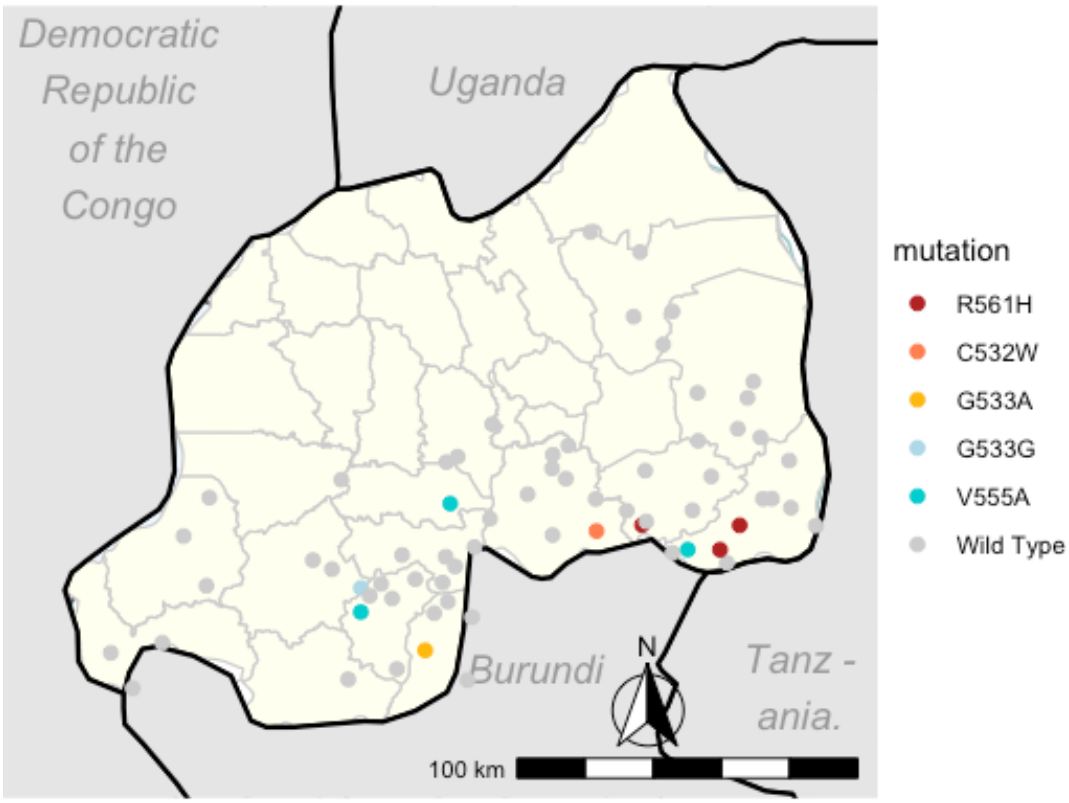
Supplementary Materials



Supplementary Figure 1: Cluster level mutation presence. Colored dot indicates presence of mutation in a cluster, not prevalence.

Supplementary Table 1: *Pfk13* haplotypes observed in sequenced samples (N=351)

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Mutation	Amino Acid Sequence	Codon Change at Mutation Site	N Samples	Percent of Population (Weighted)
Wild Type	516-DVWYVSSNLNIPRRNNCGVTSNGRIYCIGGYDGSSIIPNVEAYDHRMKAWVEVAPLN-572	NA	341	97.03%
C532W	516-DVWYVSSNLNIPRRNN**W**GVTSNGRIYCIGGYDGSSIIPNVEAYDHRMKAWVEVAPLN-572	TGT -> GGT	1	0.28%
G533A	516-DVWYVSSNLNIPRRNNC**A**VTSNGRIYCIGGYDGSSIIPNVEAYDHRMKAWVEVAPLN-572	GGT -> GCT	1	0.27%
G533G	516-DVWYVSSNLNIPRRNNC**G**VTSNGRIYCIGGYDGSSIIPNVEAYDHRMKAWVEVAPLN-572	GGT -> GGA	1	0.30%
V555A	516-DVWYVSSNLNIPRRNNCGVTSNGRIYCIGGYDGSSIIPN**A**EAYDHRMKAWVEVAPLN-572	GTA -> GCA	3	0.78%
R561H	516-DVWYVSSNLNIPRRNNCGVTSNGRIYCIGGYDGSSIIPNVEAYDH**H**MKAWVEVAPLN-572	CGT -> CAT	4	1.34%

Supplementary Table 2: Survey weighted Pfk13 Mutation Prevalence Within Administrative Districts

District	C532W	G533A	G533G	V555A	R561H	Wild Type	N Samples
Bugesera	1 (2.14%)	0	0	0	0	47	48
Gatsibo	0	0	0	0	0	11	11
Gisagara	0	0	0	0	0	27	27
Huye	0	0	0	1 (3.01%)	0	32	33
Kamonyi	0	0	0	0	0	3	3
Karongi	0	0	0	0	0	4	4
Kayonza	0	0	0	0	0	23	23
Kirehe	0	0	0	1 (1.78%)	3 (5.47%)	50	54
Ngoma	0	1 (1.63%)	0	0	1 (2.74%)	50	52
Nyamagabe	0	0	1 (5.37%)	0	0	19	20
Nyamasheke	0	0	0	0	0	3	3
Nyanza	0	0	0	1 (1.93%)	0	44	45
Nyaruguru	0	0	0	0	0	9	9
Ruhango	0	0	0	0	0	12	12
Rusizi	0	0	0	0	0	7	7
Total	1	1	1	3	4	341	351

Supplementary table 3: previous sources for *Pfk13* mutation information

Mutation	In vitro artemisinin resistance status	Date First Observed, Location (Percent), Study	Other Observations in Rwanda: Date, Location (Percent), Study	Notes
C532W	Unvalidated	2014-15, Bugesera (2.14%), **this study**	none	
G533A	Unvalidated	2014-15, Ngoma (1.63%), **this study**	• 2019, Huye District (1.5%), Bergmann et al 2021	Associated with treatment failure in India, but cannot be directly linked to ACT resistance (Mishra et al 2015)
G533G	Unvalidated	2014-15, Nyamagabe (5.37%), **this study**	none	
R561H	Validated	2014-15, Masaka (7.4%), Uwimana et al 2020	• 2014-15, Kirehe (5.47%), Ngoma (2.47%), **this study** • 2018, Masaka (19.6%), Rukara (22%), Uwimana et al 2021 • 2019, Huye District (4.5%), Bergmann et al 2021	
V555A	Unvalidated	2012, Huye District (1.2%), Tacoli et al 2015	• 2014-15, Huye (3.01%), Kirehe (1.78%), Nyanza (1.93%), **this study** • 2015, Masaka (0.39%), Rukara (1.2%), Uwimana et al 2020 • 2019 Huye District (1.5%), Bergmann et al 2021	

Supplementary Methods

PCR Protocol

PCR step 1

- Primers were manufactured by IDT and hydrated to 100 uM stock solutions in Low EDTA TE (Thermo #AAJ75793AP), then diluted to 10 uM in molecular grade water (MGW).
- DNA was centrifuged at 4000 rpm for 3 minutes before 2.5uL was transferred from the top of the tube to the reaction mixture to reduce chelex carryover.
- Non-template negative control and 3D7 positive controls were added to each plate.

PCR step 2

- 5uL of PCR product from PCR1 diluted 1:10 with molecular grade water were added immediately after dilution.

Overall

- Thermocycling was run on an Eppendorft Mastercycler 96
- PCR step 1 primers contained linkers and PCR step 2 primers contained unique barcodes to allow for multiplexing.

PCR Step 1 Master Mix

Reagents (NEB #M0491, Thermo BP28191)	**Stock Concentration**	**Volume 1x reaction**
Molecular Grade Water	NA	11.75 uL
Q5 Reaction Buffer	5x	5 uL
dNTPs	10 mM	0.5 uL
Primer F	10 uM	2 uL
Primer R	10 uM	0.5 uL

Q5 Polymerase	2000 U/mL	0.25 uL
MgCl	50 mM	3 uL
Total		**22.5 uL**
		+2.5 uL DNA

Primer Design

Forward Primer	5'-GACTCGCCAAGCTGAAGNNNNCATAGCTGATGATCTAGGGG-3'
Reverse Primer	5'-ACGTGTGCTCTTCCGATCTNNNNCTGAGGTGTATGATCGTTTAAG-3'

PCR Step 1 Thermocycling Parameters

Cycle Step		**Temperature (C)**	**Time**	**No. Cycles**
Initial Denaturation		98	30 seconds	1x
Amplification	Denaturation	95	10 seconds	30x
	Annealing	56	15 seconds	
	Elongation	72	20 seconds	
FInal Elongation		72	2 minutes	1x
Hold		4	Hold	1x

PCR Step 2 Master Mix

Reagents (NEB #M0491, Thermo #BP28191)	**Stock Concentration**	**Volume 1x reaction**
Molecular Grade Water	NA	11.75 uL
Q5 Reaction Buffer	5x	5 uL

dNTPs	10 mM	0.5 uL
Barcoded Primer F	10 mM	1.25 uL
Barcoded Primer R	10 mM	1.25 uL
Q5 Polymerase	2000 U/mL	0.25 uL
Total		**20 uL**
		+5 uL PCR step 1 product diluted 1:10 with MGW

PCR Step 2 Thermocycling Parameters

Cycle Step		**Temperature (C)**	**Time**	**No. Cycles**
Initial Denaturation		98	30 seconds	1x
Amplification	Denaturation	95	10 seconds	6x
	Annealing	59	15 seconds	
	Elongation	72	20 seconds	
Final Elongation		72	2 minutes	1x
Hold		4	Hold	1x