CSP and CPMP plates were processed together and treated as technical replicates within a single run. The standard workflow of SeekDeep (extractorPairedEnd, qluster and processClusters) was executed through customized Bash scripts. Default SeekDeep settings were applied, including “strict errors” filtering and a predefined Illumina error profile (Hathaway *et al.* 2018). Additional filtering was performed in R-Studio (2025.9.0.387). Haplotypes supported by > 50 reads within a sample and representing > 1% of the total population were retained from further analysis.

Results

Sequence analysis of *Pfkelch 13:* Propeller Domain.

For the complete sequencing of *PfK13*, the gene was sub-divided into five PCR amplified fragments, with fragments 4 and 5 covering the propeller domain. Of the 2323 samples analysed, 1807 samples (77.8%) were successfully sequenced for fragment 4 and 1802 (77.6%) for fragment 5.

373 The map in Figure 3A lists the non-synonymous propeller mutations seen in each of 16
374 provinces. Across all propeller domain sequenced, non-synonymous mutations were
375 recorded at 59 codon positions, the majority (n=43) were seen only once. Only three SNPs
376 listed by WHO as validated, candidate or potential markers of artemisinin partial resistance
377 were seen, and these are indicated in Figure 3A by an asterisk. R561H (validated) in Tshopo,
378 P441L (candidate) in North-Kivu were each observed once (previously reported in Mesia *et al.*
379 2024). The Q613E (n=6) which is a WHO potential candidate, was found in 4 provinces with
380 multiple observations in Kwilu province (n=3).

381 To compare Provinces, we took the number of non-synonymous SNPs recorded at each site
382 and expressed it as a percentage of the number of samples successfully sequenced. We used
383 this to classify Provinces into broad prevalence categories which are indicated by colour
384 shading in the map in figure 3A. The province with highest prevalence was Tanganyika where
385 9 non-synonymous SNPs were observed among the 80 samples sequenced (11.2%) (shaded
386 dark brown in the map). Haut-Lomami. Tshuapa, Mongala, Sud-Ubangi, Tshopo, North Kivu,
387 and Mai-Ndombe ranged between 6.0% and 8.2% (shaded orange) and Maniema, Kasai-
388 Central, Kwilu, Kongo-Central, and Haut-Katanga ranged between 2.4% and 5.0% (shaded
389 yellow). In Ituri, South Kivu and Lualaba samples, no non-synonymous mutations in the
390 propeller region were observed (0%) (shaded green).

391 Non-synonymous propeller SNPs that were seen multiple times are indicated in Figure 3A by
392 colour text. The A578S (n=16) was seen in 5 provinces with Haut-Katanga (n=5) and Tshopo
393 (n=6) the most abundant. S695T was observed once each in four different provinces, and
394 A621T observed three times, in three different provinces. V568M was observed three times

395 in 2 provinces (two observations in Haut-Lomami, one in Sud Ubangi). Eleven mutations were
396 observed twice, all as single observations in two different provinces.

397 Concerning synonymous SNPs in the propeller region, we observed 35 unique SNPs.
398 Approximately half of synonymous SNPs were observed only once (n=18) while others were
399 observed multiple times, for example R471R (n=19), S681S (n=14), C469C (n=10), and T478T
400 (n=5). A full list of the synonymous and non-synonymous SNPs recorded in the propeller
401 domain in each of the 16 Provinces is provided in Supplementary table 2.

402

403 **Sequence analysis of *Pfk13:* the N-terminal, BTB/POZ domain.**

404 Sequence analysis of the *Pfk13* gene N-terminal, BTB/POZ domain was carried out in samples
405 from 10 Provinces by amplification and sequencing of 3 fragments. Of the 1399 samples
406 analysed, successful sequencing result was achieved for 64.4% (N=901) for fragment 1, 69.8%
407 (N=977) for fragment 2, 72.3% (N=1011) for fragment 3. The non-synonymous SNPs recorded
408 in samples from each province are shown in the map in Figure 3B and all synonymous and
409 non-synonymous SNPs in the N-terminal BTB/POZ domain are listed by Province in
410 Supplementary Table 3 with prevalence of each SNP indicated as a percentage of the number
411 of successfully sequenced samples for the relevant fragment.

412 Most non-synonymous SNPs in the N-terminal, BTB/POZ domain were observed only once.
413 Of the total of 73 different non-synonymous mutations seen, 53 were just a single
414 observation. A notable exception was the K189T, which was seen in all Provinces in a total of
415 304 observations. The geographical trends in prevalence of K189T is shown in the colour

shaded map in Figure 4. Other common polymorphisms included K189N (n=30), R255K (n=30), and L258M (n=10).

Of the 33 synonymous mutations observed in the N-terminal, BTB/POZ domain, 7 were seen more than once, with the most prevalent being S74S (n=5), and L119L (n=18), and P471P (n=10), (Supplementary table 3).

Comparing synonymous and non-synonymous SNPs in the propeller vs N-terminal, BTB/POZ domains of *Pfkelch 13*.

Sequence data for both the propeller and N-terminal, BTB/POZ domains was available in 10 Provinces. Across all propeller domain sequences, non-synonymous and synonymous SNPs were found in equal numbers. By contrast non-synonymous SNPs were 10-fold higher than synonymous SNPs in N-terminal BTB/POZ domain sequences. The reason for this large disparity is the K189T polymorphism which was common across all Provinces. The map in Figure 4 shows the geographical distribution of the *Pfk13* K189T variant across the 10 provinces for which data was available. It is clear the highest prevalence was in the North (Mongola, Sud-Ubangi) (20.5%; 116/565), followed by the West (Kwilu, Mai-Ndombe) (16.9%; 131/773) and East (Maniema, South Kivu, Ituri, Tanganyika) (15.0%; 176/1177), whereas in the South (Haut-Lomami, Lualaba) diversity was lower (6.7%; 44/652). The counts and prevalences of non-synonymous mutations and synonymous propeller domain SNPs in the 4 regional areas are listed in supplementary tables 4A and 4B and counts and prevalences of non-synonymous mutations and synonymous N-terminal BTB/POZ domain SNPs in the 4 regional areas are listed in supplementary tables 4C and 4D.

Several cases were observed where there was a 'double mutant' *Pfk13* haplotype – some of these involved combinations of the non-propeller mutation K189T with a propeller mutation, for example K189T/Q613E and K189T/V568M, although these were often detected in mixed infections. *Pfkelch13* haplotypes carrying multiple SNPs are rare; however, one previous study from Rwanda reported a haplotype combining K189T and R561H (Fankem *et al.* 2025). In contrast, the single sample carrying R561H in the present study did not also contain the K189T mutation. Further work is needed to investigate haplotypes carrying multiple mutations and to assess potential associations between these mutations.

Sequence analysis of *Pfcrt* and *Pfmdr1*.

The sequencing of *Pfcrt* including codons 72-76 required amplification of a single fragment while sequencing of *Pfmdr1* including codons 86-184-1034-1042-1246 required amplification of 2 fragments. Detailed results for each codon in both genes are shown in Supplementary table 5. Data from six sites published previously in Mesia *et al.* 2024 are incorporated with the new data from 10 new sites in this overall analysis of 16 provinces.

To summarise haplotypes of *Pfcrt* and *Pfmdr1* mixed genotype infections were excluded. The full haplotype for *Pfcrt* and *Pfmdr1* was assembled in 73.0% (N=1695) and 53.8% (N=1250) of the samples collected, respectively. Polymorphisms at *Pfcrt* consisted of two primary haplotypes, CVIET (with 76T conferring chloroquine resistance) and CVMNK (wildtype). The proportion of samples with each haplotype are shown in the map in Figure 5 and Supplementary table 5. The highest frequency of the CVIET haplotype was seen in North Kivu (84.2%, N=117) and South Kivu (94.1%, N=48); provinces that form part of the Great Lakes region. Ituri, North of the Kivus had a lower prevalence at 17.8% (N=16) comparable to

Tshuapa located further to the West with 16.1% (N=25). The western provinces Kongo Central and Kwilu also displayed a relatively high proportion of CVIET (29.0%, N=45 and 20.9%, N=33, respectively) compared to the other provinces which had a prevalence of the CVIET haplotype below 10%.

Prevalences of mutations at *Pfmdr1* codons 86, 184, 1034, 1042, and 1246 and counts of *Pfmdr1* haplotypes are listed in Supplementary table 5. The 86Y (conferring chloroquine resistance) was at highest prevalence in South Kivu (50%) and Kwilu (43.5%). *Pfmdr1* haplotype distribution is mapped in Figure 6. The most common *Pfmdr1* haplotypes were NFSND (34.2%, N=428), highest in Sud-Ubangi (53.3%, N=8) and Tshuapa (47.0%, N=55), and NYSND (54.7%, N=684) most prevalent in Lualaba (86.5%, N=32) and Mongala (79.0%, N=15). The D1246Y was found predominantly in the East of the Country where it occurred in combination with N86Y. The haplotype YYSNY was seen in North Kivu (12.1%) and South Kivu (10.8%). and haplotype NYSNY in North Kivu (9.1%) and South Kivu (2.7%).

Discussion

In this study, we adopted an operational approach which combined parasite sampling at National resistance surveillance sentinel clinics with a scalable high-throughput genotyping platform called MOSAIC. We show that sampling only 100-200 malaria patients from one sentinel clinic per province can give broad indication of the major geographical trends in partner drug resistance. A limitation of our study is that not all 26 Provinces were covered, and that samples were collected over a six-year period (2019-2025). Better spatial precision

in mapping to first administrative level would be achieved by sampling in every Province simultaneously in the same year.

We found very few PfK13 confirmed markers of artemisinin resistance, this observation is consistent with other molecular surveillance studies in the DRC. Despite the lack of PfK13 confirmed markers of artemisinin resistance, TES studies have raised concern about efficacy of ACTs in some specific sites. Therapeutic efficacy studies of ALU and ASAQ conducted at five NMCP sentinel sites across the DRC in 2017-2018 showed clinical effectiveness in all but two sites (Moriarty *et al*. 2021). In Kasai Central the efficacy of ALU was below the minimum threshold set by the WHO and in North Kivu efficacy of ASAQ was below that threshold (Moriarty *et al*. 2021). The wildtype *Pfmdr1* N86 and wildtype *Pfcrt* K76, are both associated with parasite tolerance to Lumefantrine and were found at high frequency in Kasai Central, while mutations *Pfmdr1* 86Y and *Pfcrt* 76T both associated with increased risk of ASAQ treatment failure, were highest in North and South Kivu where reduced ASAQ efficacy was reported (Moriarty *et al*. 2021).

The recent adoption of Multi First Line (MFT) strategy in 2024 in the DRC is designed to address concerns about selection pressure for resistance to artemisinin derivatives and promote antimalarial stewardship. Our surveillance shows a high prevalence of both *Pfmdr1* 86Y and *Pfcrt* 76T in North and South Kivu suggesting ASAQ treatment is at risk, and ALU will be effective in these Provinces. Elsewhere in the Country wildtype *Pfmdr1* N86 and wildtype *Pfcrt* K76 predominate particularly in the North and South. In these areas, ALU treatment efficacy is at risk, and use of ASAQ is advised.

The North Kivu and South Kivu Pfmdr and Pfcrt profiles are similar to that described in Burundi where 84% CVIET was reported recently (Niyomwungere 2026). In Uganda Tanzania or

Rwanda, it is not as high (Wernsman Young 2025). We found a comparatively high prevalence of Pfmdr1 haplotypes containing the 86Y in Eastern DRC and this is comparatively unique in the Great Lakes Region (Wernsman Young 2025). The *Pfmdr1* 86Y and *Pfcrt* CVIET are both elevated in the same provinces, which is consistent with their co-selection, most likely through the use of 4-aminoquinolines in treatment.

Pfk13 diversity in the wider geographical context.

Eastern DRC borders with areas where WHO validated and candidate markers of *Pfk13* artemisinin (partial) resistance are well established (Uwimana A. *et al.* 2021. Straimer *et al.* 2022, Ishengoma *et al*. 2024, Conrad *et al*. 2023, Olupot Olupot *et al.* 2025, Wernsman Young *et al*. 2025, Aranda-Diaz *et al*. 2025). Of all the WHO validated and candidate markers found in the Great Lakes region in this country-wide analysis we observed only one R561H and one P441L (both were previously reported in an earlier publication (Mesia *et al* 2024)). The potential marker Q613E we observed at low prevalence in four provinces (North Kivu (0.6%), Tanganika (1.2%), Kwilu (2.1%), Kongo Central (0.6%)). We did not observe any further cases of the validated C469Y previously reported in a single isolate from Bukavu, South Kivu in 2022 (Van Loon *et al.* 2024), or the validated A675V seen at low prevalence among isolates from patients recently arrived from Ituri and attending a health facility in Kyangwali refugee camp in Uganda in 2022 (Tukwasibwe *et al.* 2024).

A wide range of non-synonymous propeller mutations not classified by the WHO, were seen. These mutations were largely rare and were widely geographically distributed across DRC. This is consistent with findings of previous studies in DRC (Onyamboko *et al*. 2025, Yobi *et al.* 2020b, Yobi *et al.* 2021, Mvumbi *et al.* 2017 Mesia *et al.* 2024, Van Loon *et al.* 2024) and likely

528 indicative of the baseline PfK13 diversity which pre-existed the widespread use of artemisinin
529 derivatives in first-line treatment. Interestingly, while found as a synonymous mutation at
530 codon C469C in the present study, the C469Y is on the WHO-list as a validated marker for
531 artemisinin partial resistance, and C469F is a candidate. Likewise, V568M an alternative
532 mutation to WHO-candidate marker V568G was observed, and alternative substitutions at 4
533 WHO-potential marker codon positions M579V instead of M579I, P527S instead of P527H,
534 K438R instead of K438N, and T508I instead of T508N. While the significance of these
535 mutations is unknown, it underlines the importance of PfK13 surveillance by full sequence
536 analysis rather than screening only for known markers of resistance.
537
538 It is perhaps surprising that we recorded no non-synonymous mutations in the propeller
539 region in parasites from South Kivu, Ituri and Lualaba. One possible explanation may be that
540 diversity was constrained because these were low transmission settings, the parasite
541 prevalence (a proxy for transmission intensity) for all sample sites is shown in table 1. South
542 Kivu and Lualaba do have low parasite prevalence and are therefore the sites most likely to
543 have low multiplicity of infection and constraints on genetic diversity. Synonymous mutation
544 in propeller sequences should be similarly constrained in the same settings but in South Kivu
545 and Lualaba rates were average. In Ituri we observed an absence of both synonymous *and*
546 non-synonymous mutations and suggest no obvious explanation for this.
547
548 Beyond the propeller region there is evidence to suggest that non-synonymous mutations in
549 the N-terminal BTB/Poz region of *Pfk13* might also be associated with slower artemisinin
550 clearance rates. A study in Nigeria found association with K189T and R255K mutations

(Amusan *et al.* 2025). Non-synonymous mutations were frequent in DRC and the K89T was widespread with a general trend in prevalence from the north to south shown in the map in figure 4. Several cases were observed where there might be a 'double mutant' *Pfk13* haplotype – these were combinations of the non-propeller mutation K189T with a propeller non-synonymous mutation previously reported by Fankem *et al.* 2025. However, the K189T was frequently observed, and the functional relevance of the possible combinations is unknown.

Partner drug resistance and geographic structure

Pfcrt and *Pfmdr1* modify parasite susceptibility to antimalarial drugs used as partners in ACTs (Yuan *et al.* 2011, Veiga *et al.* 2016). The *Pfcrt* CVIET haplotype was historically selected to very high frequency by widespread use of chloroquine treatment throughout Africa during the 1980s (Charmot *et al.* 1991). Since Chloroquine (CQ) was withdrawn, the prevalence of CVIET declined dramatically and was replaced by the CVMNK wildtype (Mohammed *et al.* 2013, Njiro *et al.* 2022). A spatiotemporal analysis of *Pfcrt* mutant prevalences across DRC in 2007 and 2013 (Deutsch Feldman *et al.* 2019) reported elevated levels of the CVIET resistance haplotype in eastern provinces particularly in Eastern DRC (North and South Kivu), and in Kinshasa in the far west. The geographical distribution of drug resistance in the DRC observed in the present study is consistent with these previous studies. CVIET remains high especially in North and South Kivu but also in the central belt encompassing Kwilu and Tshuapa across to Kongo Central, while CVMNK wildtype predominates in northern and southern regions.

Until recently, ASAQ and ALU were both recommended as first-line treatments in DRC with ASAQ distributed in rural areas and ALU in urban areas. It is possible that regional differences

in prescribing practices and drug availability may be the driver of the observed *Pfcrt* and *Pfmdr1* geographical distribution patterns observed. The use of ALU exerts selection for *Pfmdr1* N86 and *Pfcrt* CVMNK (Venkatesan *et al.* 2014, Humphreys *et al.* 2006) and the displacement of *Pfmdr1* 86Y and *Pfcrt* CVIET (once fixed by historical use of Chloroquine) by *Pfmdr1* N86 and *Pfcrt* CVMNK has been widely documented in DRC (Onyamboko *et al*. 2025 Deutsch Feldman *et al.* 2019)

Conclusion

The findings of the present study demonstrate clear geographic differences in *Pfk13, Pfcrt,* and *Pfmdr1* resistance markers across the DRC. We found high prevalence of both *Pfmdr1* 86Y and *Pfcrt* 76T in North and South Kivu suggesting ASAQ is at risk and ALU will be effective in these Provinces. In the north and south of the Country wildtype *Pfmdr1* N86 and wildtype *Pfcrt* K76 predominate, and in these areas use of ASAQ is advised. Treatment policy switched in 2024 to a MFT strategy with more ACTs, including ASAQ, ALU and in addition, Artesunate-Pyronaridine (AP), Dihydroartemisinin-Piperaquine (DHAPQ) is used in epidemic outbreaks and Quinine Clindamycin in cases of intolerance and failure of ACTs. With increasing use of MFT, sustained molecular surveillance to prepare for emerging ACT resistance and possible shifts in ACT treatment susceptibility is increasingly important. We have demonstrated that pragmatic use of the TES surveillance framework for sampling for molecular surveillance can inform sub-national tailoring of treatment and chemoprevention. More detailed sampling frameworks are needed in order to define hotspots of emerging artemisinin resistance and MOSAIC high throughput genotyping has valuable application for both.

Abbreviations

ACT – Artemisinin Combination Therapy

ALU – Artemether-Lumefantrine

AP – Artesunate-Pyronaridine

ASAQ – Artesunate-Amodiaquine

DBS – dried blood spot

DHAPQ – Dihydroartemisinin-Piperaquine

DRC – Democratic Republic Congo

MFT – Multi First Line Therapy

MOSAIC – Multi-locus high throughput Oligo-based Sequencing of targeted Amplicons on Illumina miseq for high sample Capacity

NMCP – National Malaria Control Program

Pfaat - Plasmodium falciparum amino acid transporter 1

Pfcpmp – Plasmodium falciparum conserved Plasmodium membrane protein

Pfcrt – Plasmodium falciparum chloroquine resistance transporter

Pfcsp - Plasmodium falciparum circumsporozoite protein

Pfdhfr - Plasmodium falciparum dihydrofolate reductase

Pfdhps - Plasmodium falciparum dihydropteroate synthase

Pfexo – Plasmodium falciparum exonuclease

Pfk13 - Plasmodium falciparum Kelch 13

Pfmdr1 – Plasmodium falciparum multidrug resistance protein 1

Pfubp1 - Plasmodium falciparum ubiquitin-specific protease 1

RDT – Rapid Diagnostic Test

SNP – Single Nucleotide Polymorphism

TES – Therapeutic Efficacy Studies

WHO – World Health Organization

Declarations

Ethics approval and consent to participate

Ethical Approval was obtained for the different sample collections. Specific provinces linked to the approvals are described in detail in the methodology section. The ethical approvals are listed below.

Ethical Approval was obtained in DRC (reference number ESP/CE/77C/2023), and in UK (LSHTM reference number 31393). Ethical Approval was obtained in DRC (ESP/CE/071/2019) and UK (LSHTM reference number 31398). Ethical Approval was obtained in DRC (ESP/CE/69/2020). Ethical Approval was obtained in DRC (reference; 496/CNES/BN/PMMF/2023), UK (LSHTM reference number 29281) and from the WHO (ERC reference number 0003949).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding authors on reasonable request. Later, data will be available on *LSTMH Data Compass* (https://datacompass.lshtm.ac.uk/) after publication.

Competing interests

The authors declare no competing interests.

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Author's contributions

HH and CR analysed the data and wrote the manuscript. GMK, HM1, CR and MA designed the DRC sampling framework. GMK, HM1, DMY, GML and LK were responsible for DRC sample collection and sample preparation.

HH, LW, MAS, SMI, MR and MBS worked on the development of the bioinformatic analysis pipeline.

ACP, LK, AM1, VAB, MES, IMA, KB, AM2, EFH, HH, LW, MAS, SMI, MR, MBS, LAE, RMV, RMC, CS, CR and MA all contributed to laboratory analysis, genotyping, mapping and to the work of the Plus Project Genotyping workpackage.

The PLUS Project drug resistance mapping sample collection protocol was authored by CR, MA, CN, AM1, HM2, WY, IMA, WM, GMK, HM1, CS, RG.

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Tables and figure legends

Table 1. Sampling framework covering 16 of the 26 administrative provinces of DRC.

Province	Clinic Location	Sample site parasite prevalence (pfpr2_10)	Collection Year	Samples analysed	Collection study
Tshopo	Kabondo	0.2876	2021	176	TES of AL and ASAQ 2021*
Mongola	Lisala	0.2405	2024	150	Plus Project collection
Sud-Ubangi	Gemena	0.3383	2024	150	Plus Project collection
Maniema	Kalima	0.2060	2023	179	TES of AL and ASAQ 2023
South Kivu	Katana	0.1387	2019	69	NMCP sentinel survey**
North Kivu	Rutshuru	0.1138	2020	200	TES of AL and ASAQ 2020*
Ituri	Bunia	0.2653	2024	150	Plus Project collection
Tanganyika	Kalemie	0.3076	2024	150	Plus Project collection
Kasai-Central	Mikalayi	0.3728	2021	180	TES of AL and ASAQ 2021*
Haut-Lomami	Kamina	0.2392	2019	92	NMCP sentinel survey **
Lualaba	Fungurume	0.1575	2019	68	NMCP sentinel survey **
Haut-Katanga	Kapolowe	0.2134	2021	180	TES of AL and ASAQ 2021*
Kongo-Central	Kimpese	0.3431	2020	200	TES of AL and ASAQ 2020*
Kwilu	Vanga	0.5345	2023	177	TES of AL and ASAQ 2023
Mai-Ndombe	Inongo	0.2271	2024	150	Plus Project collection
Tshuapa	Boende	0.4068	2021	179	TES of AL and ASAQ 2021*

For each province, the table shows the clinic location, the parasite prevalence at that location, the year of sampling, the originating study or surveillance activity, and the number of samples processed by MOSAIC. Where applicable publication references for TES or sentinel surveys are indicated. *Mesia et al. 2024, **Yobi et al. 2022. No samples from Haut-Uele, Bas-Uele, Nord-Ubangi, Equateur, Kasai, Kasai-Oriental, Sankuru, Lomami, Kwango or Kinshasa were included in the analysis.

Supplementary tables

Supplementary Table 1. Primer sequences and genomic reference information for all targeted amplicons in MOSAIC.

The table lists outer and nested PCR primers used to amplify *Pf* gene fragments included in the targeted sequencing panel. Primer sequences are shown in 5′→3′ orientation, with adapter overhangs indicated where applicable. Primer sources or protocol-specific modifications are indicated where relevant.

Supplementary table 2. Summary of synonymous and non-synonymous sequence variation in the *Pfk13* propeller domain in 16 Provinces.

The propeller region is covered by sequencing fragments 4 and 5, corresponding to propeller domains 1–6 as indicated. For each Province, the table shows the number of successfully sequenced samples, counts of detected non-synonymous and synonymous SNPs, and prevalence expressed as a percentage of the number of successfully sequenced samples. Listed codon changes represent all observed mutations, with numbers in parentheses indicating repeated occurrences within a site. Totals summarize data for all provinces combined.

Supplementary table 3. Summary of synonymous and non-synonymous sequence variation in the *Pfk13* N-terminal and the BTB/POZ domain.

Fragment coverage spans N-terminal *Plasmodium*-specific (codons 18–225) and apicomplexa-specific (codons 225–345) regions, and the BTB/POZ domain (codons 346–439). Data is available for 10 Provinces; the 6 Provinces not sequenced are shaded grey. For each study site, the number of successfully sequenced samples, counts of detected non-synonymous and synonymous mutations, and their prevalence among successfully sequenced samples in that site are shown. Listed codon changes represent all observed mutations, with numbers in parentheses indicating repeated occurrences within a site. Totals summarize all provinces included in the analysis combined.

Supplementary table 4. Distribution of non-synonymous and synonymous *Pfk13* mutations summarized by geographical region.

Mutations are grouped according to genomic coverage by sequencing fragments, including the N-terminal BTB/POZ domain (fragments 1–3) and the propeller domain (fragments 4–5). For each mutation, counts are shown by geographic region (North, East, South, West, as indicated in table supplemental table 2 and 3), together with the total number of occurrences (N) and overall percentage relative to all successfully sequenced samples. Domain boundaries are indicated to facilitate interpretation of mutation location.

4A. Non-synonymous SNPs in the *Pfk13* propeller domain

4B. Synonymous *Pfk13* SNPs in the *Pfk13* propeller domain

4C. Non-synonymous SNPs in *Pfk13* N-terminal, BTB/POZ domain

4D. Synonymous SNPs in *Pfk13* in *Pfk13* N-terminal, BTB/POZ domain

Supplementary table 5. Genotype and haplotype distribution of *Pfcrt* and *Pfmdr1* across study provinces.

For each site, counts (N) and percentages (%) are shown relative to successfully sequenced samples (excluding mixed infections). The *Pfcrt* section summarizes haplotype frequencies, while the *Pfmdr1* section details codon-specific genotype distributions and inferred haplotypes. Regional totals represent all successfully genotyped isolates per province.

Figure 1. Overview of the workflow in MOSAIC pipeline

MOSAIC (*Multi-locus high-throughput Oligo-based Sequencing of targeted Amplicons on Illumina MiSeq for high sample capacity*), is a high-throughput genotyping pipeline that processes blood samples through DNA extraction, qPCR analysis, targeted amplicon sequencing, and downstream bioinformatic analysis. Using MOSAIC, multiple gene fragments from a large number of samples (average runs of around 1500 samples) can be analyzed in a single sequencing run, while retaining the ability to trace each sequence back to its original sample through fragment-specific barcoding (indexing). Identification of sampling areas is in collaboration with the National Malaria Control Programme where possible.

Figure 2. Genes and fragments of *P. falciparum* targets

Schematic overview of *P. falciparum* resistance-associated loci and sequenced regions relative to the 3D7 reference. Nuclear genes are shown in amino acid coordinates with colored segments indicating regions amplified and sequenced in this study. Key resistance-associated codons are marked above each gene. The *Pfdhps* amplicon contains an internal sequencing gap due to read-length limitations of MiSeq 2×300 bp sequencing; all resistance-associated codons remain covered. The large *Pfubp1* locus is displayed with a scale break for clarity. The mitochondrial genome is shown separately in base-pair coordinates with sequenced fragments indicated. Coordinates correspond to the *P. falciparum* 3D7 reference sequences. All 3D7 reference IDs are shown in supplemental table 1.

Figure 3. Distribution of non-synonymous *PfK13* sequence variation in (A) the propeller domain and (B) the BTB/POZ domains.

Mutations seen in each province are listed, and the number of distinct *Pfk13* mutations expressed as a percentage of the total number of samples successfully sequenced. If mutations were observed more than once in a province, the observation count is indicated by the number in parentheses. Mutations seen in multiple provinces are highlighted in colour. An asterisk (*) denotes mutations classified by WHO as validated, candidate or potential markers of drug resistance.

3A. Non-synonymous mutations within the *Pfk13* propeller domain in 16 provinces of DRC

3B. Non-synonymous mutations in the *Pfk13* N-terminal BTB/POZ domains in 10 provinces of DRC.

Figure 4. Geographic distribution of the *Pfk13* K189T variant across 10 provinces of the DRC.

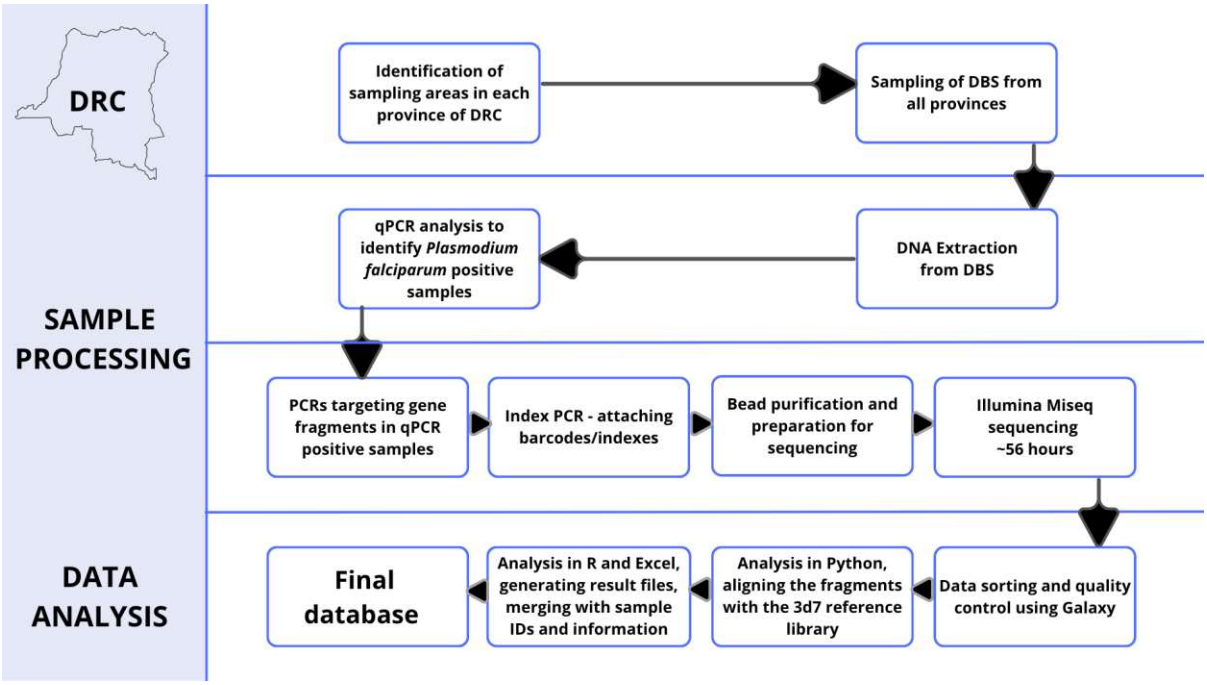
Provinces are shaded according to the observed prevalence of the K189T mutation, grouped into <20%, 20–40%, and >40% categories. Percentage values and sample sizes of successfully sequenced samples (N) indicate the proportion of sequenced isolates carrying K189T within each province. The accompanying table summarizes the number of K189N-positive samples detected per province.

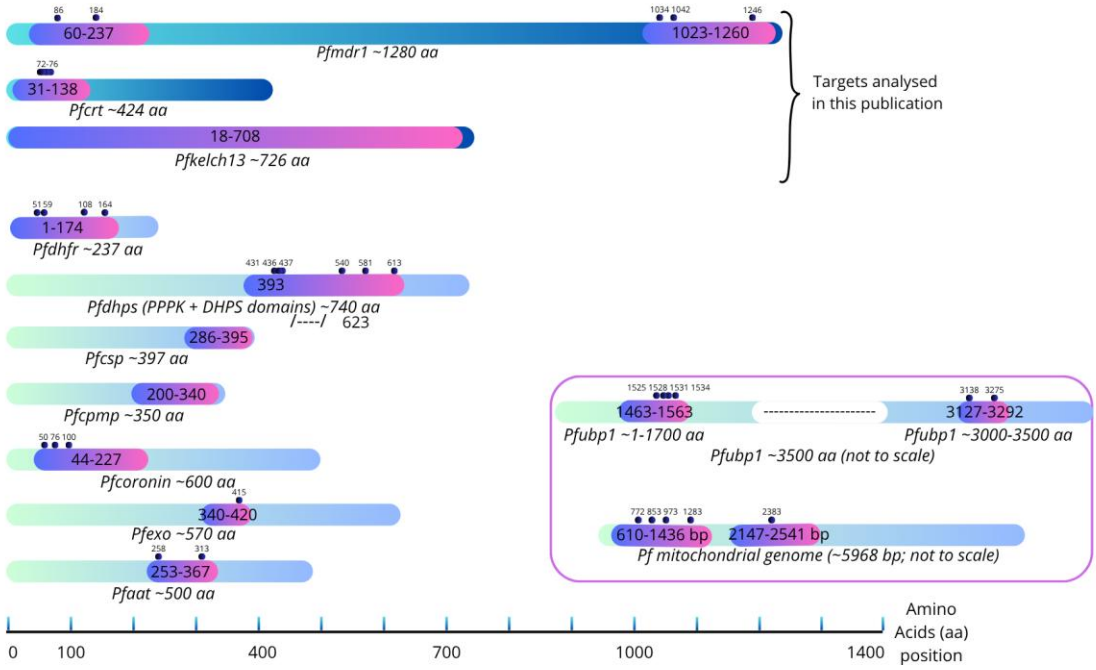
Figure 5. Provincial distribution of *Pfcrt* haplotypes across the DRC.

Pie charts indicate the proportional frequency of codon 72 – 76 haplotypes of *pfcrt*. CVMNK (blue) and CVIET (red) among isolates from each province. The number displayed within each circle indicates the total number of successfully sequenced samples per Province (N). Circle size is uniform and does not reflect sample size.

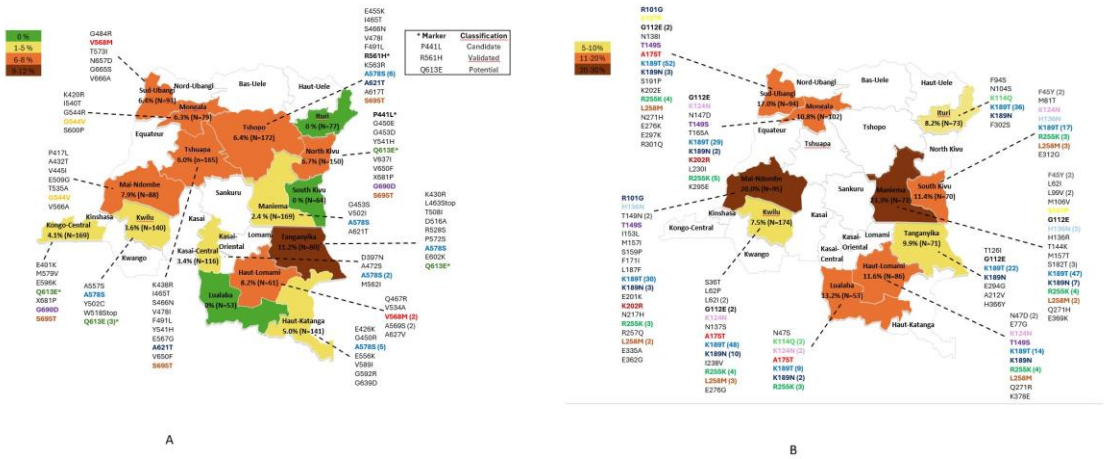
Figure 6. Provincial distribution of *Pfmdr1* haplotypes across the DRC.

Pie charts show the proportional frequency of *Pfmdr1* haplotypes for codons 86, 184, 1034, 1042, 1246. in each province, with colors corresponding to the specific haplotypes indicated in the legend. The number of successfully sequenced samples at each site is displayed within each pie chart Circle size is uniform and does not reflect sample size.

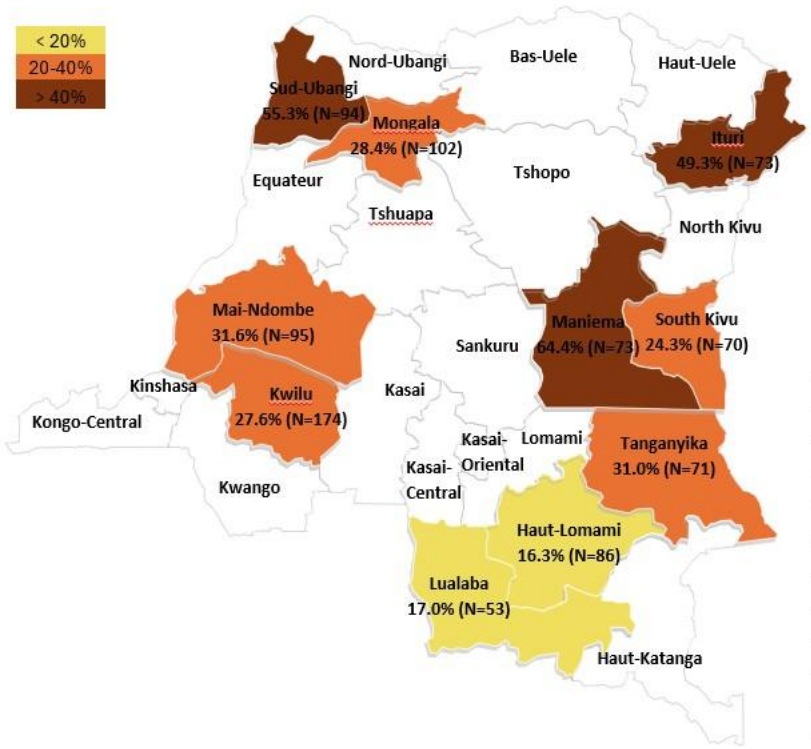



86
184
60-237
1034 1042
1246
1023-1260
Pfmdr1 ~1280 aa
72-76
31-138
Pfcrt ~424 aa
18-708
Pfkelch13 ~726 aa
Targets analysed in this publication
5159
108
164
1-174
Pfdhfr ~237 aa
431 436 437
540
581
613
393
Pfdhps (PPPK + DHPS domains) ~740 aa
/----/ 623
286-395
Pfcsp ~397 aa
200-340
Pfcpmp ~350 aa
50 76 100
44-227
Pfcoronin ~600 aa
415
340-420
Pfexo ~570 aa
258
313
253-367
Pfaat ~500 aa
1525 1528 1531 1534
1463-1563
3138 3275
3127-3292
Pfubp1 ~1-1700 aa
Pfubp1 ~3000-3500 aa
Pfubp1 ~3500 aa (not to scale)
772 853 973 1283
2383
610-1436 bp
2147-2541 bp
Pf mitochondrial genome (~5968 bp; not to scale)
0
100
400
700
1000
1400
Amino Acids (aa) position

1149

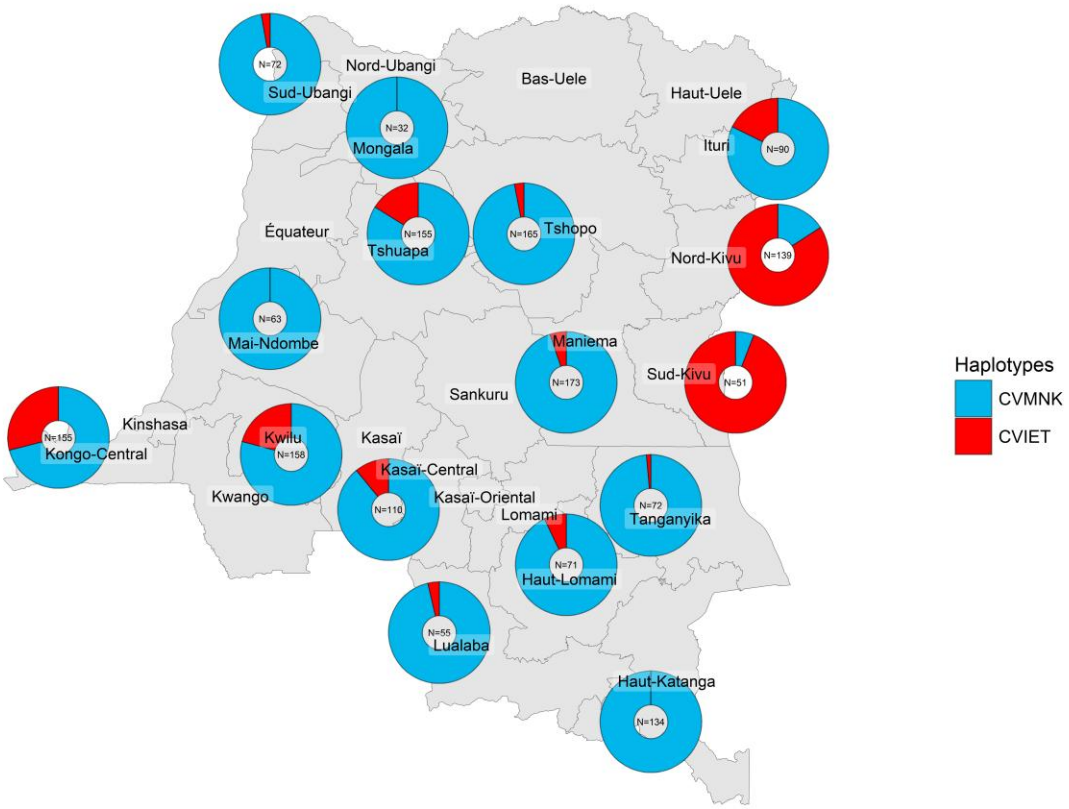

A
B

1150

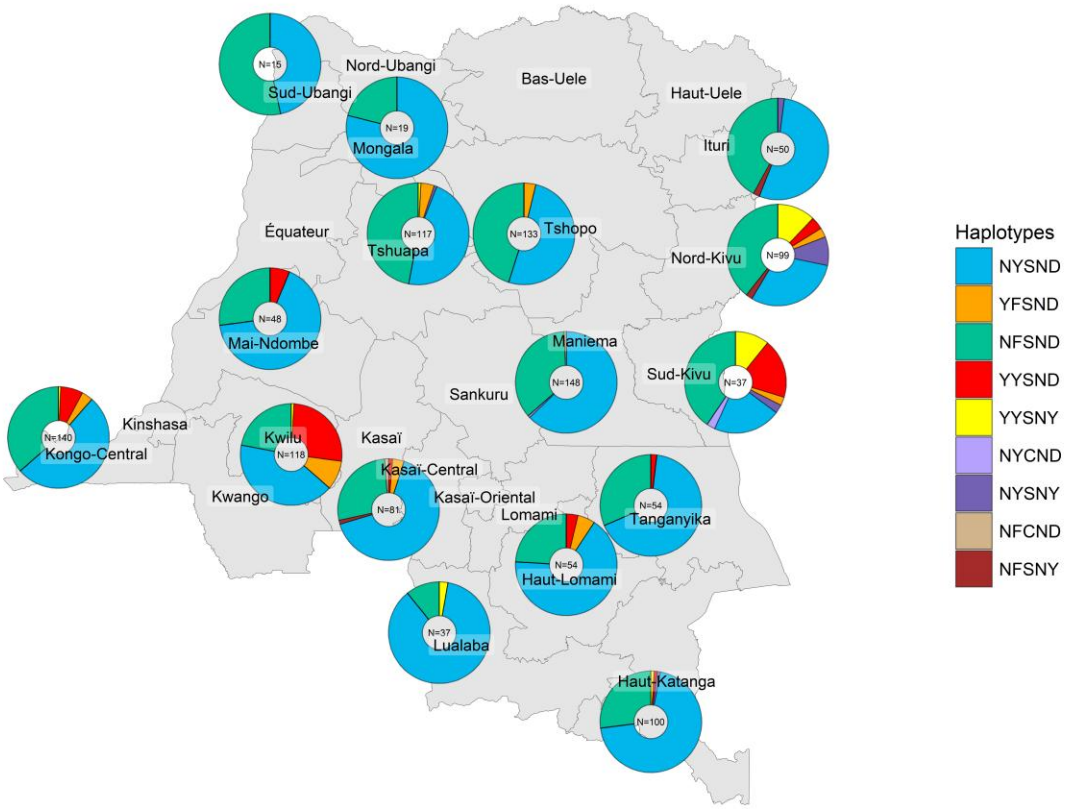


Province	K189N (n)
Sud-Ubangi	K189N (3)
Mongala	K189N (2)
Mai-Ndombe	K189N (3)
Kwilu	K189N (10)
Lualaba	K189N (2)
Haut-Lomami	K189N (0)
Tanganyika	K189N (0)
Maniema	K189N (7)
South Kivu	K189N (0)
Ituri	K189N (0)

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N=72
Nord-Ubangi
Sud-Ubangi
Bas-Uele
Haut-Uele
N=32
Mongala
Ituri
N=90
Équateur
N=155
Tshuapa
N=165
Tshopo
Nord-Kivu
N=139
N=63
Mai-Ndombe
Maniema
N=173
Sankuru
Sud-Kivu
N=51
N=155
Kongo-Central
Kinshasa
Kwilu
N=158
Kasaï
Kasaï-Central
N=110
Kwango
Kasaï-Oriental
Lomami
N=72
Tanganyika
N=71
Haut-Lomami
N=55
Lualaba
Haut-Katanga
N=134
Haplotypes
CVMNK
CVIET

1152



1153

1154

1155

1156

1157

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarytable1.xlsx
- Supplementarytable2.xlsx
- Supplementarytable3.xlsx
- Supplementarytable5.xlsx
- Supplementarytable4AD.xlsx