Detailed Laboratory Methods

DNA extraction and genotyping

Genomic DNA was isolated from dried blood spots on Whatman 903 filter paper (46 samples) or from microscopy slides (2 samples) using the QIAamp DNA blood mini kit (QIAGEN, Valencia, CA) per manufacturer's instructions. Extraction from microscopy slides was performed according to Cnops et al. protocol [1]. Genus and species specific photo-induced electron transfer (PET)-PCR was used to confirm *P. falciparum* using a Stratagene Mx3005P (Agilent, Santa Clara, CA) [2].

Extracted DNA from dried blood spots collected on Day 0 and day of failure was used for genotyping four drug resistant markers (*pfmdr1*, chromosome 10 and 13 SNPs, and K13-propeller) and testing seven neutral microsatellite loci spanning six chromosomes (TA1, chromosome 6; poly α, ch. 4; PfPK2, Ch. 12; TA109, ch. 6; and 2490, ch. 10; C2M24, ch. 2; C3M69, ch. 3) [3-5]. The microsatellite markers were PCR-amplified using primers and methods described previously [3, 4, 6]. Briefly, fluorescent labeled (HEX and FAM) PCR products were separated by capillary electrophoresis on an ABI 3130xl Genetic Analyzer and fragments were sized and scored using GeneMapper v3.7 (Applied Biosystems, Foster City, CA). When the sizes of alleles at a microsatellite marker differed by >2 base pairs they were considered different alleles. Previous laboratory studies have found that in polyclonal infections microsatellite alleles may occasionally be undetectable [7]. Due to the high prevalence of mixed strain (polyclonal) infections in highly endemic regions of Africa, we classified an infection as recrudescent if at least 6 of 7 neutral microsatellites shared alleles.

Chromosome 10 and 13

PCR for pyrosequencing. The PCR and sequencing primers for the Chromosome 10 and Chromosome 13 SNPs were synthesized at the CDC Biotechnology Core Facility. The primer sequences are shown in Table S3. The primary and nested PCRs were generated in 50ul reaction volumes, which contained 1 ul DNA, 0.5 uM of forward and reverse PCR primers, 0.2mM deoxynucleotide triphosphates, 2.0mM $MgCl_2$, 10X High Fidelity PCR Mix, and 2.6U/reaction High Fidelity Enzyme (Roche Applied Science, Penzberg, Germany). The primary PCR cycling conditions were 94°C for 2 min; 30 cycles with denaturation at 94°C for 15 s, annealing at 59°C for 30 s, and elongation at 72°C for 45 s; 1 cycle at 72°C for 8 min; and a final hold at 4°C. The thermal-cycling conditions for the nested reactions were the same as for the primary PCR reaction. PCR was performed using an Icycler Thermal cycler (Bio-Rad, Hercules, CA).

Pyrosequencing reactions. Single-stranded biotinylated PCR products were prepared for sequencing using the Pyrosequencing Vacuum Prep Tool (Biotage AB, Uppsala, Sweden). Three microliters of Streptavidin Sepharose HP beads (Amersham Biosciences, Uppsala, Sweden) was added to 40 ul binding buffer (10 mM Tris-HCl, pH 7.6, 2 M NaCl, 1 mM EDTA, 0.1% Tween 20) and mixed with 20 ul PCR product and 20 ul water for 10 min at room temperature using an Orbit Digital Shaker (Labnet International, Woodbridge, NJ). The beads containing the immobilized templates were captured on the filter probes after the vacuum was applied and then washed with 70% ethanol for 5 s, denaturation solution (0.2MNaOH) for 10 s, and washing buffer (10 mM Tris-acetate, pH 7.6) for 5 s. The vacuum was then released, and the beads were released into a PSQ96 Plate Low (Biotage AB, Uppsala, Sweden) containing 45 ul annealing buffer (20 mM Trisacetate, 2 mM MgAc2, pH 7.6) and 0.5 uM sequencing primer.

Pyrosequencing reactions were performed according to the manufacturer's instructions using the PSQ 96 SNP Reagent Kit (Biotage AB, Uppsala, Sweden), which contained the enzyme, substrate, and nucleotides. The assays were performed on the PSQ 96MA (Biotage AB, Uppsala, Sweden). The sample genotype was determined using the SNP Software (Biotage AB, Uppsala, Sweden).

Kelch K13

PCR reactions and Sanger sequencing. The kelch K13 gene was amplified using a nested PCR approach; primers used are shown in Table S3. Two µL of genomic DNA was amplified using 0.5 µM of each primer, 0.2mM dNTP, 3 and 2 mM $MgCl_2$ for the primary and secondary reactions, respectively, and 1 U Expand High Fidelity Taq (Roche) . For the primary reaction the following cycling parameters were used: 5 min at 94ºC , 40 cycles at 94ºC for 30 sec, 60ºC for 90s, 72ºC for 90s, and final extension for 10 min at 72ºC. For the nested PCR, 1µL of the primary PCR product was used as template. For the nested PCR reaction the following cycling parameters were used: 2 min at 94ºC, 40 cycles at 94ºC for 30 sec, 55ºC for 30s, 72ºC for 90s, and final extension for 10 min at 72ºC. PCR products were confirmed using a 2% agarose gel electrophoresis and Gel red (Biotium, Hayward, CA). Sanger sequencing of PCR products was performed using ABI 3730 and analyzed with Geneious Pro R7 (Biomatters, Inc. , Auckland, New Zealand) to identify specific SNPs.

Supplementary Table S1.Microsatellite data from 24 late treatment failures observed during therapeutic efficacy studies in Zaire and Uíge Provinces, Angola, 2013

Chromosome	6				4		12			6		10		2		3	
Marker Name	**TA1**				**Polyα**		**PfPK2**			**TA109**		**2490**		**C2M34**		**C3M69**	
Sample ID	1				3		7			9		12		313		383	
A111	170				162		163			173		84		219		124	
A111d28	172				162		163			173		84		219		124	
A114	150				162		175			161		84		229		166	
A114d21	169				162		193			176		84		234		132	
A115	181				158	152	175	184		185		74		230		141	
A115d28	166				165		167			185		84		238		124	
A144	178	166	169	181	138	162	184	166	187	161	176	81	84	230		124	
A144d21	181				165		175			167		84		219		143	
A145	171				171		160			186		81		238		124	
A145d21	172				171		160			185		81		238		124	
A171	165				132		173			176		84		238		124	
A171d28	163				158		187			164		84		230	238	150	
B304	175				155		166			162		74		243		124	
B304d21	172				155		166			213		84		236		140	
B312	182				132		169			165		75		247		154	
B312d21	169				152		179			161		74		247		124	
B314	160				162		160			177		81		223		141	
B314d21	160				161		160			176		81		223		141	
B416	162	168			149		167			161	164	81		219	253	143	
B416d28	163	169			149		166			161	164	81		219		143	
B421	162				149		166			176		87		215		147	
B421d28	166				174		179			161		84	81	255		139	
B422	174	162			142	159	164	187		177	165	84		234		145	
B422d28	175				158		187			176		84		234		141	
B423	159				171		166			165		84		230		139	
B423d21	172	160			161	155	169	163		164		84		223		132	
B371	174				171		172	169		177		81	84	261		124	
B371d21	172				165	152	187			176		84		232		143	
B375	171				159		169			164		81		238		124	
B375d28	163				162		172	178		176		84		259		143	
B384	160				161		163			161		84		243		136	
B384d21	160				161		163			161		84		242		136	
B385	168	165			158		184			161	185	84	94	236		143	
B385d21	169	166			158		184	160		161	185	84	94	236		143	
B386	168				145		184			176		84		230		145	
B386d14	172				158		169			173		81		223		124	
B387	169				152		175			188		74		272		136	
B387d21	163				149		182			176		77		230		124	
B398	170				161		172			179		84		239		143	
B398d21	184				142		157			164		84		225		172	
B399	168	183			155	183	175	163		164		81		227	255	138	
B399d21	184	169			155		175			164		81		227		139	
B402	169	160			150		187			176	200	84		261	236	124	150
B402d28	166				168		181			173		81		249		150	

Chromosome	**6**		**4**		**12**		**6**		**10**		**2**		**3**	
Marker Name	**TA1**		**Polyα**		**PfPK2**		**TA109**		**2490**		**C2M34**		**C3M69**	
Sample ID	1		3		7		9		12		313		383	
B404	178		152	138	172		188		84		270		131	
B404d28	160		139		172		200	188	84		270	221	130	
C560	168		149		184		176		84		238		150	
C560d28	181		155		190		185		87		232		145	
Reference Strains:														
3D7	183		152		169		173		81		262		172	
HB3	140		180		193		188		84		232		124	

Supplementary Table S2. Sequencing Primers for *pfmdr1*

Gene	Primer orientation,* name	Primer sequence	Reference
pfmdr1			
Region 1			Vinyaka et al., 2010
Primary			
	F, AL6875	5'-CCGTTTAAATGTTTACCTGCAC-3'	
	R, AL6876	5'-TGGGGTATTGATTCGTTGCAC-3'	
Secondary			
	F, AL6877	5'-GTATGTGCTGTATTATCAGGAG-3'	
	R, AL6878	5'-AGCCTCTTCTATAATGGACATG-3'	
Region 2			
Primary			
	F, AL6792	5'-GCATTTAGTTCAGATGATGAAATG-3'	
	R, AL6793	5'-CCATATGGTCCAACATTTGTATC-3'	
Secondary			
	F, AL6794	5'-TATGCATACTGTTATTAATTATGG-3'	
	R, AL6795	5'-TTCGATAAATTCATCTATAGCAG-3'	

*F, forward; R, reverse.

Supplementary Table S3. PCR and Sequencing Primers for Chromosome 10 and 13 SNPs, K13

Gene	Primer orientation,* name	Primer sequence
Chromosome 10 and 13 SNPs*:*		
Chromosome 10		
Primary PCR		
	F, PCH10F1	5'- TGTATGAATAGGGATTGTCC-3'
	R, PCH10R1	5'- GCTATATCTTTTTTAATTTTTGCTCGTC-3'
Secondary PCR		
	F, PCH10F2	5'- GACGAGCAAAAATTAAAAAAGATATAGC-3'
	R, PCH10R2-BIOT	5'- TTAGTGAAAATTATATGTAATGGGTG-3'
Sequencing Primer	F, Pyro10SeqF	5'- TTTTATAAAAGAACTATGCA-3'
Chromosome 13		
Primary PCR		
	F, PCH13F1	5'-GAATAAAGATGATATGAACGATG-3'
	R, PCH13R1	5'- CTAATTAAGGGATATGTAGCATC-3'
Secondary PCR		
	F, PCH13F2	5'- AGGAAGACAACGGTGACGATCTA-3'
	R, PCH13R2-BIOT	5'- TTCTAAACAAATACAACATTGCATGAC -3'
Sequencing Primer	F, Pyro13SeqF	5'- TTAGTAAAGTAGATCATTCC-3'
Kelch K13*:*		
Primary PCR		
	F, K13P1	5'- GGGAATCTGGTGGTAACAGC-3'
	R, K13R1	5'- CGGAGTGACCAAATCTGGGA-3'
Secondary PCR & Sequencing primers		
	F, K13S1	5'- GTGAAAGTGAAGCCTTGTTG-3'
	R, K13S2	5'-TTCATTTGTATCTGGTGAAAAG -3'

Supplementary Information References

1. Cnops L, Van Esbroeck M, Bottieau E, Jacobs J: **Giemsa-stained thick blood films as a source of DNA for Plasmodium species-specific real-time PCR.** *Malar J* 2010, **9:**370.
2. Lucchi NW, Narayanan J, Karell MA, Xayavong M, Kariuki S, DaSilva AJ, Hill V, Udhayakumar V: **Molecular diagnosis of malaria by photo-induced electron transfer fluorogenic primers: PET-PCR.** *PLoS One* 2013, **8:**e56677.
3. Anderson TJ, Haubold B, Williams JT, Estrada-Franco JG, Richardson L, Mollinedo R, Bockarie M, Mokili J, Mharakurwa S, French N, et al: **Microsatellite markers reveal a spectrum of population structures in the malaria parasite Plasmodium falciparum.** *Mol Biol Evol* 2000, **17:**1467-1482.
4. Anderson TJ, Su XZ, Bockarie M, Lagog M, Day KP: **Twelve microsatellite markers for characterization of Plasmodium falciparum from finger-prick blood samples.** *Parasitology* 1999, **119 (Pt 2):**113-125.
5. McCollum AM, Mueller K, Villegas L, Udhayakumar V, Escalante AA: **Common origin and fixation of Plasmodium falciparum dhfr and dhps mutations associated with sulfadoxine-pyrimethamine resistance in a low-transmission area in South America.** *Antimicrob Agents Chemother* 2007, **51:**2085-2091.
6. Griffing SM, Mixson-Hayden T, Sridaran S, Alam MT, McCollum AM, Cabezas C, Marquino Quezada W, Barnwell JW, De Oliveira AM, Lucas C, et al: **South American Plasmodium falciparum after the malaria eradication era: clonal population expansion and survival of the fittest hybrids.** *PLoS One* 2011, **6:**e23486.
7. Greenhouse B, Myrick A, Dokomajilar C, Woo JM, Carlson EJ, Rosenthal PJ, Dorsey G: **Validation of microsatellite markers for use in genotyping polyclonal Plasmodium falciparum infections.** *Am J Trop Med Hyg* 2006, **75:**836-842.