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ABSTRACT

Title: The Effect of Drug Pressure and Transmission Setting on Sulfadoxine-Pyrimethamine Resistant *Plasmodium falciparum* Haplotype Prevalence and Selective Sweep Characteristics, in Malawi

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Background: The continued expansion of resistance to anti-malarial chemotherapies is a threat to public health, and to malaria control and elimination. The reexpansion of drug sensitive parasites after the removal of drug pressure has renewed interest in epidemiological factors affecting resistance haplotype dynamics, in the hopes that previously abandoned drugs might once again find clinical utility.

Objectives: Estimate the effect of changes in drug pressure and different malaria transmission settings on sulfadoxine-pyrimethamine (SP)-resistant haplotype prevalence and characteristics of selective sweeps.

Methods: DNA was extracted from dried blood spots representing malaria infections from three time periods (high-SP use 1999-2001, transition-period 2007-2008, low-SP use 2012) of drug pressure in Malawi and three transmission settings (urban-low, rural-moderate, rural-high). Pyrosequencing and microsatellite genotyping were performed on all samples to determine haplotype prevalence and sweep characteristics. Changes in

haplotype prevalence were assessed via Chi-squared tests and changes in sweep characteristics via permutation.

Results: We observed the persistence of the DHFR 51I/59R/108N and DHPS 437G/540E haplotypes, five years after reduction in SP pressure as well as an increase in the prevalence of DHPS 437G/540E/581G haplotype. Selective sweeps indicated little to no fitness cost to the DHFR 51I/59R/108N and DHPS 437G/540E haplotypes in the absence of strong SP pressure. A decline in polyclonal infections was found across the three time periods. No significant difference in haplotype prevalence was found between transmission settings. Sweep characteristics could suggest divergent evolutionary history in the rural-moderate transmission setting.

Conclusions: There is little to no fitness cost of SP-resistance in the absence of strong SP pressure in these three transmission settings within Malawi. The reexpansion of SP sensitive parasites in the region is not expected under current epidemiological conditions. Reduction in the amount of malaria in the region could further reduce the likelihood of reexpansion through the elimination of rare haplotypes due to genetic drift.

The Effect of Drug Pressure and Transmission Setting on Sulfadoxine-Pyrimethamine Resistant *Plasmodium falciparum* Haplotype Prevalence and Selective Sweep Characteristics, in Malawi

by
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for my parents Patricia and Nicholas Artimovich

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LIST OF ABREVIATIONS

ACT	Artemisinin based Combination Therapies
pfdhfr-ts	Dihydrofolate Reductase-thymidylate synthase (gene)
pfdhps	Dihydropteroate Synthase (gene)
DHFR	Dihydrofolate Reductase
DHPS	Dihydropteroate Synthase
EIR	Entomological Inoculation Rate
ICEMR	International Center for Excellence in Malaria Research
IPT	Intermittent Preventive Treatment
IPTp	Intermittent Preventive Treatment in pregnancy
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
SP	Sulfadoxine-Pyrimethamine
ST	Sulfamethoxazole-Trimethoprim
WHO	World Health Organization

I. INTRODUCTION

The continued expansion of *Plasmodium falciparum* resistance to antimalarial chemotherapies is a threat to public health and to the successful implementation of malaria control and elimination strategies. The expansion of artemisinin resistance into Africa would jeopardize the lives of millions throughout the continent. However, the return of drug sensitivity to some regions after drug policy changes has sparked hope that previously abandoned drugs, such as chloroquine and sulfadoxine pyrimethamine (SP), might once again find clinical utility. Malawi was the first African nation to adopt SP as the first line treatment of uncomplicated malaria, in 1993, in response to failing chloroquine efficacy. Within a decade of the removal of chloroquine pressure, chloroquine sensitive parasites reemerged in Malawi. The return of drug sensitivity to the region was shown to be due to reemergence of diverse sensitive parasites that had survived chloroquine selective pressure.

Within a decade of the switch to SP, SP treatment failures began to increase in Malawi. A corresponding increase in the prevalence of resistance polymorphisms within dihydrofolate reductase-thymidylate synthase (*pfdhfr-ts*) and dihydropteroate synthase (*pfdhps*) was also observed. In 2007, SP was replaced by an artemisinin-based combination therapy (ACT). Whether SP resistance in Malawi will decline, similar to chloroquine resistance, in the absence of drug pressure remains unknown. In settings with high malaria transmission (such as Malawi), greater parasite diversity, higher recombination rates, and more clinically immune individuals potentially harboring drug-

sensitive parasites are reported, which may affect the dynamics of drug resistance polymorphisms in the absence of drug pressure.

The following study estimates the effects of changes in SP drug pressure and variation in transmission setting on the prevalence of SP resistant haplotypes and the characteristics of the associated selective sweeps. In the face of emerging artemisinin resistance, such research may reveal whether, in the absence of drug pressure, SP-sensitive parasites have re-expanded in the population to the point where SP could once again be an effective treatment for malaria.

A. Specific Aims

1. Aim 1

Estimate the effect of drug pressure on the prevalence of sulfadoxine-pyrimethamine resistant haplotypes and characteristics of selective sweeps.

2. Aim 2

Estimate the effect of transmission setting on the prevalence of sulfadoxine-pyrimethamine resistant haplotypes and characteristics of selective sweeps.

B. Overview

Chapter II of the dissertation will present pertinent background information needed to understand the subject matter of the thesis research. Chapter III describes complete methodologies used in the course of the dissertation research. Chapters IV and V represent manuscripts that present the results of the thesis research, as well as specific

methods and discussions pertaining to each aim. Chapter VI is a general discussion of the research findings and implications.

II. BACKGROUND

A. Etiology and burden of malaria worldwide

The efficacy of anti-malarial drugs is essential to the health and survival of children in malaria endemic countries. In 2012, 207 million cases of malaria were reported worldwide, resulting in 627,000 deaths [1]. In Malawi alone, in 2012, there were approximately 143,100 malaria cases requiring hospitalization and resulting in over 5,500 deaths, most among children under the age of five years [1].

Malaria is caused by protozoan parasites of the genus *Plasmodium*. Several species of human malaria exist, of which *P. falciparum* is the most deadly. *P. falciparum* enters the human bloodstream through the bite of an infected female *Anopheles* mosquito. The parasites travel to the liver where they undergo asexual replication before being released into the blood stream. The haploid parasites then invade host red blood cells and undergo further asexual replication. Some parasites, however leave the asexual replication pathway and instead differentiate into male and female gametocytes that are ingested by another female mosquito where they undergo sexual reproduction within the mosquito midgut. Within the mosquito midgut the parasite develops into oocysts, which burst and released the asexual sporozoites. The sporozoites travel to the mosquito salivary gland where they are transmitted to the next human host when the mosquito takes another blood meal.

The burden of malaria disease is most heavy in sub-Saharan African nations, such as Malawi, which have an ideal climate for the mosquito vector, but often lack economic resources to properly control malaria. Treatment of clinical malaria with anti-malarial

drugs has been the focus of initial control efforts in Malawi. Through the 1970's, chloroquine was a safe and effective treatment for clinical malaria in Africa, however, the emergence and migration of chloroquine resistant parasites from SE Asia to Africa in 1997, led to the rapid expansion of chloroquine resistance through African countries utilizing chloroquine [2]. Malawi was the first African nation to switch from chloroquine to SP treatment in 1993 in response to failing chloroquine efficacy. The spread of SP-resistance from Southeast Asia, to Africa forced another drug policy change in Malawi, in 2007, to a more costly artemisinin-based combination therapy (ACT), artemether-lumefantrine, though SP is still used for intermittent preventive treatment in pregnancy (IPTp). With no new or more efficacious anti-malarial drugs on the immediate horizon and the artemisinin resistance becoming firmly established in Southeast Asia [3], Malawi and other malaria endemic countries are vulnerable to the next wave of anti-malarial resistance.

B. Evidence for the reemergence of drug sensitive parasites

In 2006, Laufer and colleagues reported the return of chloroquine clinical efficacy in Malawi, twelve after chloroquine was removed as the first-line therapy for treatment of clinical malaria [4]. A follow-up study demonstrated that the return of chloroquine sensitivity in Malawi resulted from a re-emergence of diverse sensitive parasites that had survived chloroquine selective pressure, possibly in clinically immune hosts. The reduction in resistant chloroquine parasites was the result of negative selection against chloroquine resistance alleles in the absence of the chloroquine selective pressure [5].

Evidence for directional selection of antimalarial drug resistance haplotypes has been demonstrated in the form of selective sweeps [6-8]. An allele which provides a fitness advantage for its host will be favored and increase in frequency within a population. As the selected allele increases in frequency, so do flanking neutral markers in linkage with the selected allele (i.e. genetic hitchhiking), leading to a regional reduction in the heterozygosity of neutral markers flanking the advantageous allele (a selective sweep). When several resistance alleles are inherited together, we refer to them as a resistance haplotype. Aside from providing evidence for positive directional selection of a specific resistance haplotype, the characteristics of a selective sweep are sometimes used to infer the strength of selection and how selection at the locus varies between regions and time points.

C. Origin, distribution and molecular epidemiology of sulfadoxine-pyrimethamine resistance

Polymorphisms in the genes dihydrofolate reductase-thymidylate synthase (*pfdhfr-ts*) and dihydropteroate synthase (*pfdhps*) have been shown to be the causative variants conferring SP resistance in *P. falciparum* [9]. Both DHFR and DHPS are enzymes in the parasite folate biosynthetic pathway. Inhibition of these enzymes through the use of sulfadoxine (DHPS) and pyrimethamine (DHFR), prevents the parasite from synthesizing folate co-factors needed in protein synthesis, DNA repair, and DNA replication [10]. Multiple origins of the resistance polymorphisms have been identified; however the most highly resistant haplotypes, those with three or more *pfdhfr-ts* resistance polymorphisms, or 2 or more *pfdhps* resistance polymorphisms have only a

few origins. DHFR (51I/59R/108N), the DHFR 'triple mutant' and DHPS 437G/540E, the DHPS 'double mutant', found in Africa, has been shown to have a single origin in Southeast Asia. In both cases the highly resistant haplotypes migrated to Africa and spread throughout the continent [11]. These resistance haplotypes have been shown to act in synergy with one and other, with each additional mutation within a haplotype conferring a greater degree of resistance (i.e. DHPS 437G/540E, exhibits more resistance than DHPS 437G alone). While the Asian DHFR 51/59R/108N and DHPS 437G/540E predominate in Malawi, the local independent emergence of a highly resistant DHPS 437G/540E/581G haplotype has been shown in several African nations [12]. Evidence of positive directional selection of resistance haplotypes in *pfdhfr-ts* and *pfdhps* has been shown in other African populations under SP pressure in the form of selective sweeps [8,13,14]. In the absence of SP pressure, resistance polymorphisms in *pfdhfr-ts* and *pfdhps* may confer a disadvantage to the parasite, allowing them to be outcompeted by sensitive parasites with fully functional DHFR and DHPS enzymes [15,16]. Under these circumstances, negative selection would then begin to act to reduce the prevalence of the resistant genotypes, while mutation and recombination would begin to increase heterozygosity in markers flanking the resistance haplotypes, degrading the selective sweep.

D. Factors effecting resistant haplotype population dynamics

Two studies conducted during high SP-use in Malawi, provide some insight into haplotype prevalence during this time. Kublin *et al.* found a 60% prevalence of DHFR-51I/59R/108N and DHPS 437G/540E mutants between 1997-1999. Lin *et al.* found an

81% prevalence of DHFR51I/108N and DHPS 437G/540E mutants in pregnant women, between 2003-2006 [17,18]. These studies suggest an increase in prevalence of highly resistant haplotypes, though differences in study participant demographics and methodologies prevent a direct comparison. The prevalence of SP- resistant haplotypes, after the reduction in SP drug pressure (in 2007), in Malawi has not yet been reported. A study in Kenya estimated changes in SP haplotype prevalence two years after a drug policy change from SP to ACTs and found a reduction in the prevalence of quintuple variants DHFR 51I/59R/108N and DHPS 437G/540E as compared to 51I/59R/108N variants, suggesting that the reduction of drug pressure may have led to a reduction in highly resistant haplotypes first, lending credence to the possibility of a fitness cost to resistance polymorphisms in these enzymes [19]. A significant increase in the number of sensitive alleles was also noted. We hypothesize that Malawian parasites will exhibit similar changes in resistance haplotype prevalence as a result of reduced SP drug pressure. The extent of any change in haplotype prevalence in Malawi following drug pressure reduction will be vital for informing public health policy and modeling of resistance haplotype population dynamics.

The rate of re-emergence of drug sensitive parasites depends in part on the extent of reduction of drug pressure [20]. Absence of drug pressure alone, however, may not be enough to cause a re-emergence of sensitive parasites; as evidenced by the persistence of SP-resistant parasites in Venezuela and chloroquine-resistant parasites in Cambodia, years after the removal of drug pressure [21,22]. A study of the frequency of Peruvian DHFR and DHPS resistance haplotypes showed a decline in DHFR 51I/108N/164L haplotypes within five years of SP removal as well as a decline in DHPS

437G/540E/581E [23]. A study in Kenya observed differential change in genotype prevalence between rural-moderate and rural-high transmission settings two years after replacing SP with ACTs [19]. Malaria parasites from areas of high transmission tend to be more diverse and have higher recombination rates [22,24,25]. In the absence of selective pressure, high parasite diversity and recombination rates begin to increase the level of heterozygosity observed around resistance loci. Recombination between resistant and sensitive parasites from diverse lineages will begin to increase heterozygosity within the resistant parasite population, decreasing the width and depth of the selective sweep [26,27]. Higher malaria transmission also results in a larger proportion of clinically immune hosts that can serve as a reservoir of drug-sensitive parasites that can re-expand after removal of drug pressure [28]. Areas with low malaria transmission tend to have lower levels of parasite diversity, leading to higher rates of inbreeding and more rapid rates of fixation of polymorphisms, possibly explaining why re-emergence of sensitive parasites was not seen in the low transmission settings of Cambodia or Venezuela [24]. The rate of malaria transmission throughout Malawi is higher than that observed in S. America and SE. Asia, yet relatively higher and lower transmission settings can be found in rural and urban environments within Malawi [29].

E. Study opportunity and significance

This study affords the opportunity to estimate the effect of a reduction in SP drug pressure and the effect of transmission setting on the prevalence of SP resistance haplotypes and the characteristics of their associated selective sweeps. Should artemisinin resistance spread from Southeast Asia to Africa, a return to previous first line treatments

such as chloroquine and SP may be a viable option for malaria treatment and control. This study will provide data about the dynamics of drug resistance polymorphisms in the Malawian parasite population. Such information may aid in the development of predictive models of SP-resistant parasite population dynamics, so that Malawi and other African nations with similar malaria epidemiology can make more informed decisions about anti-malarial use. Evidence of a transmission setting specific effect on resistance haplotype dynamics will further inform predictive models and allow for the development of transmission setting specific public health protocols. Conversely, evidence that transmission settings, similar to those in Malawi, do not significantly affect resistance haplotype dynamics could prevent unnecessary, costly regional control measures.

III. METHODS

A. Study Samples

1. High SP-use Period (1999-2001)

Samples representing time periods when SP was used as the first-line treatment for malaria in Malawi were selected from among participants in an SP efficacy study conducted in Ndirande district Blantyre, Malawi [18]. From among the efficacy study participants, 689 filter paper blood spots collected during the years 1999-2001 were used for evaluation in this study. Each blood spot represents a different patient's initial infection on the day they were admitted to the study, with uncomplicated malaria, prior to any treatment with SP [18].

2. SP-Transition Period (2007-2009)

Samples representing transition period, following the removal of SP as the first line treatment of uncomplicated malaria, were selected from among participants in a chloroquine combination therapy study conducted in Ndirande district Blantyre, Malawi. Children aged 6 months to 5 years who were admitted to the Ndirande Health Center with any signs or symptoms suggestive of malaria were included in the study. From among the participants in the study we selected all 623 primary infections [30]. Blood spots were collected upon a participant's admittance to the study, prior to any chemotherapy.

3. Low SP-use Period (2012)

Samples representing a time period, 5 years after SP was replaced as the first-line treatment for uncomplicated malaria were selected from a malaria surveillance study conducted by the International Center for Excellence in Malaria Research (ICEMR): Malawi, at Ndirande starting in 2012. All individuals admitted to Ndirande Health Center, with temperature ≥37.5 °C or history of febrile illness or other symptoms that could indicate malaria infection were included in the surveillance study. RDT-positive filter paper blood spots (n=968), gathered between January 2012 and December 2012, were included in this study.

4. Urban-low transmission setting

Ndirande, Blantyre served as the urban-low transmission setting in this study (Figure III.1). Blantyre is located in the southern highlands. Transmission intensity is difficult to estimate without entomological inoculation rates (EIRs). When EIRs are unavailable for a particular region, the amount of malaria in a region can be used as a surrogate for transmission intensity [31]. Recent, unpublished data from ICEMR: Malawi cross-sectional survey found 8.4% of all individual samples in the community were qPCR positive for *P. falciparum* parasites.

5. Rural-moderate transmission setting

Thyolo was the rural-moderate transmission setting in this study (Figure.III.1). Thyolo and Chikwawa share the same latitude; however, Thyolo is located at a higher

elevation, more equivalent to Ndirande. ICEMR: Malawi cross-sectional survey found 14.3% of participants to be parasitemic.

6. Rural-high transmission setting

Chikwawa served as the rural-high transmission setting in this study (Figure III.1). Chikwawa is located in a river delta at low elevation in southern Malawi. ICEMR: Malawi cross-sectional survey found 29.6% of participants to be parasitemic, over three times higher than Ndirande and two times higher than Thyolo. A study of EIR in Chikwawa 2002-2003 was 183 infectious bites per person per year [32]

Figure III.1: Map of Southern Malawi.

B. Genotyping of resistance loci

Samples were extracted from filter paper blood cards using a Qiagen BioRobot (Qiagen, Valencia, CA) following the Investigator Bloodcard Protocol. Parasite genotypes at polymorphic sites within the genes encoding DHFR and DHPS were

determined via pyrosequencing. Single nucleotide polymorphisms (SNPs) within codons 51, 59, 108, of DHFR and codons 437, 540, and 581 of DHPS were genotyped for all samples using primers and amplification methods adapted from Zhou *et al.* [33]. Pyrosequencing was performed on a PyroMark Q96 MD system (Biotage, Charlotte, NC). Allele frequency was adjusted based on a standard curve [34]. Polyclonal infections are common in Malawi. An allele with a relative frequency of 80% or greater within a given infection was designated as the predominant allele. Haplotypes were constructed using only the predominant allele. Samples without a predominant allele at two or more codons were labeled "mixed-genotype." Samples that were mixed at a single codon were treated as containing both possible haplotypes.

C. Genotyping Microsatellites

To determine if reduced heterozygosity around drug resistance genes was the result of selection rather than demographic processes, we measured expected heterozygosity in 6 unlinked neutral loci (TA81, TA40, pfPK2, PolyA, TA87, ARA2) located throughout the *P. falciparum* genome [35]. These unlinked microsatellites were amplified using previously published primers and amplification conditions [35]. When multiple peaks were identified within the same sample, peaks that were less than 1/3 the height of the tallest peak were ignored and the tallest peak was designated as the predominant allele [36]. Samples without a predominant allele at a given marker were designated polyclonal. Only samples with a predominant allele were included in estimates of expected heterozygosity.

Microsatellites flanking the *pfdhfr-ts* and *pfdhps* genes were genotyped for all samples. Eleven polymorphic microsatellites flanking *pfdhfr-ts* were genotyped; five downstream (+50kb, +20 kb, +1.48 kb, +0.52 kb, +0.2 kb), and six upstream (-0.3 kb, -1.2 kb, -3.8 kb, -4.5 kb, -10 kb, -30 kb) using previously described primers and protocols [7,27]. Eight polymorphic microsatellites flanking *pfdhps* were genotyped; four downstream +9.008 kb, +1.407 kb, +0.505 kb, +0.034 kb), and four upstream (-0.132 kb, -2.849 kb, -7.489 kb, -11.069 kb) of the gene, using primers described by Vinayak *et al*.[37]. PCR cycling conditions were optimized from the original protocols (Table III.1). For microsatellites flanking *pfdhfr-ts*, a 15µl total reaction volume was used, with 7.5µl 2X Promega Master Mix, 5.3µl ultra-pure water, 0.6µl of each primer and 1µl DNA template. Cycling conditions varied between markers. For *pfdhps* flanking microsatellites +1.407kb, + 0.034kb, +0.505kb and -7.489kb, 7.5µl 2X Promega Master Mix, 6.2µl ultra-pure water, 0.15µl of each primer and 1µl DNA template was used for the primary (external) PCR. For all other *pfdhps* microsatellites 7.5µl 2X Promega Master Mix, 5.3µl ultra-pure water, 0.6µl of each primer and 1µl DNA template was used per reaction. Cycling conditions varied between markers and are shown below. Fragment size was visualized using an Applied BioSystems 3730XL high-throughput 96-capillary DNA sequencer. Analysis of electropheragrams was performed using Genemapper software (version 4.0; ABI). A Perl script was used to assign the raw electropheragram scores to an integer allele size based on the expected repeat length and variation seen in the positive controls.

D. Estimating haplotype prevalence

The prevalence of each haplotype was estimated as the number of each haplotype observed among the successfully genotyped samples for each resistance gene, divided by the total number of genotyped samples. A resistant haplotype was defined as containing any number or combination of resistance alleles at the genotyped codons within either of the resistance genes of interest. The sensitive haplotype was defined as parasites with sensitive alleles at all codons within both genes. If a parasite had sensitive alleles at all loci for only one of the resistance genes (*pfdhfr-ts* or *pfdhps*), the parasite was referred to as a *pfdhfr-ts* sensitive or *pfdhps* sensitive. Samples that were mixed-genotype at a single locus (e.g. 51I/C59R/108N) were treated as having both haplotypes. Samples with mixed-genotype at two or more loci within the same gene were excluded because a haplotype could not be determined. Chi-squared tests with Yate's correction, where appropriate, were used to determine whether haplotype prevalence differed significantly between the three time periods or transmission settings.

E. Estimating Expected heterozygosity

Expected Heterozygosity (H_e), a measure of genetic diversity at each microsatellite locus, was calculated using the standard equation for H_e and variance:

$$H_e = \left(\frac{n}{n-1}\right)\left(1 - \sum p_i^2\right),\ \frac{2(n-1)}{n^3}\left\{2(n-2)\left[\sum p_i^3 - \left(\sum p_i^2\right)^2\right]\right\}.$$

The analysis focused on sweep characteristics flanking *pfdhfr-ts* 51I/59R/108N and *pfdhps* 437G/540E due to limited prevalence of the other haplotypes in the later time

periods. H_e (± 1 standard deviation) was calculated for each group. Samples without a predominant genotype or samples with missing data were excluded from expected heterozygosity calculations. This was done to ensure that the proportion of missing samples per locus would not affect the permuted distributions. Statistical significance was determined via permutation. Diversity ratios were calculated for each between-group comparison. Calculations for H_e, standard deviation and permutations were conducted in R[38]

Table III. 1: Primers and PCR cycling conditions.

Distance (kb)	Primers Forward	Primers Reverse	Length	Repeat Motif
-30	TTAGTGACAGTGAACAGGTA	AAATTTTCAAATCCAATTAC	150	(AT)14
-10	CAGAAGGGTTATAATAAGAT (outer)	TACAAATGAAGGTCGATTTT	262	(AT)10, (TTTA)7
	FAM-CGACATACATACATTTATCA (inner)			
-4.5	TTCTACGAATTATTTTTCCA	HEX-ACAAGTTAAAAGACGAAACA	198	(AT)12
-3.8	ACAGTTATAAGATTTAATGCAA	HEX-ACTGATGAAATTGTAAATGA	200	(AT)16
-1.2	ATTGAAACTAGCTCAACAAA	FAM-TTATAAGATTCCCATAGACA	215	(AT)6, (AT)5, (AT)15, (T)14
-0.3		GGCATAAATATCGAAAAC (outer)	106	(AT)15
	ATTCCAACATTTTCAAGA	HEX-TCCATCATAAAAAGGAGA (inner)		
DHFR	**DHFR**	**DHFR**	**DHFR**	**DHFR**
0.2	TATGAACAAATGATGACAAA (inner)	FAM-ATTTCATGTTCAGGTAAAAA (inner)	176	(T)10, (AT)16, (T)26
0.52	TAAAGAAGGCATAATTTTCA (inner)	HEX-CATTGAGATAAATAAGTGTTCA (inner)	108	(AT)17
1.48	TGGGACATATTTTTGATTAG (inner)	FAM-ACTTAAAATTGCCTTTACCT (inner)	203	(AT)9
20	GATGAAAATTTGCTTACTTAC (outer)	TGGTTCAATAAACGAGACC	285	(ATG)7
	FAM-TGTGCACATGAAATTTGTTC (inner)			
50	AATGTACTACAGTAAAACAGA (outer)	AATACAAGTGGAAAGTTACC	155	(AT)14
	AGCATATATGATGTAATAAGG (inner)			

Distance (kb)	Primer Forward	Primer Reverse	Length	Repeat Motif
-11.069	AACTTATACGTATCTAAAG	TGCGGGTATAATACATTA (inner)	221	(T)18, (AT)12
		CGATAAAATATTACCATTCAGGAT (outer)		
-7.489	TTTTAACTTGTATCAAGAAAT	TACAGCACTTAATGTAAATGGAG (inner)	167	(AT)14
		GGGTTTATATATTTGTGC (outer)		
-2.849	ATGTTTGAACCCCTTAATTTA	CACATGTAAATGCATATTTATG	195	(T)10, (AT)15
-1.504	TGTCTTGAAGGACAACACATAGATG	CATAATATGAAGAGACTGAAAGTT (inner)	198	(TA)24 & (TATT)8
		GTATAAGTTTTTTGCTTAATATGTTC (outer)		
-0.132	AAATATTTGCGCCAAACTTT	TAGATTTCTTTACGCAAAAT (inner)	138	(AT)10
		GTATTAGTTCTTGTATAGTTTCC (outer)		
DHPS	**DHPS**	**DHPS**	**DHPS**	**DHPS**
0.034	GACCAAGTGTAATTTAC (outer)	AGAGTACTTGACATATAATGAGCATG	152	(AT)26 and (TA)6
	GGAAAGTGCAAACATGT (inner)			
0.505	AGGAAAGTGTACGACGTTTATTGAATG	AGGACTGATCATATTACCAAG (inner)	151	(AT)13
		TGACTGCATGTACACCGAAGG (outer)		
1.407	GCATTCACACCAGTCTGCCTTCAA	AGGAGGTTTCCCTTCACTCCATCT (inner)	248	(A)20, (AT)17
		GATGTAGATACTATTAAGGAGG (outer)		
9.008	TGGAATTCATATTAATTGTAC	GAATAAATTAATTACACACGGAA (inner)	116	(AATA)9 & (AT)9
		TGTGAAGAGAATTATCAGGAATG (outer)		

DHFR-Primary 24 (+1.48, +50, -0.3)

1.	94C	2 minutes
2.	94C	30 seconds
3.	42C	30 seconds
4.	40C	30 seconds
5.	65C	40 seconds
6.	Repeat to step 2, 24x	
7.	65C	15 minutes
8.	4C	Hold

DHFR-Primary 35 (-10, +20, +50)

1.	94C	2 minutes
2.	94C	30 seconds
3.	42C	30 seconds
4.	40C	30 seconds
5.	65C	40 seconds
6.	Repeat to step 2, 34x	
7.	65C	15 minutes
8.	4C	Hold

DHFR-Unested 35 (+0.52, +0.2, +4.5, -1.2, -30)

1.	94C	2 minutes
2.	94C	30 seconds
3.	50C	30 seconds
4.	60C	30 seconds
5.	Repeat to step 2, 4x	
6.	94C	30 seconds
7.	45C	30 seconds
8.	60C	30 seconds
9.	Repeat to step 6, 34x	
10.	4C	Hold

DHFR-Secondary 15 (+1.48)

1.	94C	2 minutes
2.	94C	20 seconds
3.	45C	20 seconds
4.	65C	30 seconds
5.	Repeat to step 2, 14x	
6.	65C	15 minutes
7.	4C	Hold

DHFR-Secondary 35 (-7.5)

1.	94C	2 minutes
2.	94C	20 seconds
3.	45C	20 seconds
4.	65C	30 seconds
5.	Repeat to step 2, 34x	
6.	65C	15 minutes
7.	4C	Hold

DHFR-Secondary 15 (-10 , +1.48 +20, +50kb)

1.	94C	2 minutes
2.	94C	20 seconds
3.	45C	20 seconds
4.	65C	30 seconds
5.	Repeat to step 2, 14x	
6.	65C	15 minutes
7.	4C	Hold

DHPS-Primary 26 (+ 0.034, -11.069, +9.008, -0.132, +0.505)

1.	**94°C**	**2 min**
2.	**94°C**	**30 sec**
3.	**42°C**	**30 sec**
4.	**40°C**	**30 sec**
5.	**65°C**	**40 sec**
Repeat sept 2, 25x		
6.	**65°C**	**2 min**
7.	**4°C**	**HOLD**

DHPS-Unnested 30 (-2.849)

1.	**94°C**	**2 min**
2.	**94°C**	**30 sec**
3.	**50°C**	**30 sec**
4.	**60°C**	**30 sec**
Repeat step 2, 15x		
5.	**94°C**	**30 sec**
6.	**45°C**	**30 sec**
7.	**60°C**	**30 sec**
Repeat step 2, 15x		
8.	**4°C**	**HOLD**

DHPS-Secondary: All markers

1.	**94°C**	**2 min**
2.	**94°C**	**20 sec**
3.	**45°C**	**20 sec**
4.	**65°C**	**30 sec**
Repeat sept 2, 15x		
5.	**65°C**	**2 min**
6.	**4°C**	**HOLD**

DHPS-Primary 26 (-7.4

1.	**94°C**	**2 min**
2.	**94°C**	**30 sec**
3.	**40°C**	**30 sec**
4.	**40°C**	**30 sec**
5.	**65°C**	**40 sec**
Repeat sept 2, 25x		
6.	**65°C**	**2 min**
7.	**4°C**	**HOLD**

DHPS-Unnested 26 (+

1.	**94°C**	**2 min**
2.	**94°C**	**30 sec**
3.	**50°C**	**30 sec**
4.	**40°C**	**30 sec**
5.	**65°C**	**40 sec**
Repeat step 2, 25x		
6.	**65°C**	**2 min**
7.	**4°C**	**HOLD**

IV. PERSISTENCE OF SULFADOXINE-PYRIMETHAMINE RESISTANCE DESPITE REDUCTION OF DRUG PRESSURE IN MALAWI. [1]

A. ABSTRACT

***Background*:** In 2007, Malawi replaced sulfadoxine-pyrimethamine (SP) with artemisinin-based combination therapy as the first-line treatment for uncomplicated *Plasmodium falciparum* malaria in response to failing SP efficacy. Here we estimate the effect of reduced SP drug pressure on the prevalence of SP-resistant parasites and the characteristics of the associated selective sweeps flanking the resistance loci.

***Methods**:* Samples from high SP use (1999-2001), transition (2007-2008) and low SP use (2012) time periods were genotyped for resistance markers at *pfdhfr-ts* codons 51, 59, 108, and *pfdhps* 437, 540 and 581. Expected heterozygosity was estimated to evaluate genetic diversity flanking *pfdhfr-ts* and *pfdhps.*

Results: An increase in the prevalence of the resistant haplotypes DHFR 51I/59R/108N and DHPS 437G/540E occurred under sustained drug pressure with no change in haplotype prevalence five years after reduction in SP drug pressure. The DHPS 437G/540E/581G haplotype was observed in 2007 and increased during a period of reduced SP pressure. Changes to the sweep characteristics flanking *pfdhfr-ts* and *pfdhps* were minimal.

***Conclusions**:* In contrast to the rapid and complete return of chloroquine sensitive falciparum malaria after chloroquine was withdrawn from Malawi, a re-emergence of SP efficacy is unlikely.

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[1] Artimovich. E, Schneider. K, Taylor.T.E, Kublin. J, Dzinjalamala. F, Laufer. M.K, Plowe. C.V D, Takala-Harirson. S. Persistence of sulfadoxine-pyrimethamine resistance despite reduction of drug pressure in Malawi. Submitted to Journal of Infectious Disease

Key words: Malaria, Sulfadoxine-pyrimethamine, resistance, selective sweeps, pyrosequencing, DHFR, DHPS

B. INTRODUCTION

The continued expansion of *Plasmodium falciparum* resistance to antimalarial chemotherapies, is a threat to public health, and malaria control and elimination strategies. The return of drug sensitivity to some regions after drug policy changes has renewed interest that previously abandoned drugs, such as chloroquine and sulfadoxine pyrimethamine (SP), might once again find clinical utility. Malawi was the first African nation to adopt SP as the first-line treatment of uncomplicated malaria, in 1993, in response to high levels of chloroquine resistance in Malawi. Within a decade of the removal of chloroquine pressure, chloroquine-sensitive parasites reemerged and predominated in Malawi [4]. Reemergence was shown, through analysis of selective sweeps, to be due to the re-emergence of diverse sensitive parasites that had survived chloroquine selective pressure, possibly in clinically immune hosts [5,6]. Within a decade of the switch to SP, treatment failures began to increase in Malawi associated with an increase in the prevalence of resistance polymorphisms within dihydrofolate reductase-thymidylate synthase (*pfdhfr-ts*) and dihydropteroate synthase (*pfdhps*), the genes responsible for SP resistance [18]. In 2007, SP was replaced by an artemisinin-based combination therapy (ACT). SP was still used after 2007 for intermittent preventive treatment of malaria in pregnancy (IPTp) (25). Whether SP resistance in Malawi will decline, similar to chloroquine resistance, in the absence of drug pressure remains unknown.

Evidence for directional selection of antimalarial drug resistance polymorphisms has been demonstrated in the form of selective sweeps [7,39,40]. An allele that provides a fitness advantage will be favored and increase in frequency in a population. As the selected allele increases in frequency, so do flanking neutral markers in linkage with the selected allele, leading to a regional reduction in the heterozygosity of neutral markers flanking the advantageous allele. This phenomenon is referred to as a selective sweep[41]. Aside from providing evidence for positive directional selection of a specific resistance haplotype, the characteristics of a selective sweep can be used to infer the strength of selection and how epidemiological factors affect selection on temporal and spatial scales.

Selective sweeps flanking *pfdhfr-ts* and *pfdhps* have been shown in other African populations under SP pressure [42]. In the absence of SP pressure, resistance polymorphisms in the *pfdhfr-ts* and *pfdhps* genes may confer a disadvantage to the parasite, allowing them to be outcompeted by sensitive parasites with fully functional DHFR and DHPS enzymes [15,16,43]. Under these circumstances, negative selection would begin to act to reduce the prevalence of the resistant genotypes, while recombination, and mutation would begin to increase heterozygosity in markers flanking the resistance-conferring genes, degrading the selective sweep.

The purpose of this study was to estimate how changes in SP drug pressure affected both the prevalence of SP resistant haplotypes and the characteristics of the associated selective sweeps. We aimed to test the hypothesis that SP drug pressure positively selected for SP resistant haplotypes during a period of high SP-use and that a subsequent reduction in SP drug pressure, following the switch to ACTs in Malawi,

selected against these highly resistant parasites and allowed for the expansion of SP-sensitive parasites.

C. METHODS

1. Study samples: All study samples were collected from Ndirande district within the city of Blantyre, Malawi. Samples from episodes of symptomatic malaria were analyzed from three time points. Samples from 1999-2001 (n=689) and 2007-2009 (n=623) represented the first clinical malaria infections in therapeutic efficacy studies. Samples from 2012 (n=968) were from episodes of symptomatic malaria that were identified at the government health center [18,30].

2. Genotyping of resistance loci: DNA extraction from filter paper blood cards was performed on all collected samples, using a Qiagen BioRobot (Qiagen, Valencia, CA) following the Investigator Bloodcard Protocol. Parasite genotypes at polymorphic sites within *pfdhfr-ts* and *pfdhps* were determined via pyrosequencing. Single nucleotide polymorphisms (SNPs) within codons 51, 59, 108, of *pfdhfr-ts* and codons 437, 540, and 581 of *pfdhps* were genotyped for all samples using primers and amplification methods adapted from Zhou *et al*. [33]. Pyrosequencing was performed on a PyroMark Q96 MD system (Biotage, Charlotte, NC). Allele frequency was adjusted based on a standard curve [34]. When a polyclonal infection was present, an allele with a relative frequency of 80% or greater within a given infection was designated as the predominant allele. Haplotypes (series of *pfdhfr-ts* or *pfdhps* alleles) were constructed using only the predominant allele. Samples that were mixed at a single codon were treated as containing

both possible haplotypes. Samples without a predominant allele at two or more codons were labeled "mixed-genotype."

3. Genotyping of microsatellites: To determine if reduced heterozygosity around drug resistance genes was the result of selection or demographic processes, we measured expected heterozygosity in 6 unlinked neutral loci (TA81, TA40, pfPK2, PolyA, TA87, ARA2) located throughout the *P. falciparum* genome, using primers and protocols described by Anderson and colleagues in 1999 [35]. When multiple peaks were identified within the same sample, peaks that that were at least one-third the height of the tallest peak were called and the tallest peak was designated as the predominant allele [44]. Samples without a predominant allele at a given marker were designated as polyclonal. Only samples with a predominant allele were included in estimates of expected heterozygosity.

Microsatellites flanking the *pfdhfr-ts* and *pfdhps* genes were genotyped for all samples. Eleven polymorphic microsatellites flanking *pfdhfr-ts* were genotyped; five downstream (+50 kb, +20 kb, +1.48 kb, +0.52 kb, +0.2 kb), and six upstream (-0.3 kb, -1.2 kb, -3.8 kb, -4.5 kb, -10 kb, -30 kb) using previously described primers and protocols [8,27]. Eight polymorphic microsatellites flanking *pfdhps* were genotyped; four downstream (+9.008 kb, +1.407 kb, +0.505 kb, +0.034 kb), and four upstream (-0.132 kb, -2.849 kb, -7.489 kb, -11.069 kb) of the gene, using primers described by Vinayak *et al.* [37]. PCR cycling conditions were optimized from the original protocols (Supplementary Table VII.1). Fragment size was visualized using an Applied BioSystems 3730XL high-throughput 96-capillary DNA sequencer. Analysis of electropheragrams

was performed using Genemapper software (version 4.0; ABI). A Perl script was used to assign the raw electropheragram scores to an integer allele size based on the expected repeat length and variation seen in the positive controls.

4. Statistical analysis

a. DHFR and DHPS Haplotype prevalence: The prevalence of each haplotype was estimated as the number of each haplotype observed among the successfully genotyped samples for each resistance gene, divided by the total number of successfully genotyped samples. Only samples with a genotyped allele at all assayed codons were used in the analysis. A SP-resistant haplotype was defined as containing any number or combination of resistance polymorphisms (polymorphisms that confer pyrimethamine or sulfadoxine resistance) at the genotyped codons within either of the resistance genes of interest. The SP-sensitive haplotype was defined as the wildtype (no mutations at any of the genotyped *pfdhrf-ts* and *pfdhps* codons). Samples with mixed-genotype at two or more codons within the same gene were excluded because a haplotype could not be determined. DHFR and DHPS haplotypes were treated and reported separately. Chi-squared tests, with Yate's correction where appropriate, were used to determine whether haplotype prevalence differed significantly between the three time periods.

b. Expected heterozygosity: Expected Heterozygosity (H_e), a measure of genetic diversity at each microsatellite locus, was calculated using the following standard equation for H_e and variance.

$$H_e = \left(\frac{n}{n-1}\right)\left(1-\sum p_i^2\right), \frac{2(n-1)}{n^3}\left\{2(n-2)\left[\sum p_i^3 - \left(\sum p_i^2\right)^2\right]\right\}.$$

H_e (± 1 standard deviation) was calculated for three groups: high SP-use time period from 1999-2001, transition time period from 2007-2008, and low SP-use time period in 2012, for both the *pfdhfr-ts* and *pfdhps* genes. Samples without a predominant genotype or samples with missing data were excluded from expected heterozygosity calculations. Statistical significance was determined via permutation [5]. Diversity ratios were calculated for $He_{highSP(1999\text{-}2001)}/He_{TransSP(2007\text{-}2008)}$, $He_{highSP(1999\text{-}2001)}/He_{LowSP(2012)}$, $He_{TransSP(2007\text{-}2008)}/He_{LowSP(2012)}$. Calculations for H_e, standard deviation and permutations were conducted in R [38].

D. RESULTS

1. Haplotype Prevalence: Complete *pfdhfr-ts* haplotypes were obtained for 394 samples from the high SP-use time point, 563 samples from the SP-transition period, and 549 samples from the low SP-use time period. For *pfdhps* 550 produced complete haplotypes for the high SP-use time period, 556 samples produced complete haplotypes from the SP-transition time period, and 546 samples produced complete haplotypes from the low SP use time period. Failure of samples to be amplified and produce complete haplotypes was likely due to sample age/quality. The analysis focused on sweep characteristics flanking DHFR 51I/59R/108N and DHPS 437G/540E haplotypes due to the small number of double and single mutant haplotypes for both *pfdhfr-ts* and *pfdhps* in the later time periods.

A statistically significant increase in the prevalence of the DHFR 51I/59R/108N was observed between the period of high SP-use and the SP-transition period (87% to 97% $p<0.0001$) (Figure IV.1A). No change in DHFR triple mutant haplotype prevalence

over the 5 years between the SP-transition period and low SP-use period was observed (p=0.94). Concurrent with the increase in DHFR 51I/59R/108N haplotypes was a decline in the prevalence of the less resistant DHFR 51I/108N and 59R/108N. Unlike the increase in DHFR 51I/59R/108N, the decrease in DHFR 51I/108N and 59R/108N haplotypes continued through the low SP-use time point. The decrease was significant for both double mutant haplotypes ($p<0.0001$), though the decrease from the transition period to low SP use periods were borderline significant (51I/108N p=0.01 and 59R/108N p=0.05). No parasites with sensitive alleles at all DHFR codons were observed in this study. In addition, there was a decrease in the prevalence of the mixed genotype infections (4% to 0%, $p<0.0001$) between high SP-use and the SP-transition time periods but no significant change in mixed genotype prevalence between the SP-transition time period and the low-SP use time period.

The DHPS 437G/540E haplotype prevalence increased from 84% to 95% (437G/540E, $p<0.0001$) between the high SP-use and the SP-transition samples sets, but there was no significant change in haplotype prevalence between the SP-transition and the low SP-use time period (95%-97%) (Figure IV.1B). The DHPS 437G/540E/581G haplotype was not observed in the high SP-use time period, but appeared at low prevalence, 2% and 4% respectively, in the subsequent periods ($p<0.0001$); with the between time period p values of (p=0.0044) from high SP-use to SP-transition and (p=0.039) from SP-transition to low SP-use. The prevalence of the haplotype containing all sensitive alleles within DHPS decreased between the high SP-use and SP-transition time periods ($p<0.0001$) and there was no change between the SP-transition and low SP-use samples sets. No change in haplotype prevalence was observed for DHPS single

mutants 437G across any of the time periods. A reduction in the prevalence of DHPS 540E was seen between high SP-use and SP-transition period (p=0.0044). Similar to the findings at *pfdhfr-ts*, a decrease in the prevalence of mixed genotype infections was found between the high SP-use and SP-transition time period (p<0.0001), though, there was no change in haplotype prevalence after the switch from SP to ACTs.

2. Characteristics of Selective Sweeps: Due to the lack of SP sensitive parasites in the data set, changes in H_e at flanking loci were compared to the average H_e of the unlinked loci, and +/- 1 standard deviation was used to assess change in H_e relative to the average unlinked H_e for the parasite population (see Supplementary Figure VII.1). Average H_e at unlinked microsatellites was 0.872 for high SP-use, 0.897 for SP-transition, and 0.904 for low SP-use time periods. These levels were not significantly different from each other (p=0.55), though the difference between individual markers was sometimes significant (see Supplementary Figure VII.1). None of the unlinked loci had H_e less than 0.8, consistent with estimates in other studies that have estimated population demographic history [5,22,27,35,45].

A reduction in expected heterozygosity (H_e) flanking the DHFR 51I/59R/108N (Figure IV.2A), consistent with a selective sweep, was observed for all time periods as compared to the average H_e of the unlinked neutral microsatellites. At *pfdhfr-ts*, H_e returned to that of unlinked markers at 30kb downstream, during the transition and low SP use periods. Comparing the sweep characteristics from the three time points, we observed asymmetrical changes in H_e across *pfdhfr-ts* flanking microsatellites. Upstream changes did not show a consistent trend toward sweep expansion under continued drug

pressure, though a reduction in H_e was observed at the distal downstream edge of the sweep. An increase in H_e after the reduction of SP-pressure occurred at the most distal upstream edge of the sweep. However, no further degradation in sweep characteristics was found between SP-transition and low SP-use periods.

Analysis of microsatellites flanking *pfdhps* also indicated the presence of selective sweeps flanking the locus in all three time periods (Figure IV.2B). H_e at the genotyped flanking microsatellites did not return to average H_e of unlinked loci. The sweep flanking *pfdhps* extended at least 10kb in either direction. Evidence of sweep degradation was found at the extreme edges of the observed sweep. However, overall sweep characteristics did not show a consistent increase or decrease in H_e between time periods.

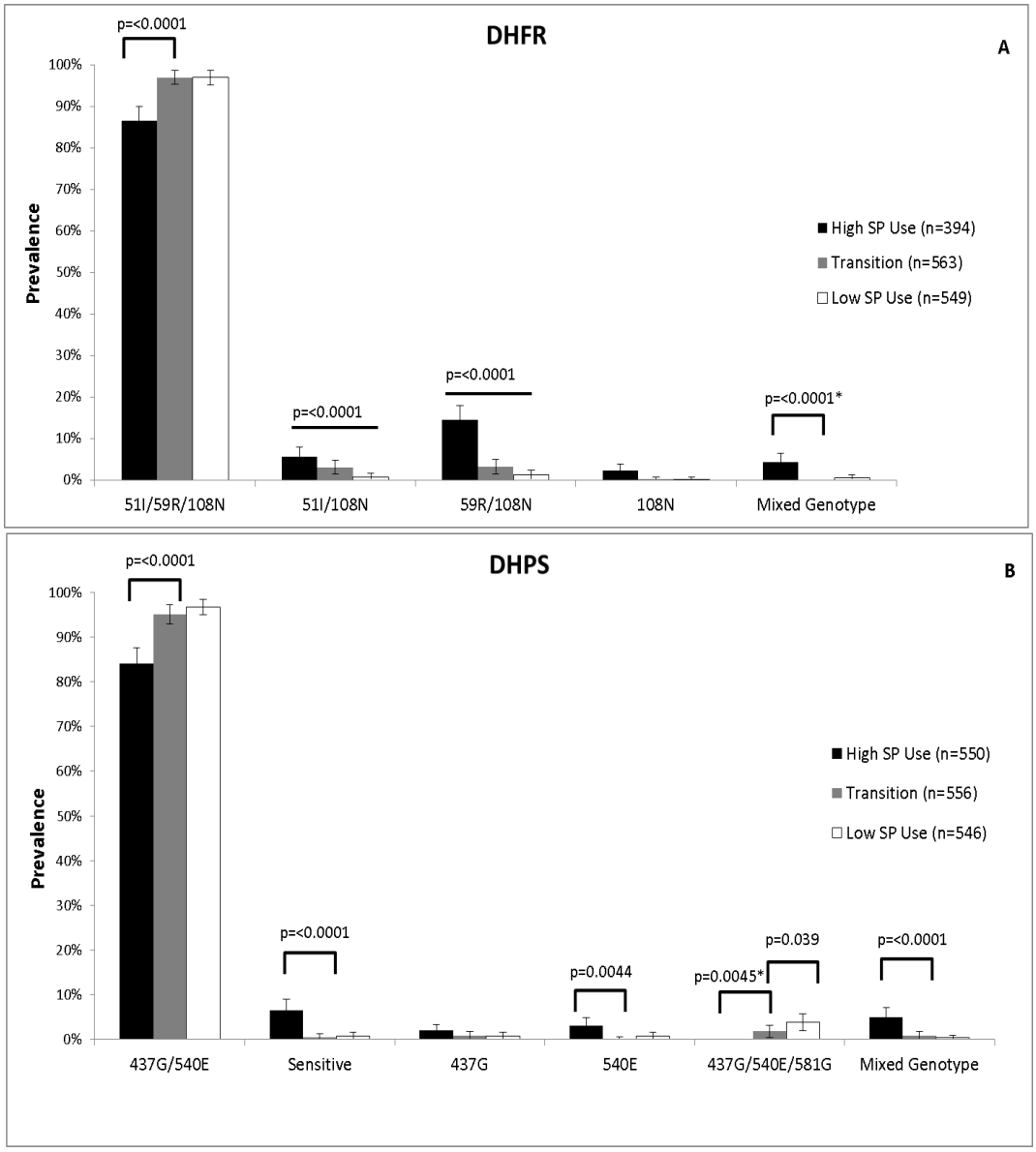

Figure IV. 1: Effect of drug pressure on SP resistant haplotype prevalence. Change in dihydrofolate reductase (DHFR) (A) and dihydropteroate synthase (DHPS) (B), haplotype prevalence over three time periods in the history of SP use in Ndirande, Malawi. Prevalence is calculated as the percentage of individuals with a given haplotype. The three time points included: High SP-use (1999-2001), SP-transition (2007-2008) and low SP-use (2012). Black bars indicate significance with the p values written above. Error bars represent 95% CI.* indicates use of Yates's correction.

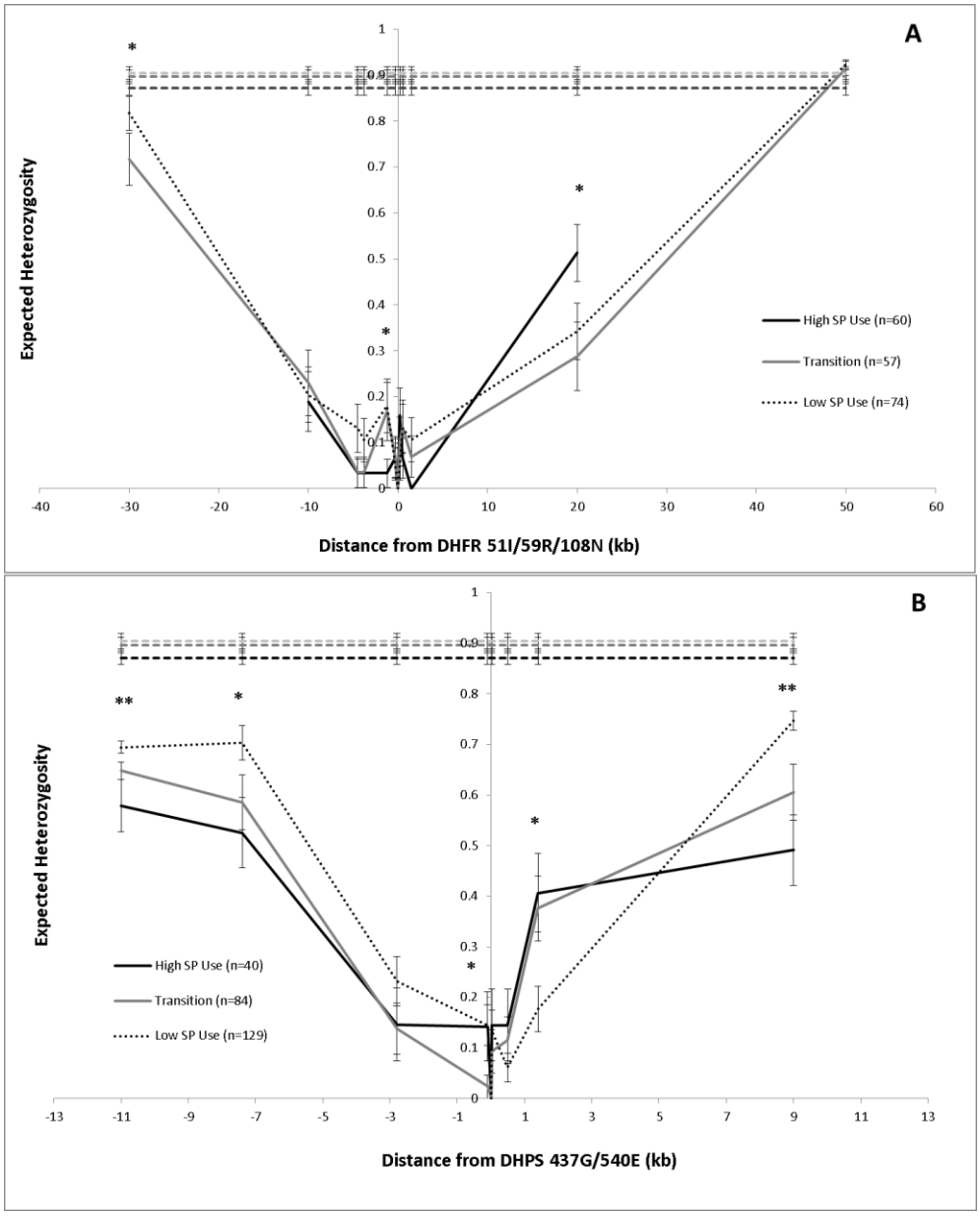

Figure IV.2: Effect of drug pressure on SP resistant haplotype selective sweeps characteristics. Expected heterozygosity in microsatellite loci flanking *dhfr-ts* (A) and pfdhps (B). Samples with missing data were excluded. Error bars represent +/- 1 standard deviation. Dashed lines represent average genomic level H_e, based on unlinked loci. Alpha <0.05 * indicates a significant difference between two time periods. ** indicates a significant difference between all three time periods, based on permutation.

Haplotype Origin: Mapping of microsatellite haplotypes indicated the *pfdhfr-ts* and *pfdhps* haplotypes to be of Southeast Asian origins, as compared to V1/S, 3D7 and HB3 control strains (Supplementary Figure VII.3). Analysis of DHPS 473G/540E/581G haplotype indicated it arose from the DHFR 437G/540E genetic background present in Malawi.

To determine whether the reduction in the prevalence of mixed genotypes was evidence of a population level reduction in number of polyclonal infection (those with two or more parasite clones), or evidence of only a reduction in genetic diversity at the resistance loci, we assessed the change in the number of samples scored as polyclonal based on the presence of multiple alleles at the unlinked microsatellites. We found a 37% reduction in the absolute number of samples scored polyclonal between high SP and low SP use time periods ($p<0.0001$). An intermediate reduction in polyclonal samples (33%) was also observed between the high and transition period ($p<0.0001$). The difference in polyclonal infections between transition and low SP use time periods was not significant ($p=0.41$).

E. DISCUSSION

Our study demonstrates the persistence of SP resistant malaria and the presence of selective sweeps, five years after the removal of SP as the first-line treatment of uncomplicated malaria in Malawi. Our findings suggest little to no fitness cost of the DHFR triple and DHPS double mutant haplotypes in the absence of strong SP pressure, under current epidemiological conditions. Our findings also present evidence that the

DHPS 437G/540E/581G triple mutant haplotype may be increasing in prevalence despite the switch to ACTs.

As we hypothesized, we have shown an increase in the prevalence of the most highly resistant DHFR and DHPS haplotypes, with a corresponding decrease in the prevalence of less resistant and of sensitive haplotypes, over the period of persistent high SP-use (1999-2007), consistent with selection for the highly resistant haplotypes by SP drug pressure. However, in contrast with the pattern observed with chloroquine resistance, we did not see a decrease in the prevalence of highly SP-resistant parasites after SP removal as the first-line treatment for uncomplicated malaria. Near fixation of DHFR 51I/59R/108N and DHPS 437G/540E may contribute to the persistence of these highly resistant haplotypes, as few sensitive alleles and no sensitive parasites were found to compete with resistant parasites. Over a greater period of time, any slight fitness cost to the DHFR 51I/59R/108N and DHPS 437G/540E haplotypes might become more apparent.

Microsatellite analysis was successful in identifying and defining the characteristics of the selective sweeps flanking *pfdhfr-ts* and *pfdhps* within the three time periods, however, the changes in sweep characteristics (coupled with prevalence findings) did not suggest a strong fitness cost of the SP resistant haplotypes At *pfdhfr-ts* it appears that selective pressure was maintained and caused a further reduction in H_e 20kb downstream during the time of transition from SP to ACTs. After SP drug pressure was relaxed in 2007-2008 an increase in H_e occurred in the low SP-use samples in both distal and proximal markers. The increase in H_e is significant only at the upstream of *pfdhfr-ts*. If this observation were made along with a reduction in SP-resistant haplotype

prevalence, it would suggest a fitness cost to the DHFR triple mutant haplotype. However, we did not find a reduction in prevalence, indicating that this increase in H_e may simply be the natural decay of the sweep due to recombination over time. The *pfdhfr-ts* sweep characteristics do not suggest a strong fitness cost of the triple mutant haplotype in the absence of SP pressure. Asymmetry in H_e, as seen flanking *pfdhfr-ts* and *pfdhps* have been reported elsewhere, and may be due to differences in mutation rates at each microsatellite locus. [27,42,46] The removal of samples with missing data (to prevent biased estimates of significance) from the analyses reduced sample size and could have biased our findings against rare low-frequency haplotypes and artificially reduced H_e. However, when sweeps were constructed using samples with varying degrees of missing data, there was no effect on the overall sweep characteristics to suggest that the exclusion of missing data affected the study conclusions. In addition, Hale *et al.* has demonstrated that only 25-30 samples are needed to accurately estimate population level genetic diversity.[47]

The decline of the DHFR double mutant haplotypes, without an increase in DHFR sensitive parasites, implies a continued selection against the DHFR double mutant haplotypes in favor of the DHFR triple mutant, rather than a fitness cost to the DHFR double mutants, relative to the sensitive parasites. A limitation of estimating haplotype prevalence (as opposed to allele frequency) is the inability to directly attribute an increase in one haplotype to a decrease in another haplotype. Two recently published methods for determining multiplicity of infection allow for the more accurate assessment of resistant allele/haplotype frequency (as opposed to haplotype prevalence) when assessing resistance allele dynamics [48,49]. However, the low prevalence of

mixed-genotype infections and no change in the proportion of polyclonal infections in 2007-2008 and 2012 suggests that using these methods would be unlikely to have a significant effect on our estimates of haplotype prevalence in this setting.

The decline in mixed genotype infections, and the reduction in polyclonal infections in the absence of any change in average H_e at unlinked loci, is consistent with a reduction in malaria transmission levels in Malawi throughout the three study time periods. It is likely that sustained drug pressure through 2007, as well as an overall reduction in malaria transmission through malaria control measures and access to effective antimalarial medication, led to the reduction in mixed-genotype and polyclonal infections. A consequence of reducing the amount of malaria in a region may be the extinction of rare haplotypes (such as SP-sensitive) due to genetic drift. With little or no selective pressure against the SP resistant haplotypes, these haplotypes may reach fixation, and further reduce the likelihood of a re-emergence of SP-sensitive haplotypes. A minimal metabolic cost to the resistant haplotypes may lead to many decades of persistent SP resistance, and prohibit the future clinical use of SP.

Our finding of continued high rates of SP resistant parasites despite cessation of SP use for the treatment of malaria could also be due in part to antifolate drug pressure that continues from other sources. Use of the antibiotic sulfamethoxazole-trimethoprim as prophylaxis in people living with HIV and for treatment of acute illnesses and low level SP pressure through SP-IPTp exposure may be sources of continued selection for SP-resistance mutations, as they both act upon the same folate enzymes DHFR and DHPS and laboratory evidence suggests cross-resistance.[50] However, there is no evidence sulfamethoxazole-trimethoprim has any impact on SP-resistant allele

prevalence, or that SP resistance impacts sulfamethoxazole-trimethoprim efficacy [51,52].

Our data confirm the findings by Taylor *et al.* that the Malawian 581G allele arose on the DHPS double mutant genetic background, but indicate that the 581G haplotype emerged by 2007, three years earlier than previously reported [12]. The increase in DHPS 437G/540E/581G prevalence, across the three time periods, suggests continued selection for this haplotype. Too few DHPS triple mutants (437G/540E/581G) were found in 2012 to meaningfully assess the extent of recent selection of this haplotype. The increase in DHPS 437G/540E/581G haplotype could threaten the efficacy of ongoing SP-IPTp treatment in the nation as the presence of 581G allele is associated with SP-IPTp failure [53]. Ongoing molecular monitoring of the 437G/540E/581G haplotype prevalence and SP-IPTp treatment should be performed to determine the impact (in any) this selection could have for IPT efficacy in Malawi.

These data suggest that, in contrast to the rapid and complete return of fully chloroquine sensitive parasites after cessation of chloroquine use, a similar resurgence of SP sensitivity in Malawi or in similar epidemiological settings is unlikely. Not only did high level SP-resistance persist for five years after the removal of SP as the first line treatment of uncomplicated malaria, but the prevalence of the DHPS 437G/540E/581G haplotype increased. In the face of expanding ACT resistance in Southeast Asia with a risk of spread to Africa, other antimalarial drug classes, perhaps including chloroquine, must be explored, as SP will likely remain an ineffective treatment for uncomplicated malaria in Malawi.

V. SULFADOXINE-PYRIMETHAMINE RESISTANT HAPLOTYPE PREVALENCE AND SELECTIVE SWEEP CHARACTERISTICS IN THREE TRANSMISSION SETTINGS IN MALAWI[2]

A. ABSTRACT

Background: Persistence of sulfadoxine-pyrimethamine (SP) resistance has been described in an urban-low transmission setting in Malawi. Higher transmission settings are associated with greater genetic diversity and genetic recombination, which could lead to a more rapid re-emergence of SP sensitive parasites, as well as more rapid degradation of selective sweeps. In this study, we investigated the impact of rural-moderate and rural-high transmission settings on haplotype prevalence and selective sweep characteristics in Malawi.

Methods: Samples from urban-low, rural-moderate, and rural-high transmission settings were genotyped for resistance markers within *pfdhfr-ts* and *pfdhps*. Expected heterozygosity (H_e) was estimated to evaluate genetic diversity.

Results: No difference in the prevalence of highly resistant DHFR 51I/59R/108N and DHPS 437G/540E was found between transmission settings. Differences in H_e flanking *pfdhfr-ts* and *pfdhps* were seen between rural-moderate and the other settings, as well as some shared haplotypes between rural-high and urban-low settings.

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[2] [2]Artimovich. E, Kapito-Tembo. A, Seydel. K, Pensulo .P,Brown. S, Joshi. S, Schneider. K, Taylor.T.E, Kublin. J, Dzinjalamala. F, Laufer. M.K, Nyirenda,O, Mathanga. D, Takala-Harirson. S. Sulfadoxine-pyrimethamine resistant haplotype prevalence and selective sweep characteristics in three transmission settings in malawi. In preparation for submission

Conclusions: Our results do not show an effect of local variation in transmission setting, as measured by percent parasitemia, on SP resistant haplotype prevalence.

B. INTRODUCTION

The potential expansion of artemisinin-resistant *Plasmodium falciparum,* from Asia to Africa, has heightened interest in identifying factors affecting resistance allele dynamics, including the re-emergence of drug sensitive malaria parasites. Chloroquine and sulfadoxine-pyrimethamine (SP) were both once used as safe and effective primary treatment for uncomplicated malaria. Successive waves of anti-malarial resistance, from Southeast Asia prompted public health organizations to abandon chloroquine, and then SP, in favor of the artemisinin-based combination therapies (ACTs).

After the removal of chloroquine drug pressure in Malawi in 1993, chloroquine sensitive parasites reexpanded in the population, eventually outcompeting the chloroquine resistant strains [4]. The return of chloroquine resistance was shown, via the analysis of selective sweeps, to be the result of the re-emergence of genetically diverse sensitive parasites that had survived selective pressure [5]. This relatively rapid, and nationwide re-emergence of genetically diverse drug-sensitive parasite in Malawi was unexpected, as drug-resistance had persisted in South America and Southeast Asia many years after reduction in drug pressure.[2-4]

In late 2007, Malawi replaced SP with an ACT in response failing SP efficacy. We have recently shown the persistence of the highly resistant haplotypes of dihydrofolate reductase and dihydropteroate synthase, DHFR 51I/59R/108N and DHPS 437G/540E, five years after the reduction of SP drug pressure. Selective sweep analysis

suggested little to no fitness cost of SP resistance in the urban setting of Ndirande where local transmission intensity is low. Whether this persistence is nationwide or regionally specific is yet unknown.

Areas of higher malaria transmission are associated with a greater proportion of clinically immune individuals, greater parasite genetic diversity, and higher recombination rates; whereas areas of lower transmission are associated with more rapid fixation of alleles [22,27]. Thus in a higher transmission setting we might expect a more rapid resurgence of sensitive parasites in the absence of drug pressure, as well as more rapid degradation of the associated selective sweeps. Thus, we hypothesized that in this study we estimate the prevalence of DHFR and DHPS resistance haplotypes in urban-low, rural-moderate, and rural-high transmission settings within Malawi and the characteristics of the associated selective sweeps.

C. METHODS

Samples were collected as part of a malaria surveillance study conducted in three district health centers: rural-high (Chikwawa), rural-moderate (Thyolo) and urban-low (Ndirande), in Malawi. Samples were collected from individuals presenting at the district health centers with uncomplicated malaria. Samples consisted of blood spots, collected on filter papers, representing a patient's initial infection on the day they were admitted to the study, prior to treatment. Entomological inoculation rates were not available for all sites. We used percent parasitemia from a community-based cross-sectional survey as a surrogate measure of transmission intensity; 8.4%, 14.3%, and 29.6% of individuals

surveyed in the urban-low, rural-moderate, and rural-high settings, respectively, were qPCR positive for *P.falciparum* parasites.

DNA was extracted from filter paper blood cards using a Qiagen BioRobot (Qiagen, Valencia, CA) following the Investigator Bloodcard Protocol. Parasite genotypes at polymorphic sites within *pfdhfr-ts* and *pfdhps* genes were determined via pyrosequencing. Single nucleotide polymorphisms (SNPs) within codons 51, 59, 108, of *pfdhfr-ts* and codons 437, 540, and 581 of *pfdhps* were genotyped for all samples using primers and amplification methods adapted from Zhou *et al*. [33]. Pyrosequencing was performed on a PyroMark Q96 MD system (Biotage, Charlotte, NC). Allele frequency was adjusted based on a standard curve [54]. An allele with a relative frequency of 80% or greater within a given infection was designated as the predominant allele. Haplotypes were constructed using only the predominant allele. Samples without a predominant allele at two or more codons were labeled "mixed-genotype." Samples that were mixed at a single codon were treated as containing both possible haplotypes.

To show that reduced heterozygosity around drug resistance genes was the result of selection rather than demographic processes, we measured expected heterozygosity in 6 unlinked neutral loci located throughout the *P. falciparum* genome.[44] These unlinked microsatellites were amplified using previously published primers and amplification conditions.[44] When multiple peaks were identified within the same sample, peaks that were less than 1/3 the height of the tallest peak were ignored and the tallest peak was designated as the predominant allele.[21,44] Only samples with a predominant allele were included in estimates of expected heterozygosity.

Eight polymorphic microsatellites flanking *pfdhfr-ts* were genotyped; four downstream (+50 kb, +20 kb, +1.48 kb, +0.2 kb), and four upstream (-0.3 kb, -1.2 kb, -10 kb, -30 kb) using previously described primers and protocols.[7,27] Eight polymorphic microsatellites flanking *pfdhps* were genotyped; four downstream (+9.008 kb, +1.407 kb, +0.505 kb, +0.034 kb), and four upstream (-0.132 kb, -2.849 kb, -7.489 kb, -11.069 kb) of the gene, using primers described by Vinayak *et al*. [37] Fragment size was visualized using an Applied BioSystems 3730XL high-throughput 96-capillary DNA sequencer. Analysis of electropheragrams was performed using Genemapper software (version 4.0; ABI). A Perl script was used to assign the raw electropheragram scores to an integer allele size based on the expected repeat length and variation seen in the positive controls.

The prevalence of each haplotype was estimated as the number of each haplotype observed among the successfully genotyped samples for each resistance gene, divided by the total number of genotyped samples. A resistant haplotype was defined as containing any number or combination of resistance alleles at the genotyped codons within either of the resistance genes of interest. The sensitive haplotype was defined as parasites with sensitive alleles at all codons within both genes. Samples with mixed-genotype at two or more loci within the same gene were excluded because haplotype phase could not be determined. Chi-squared tests with Yate's correction were used, where appropriate. Expected Heterozygosity (H_e), a measure of genetic diversity at each microsatellite locus, was calculated using the standard equations for H_e and variance: The analysis focused on

$$H_e = \left(\frac{n}{n-1}\right)\left(1 - \sum p_i^2\right), \qquad \frac{2(n-1)}{n^3}\left\{2(n-2)\left[\sum p_i^3 - \left(\sum p_i^2\right)^2\right]\right\}$$

sweep characteristics flanking DHFR 51I/59R/108N and DHPS 437G/540E due to

limited prevalence of other haplotypes in all three settings. H_e (± 1 standard deviation) was calculated for three groups: rural high, rural-moderate and urban-low settings, for both *pfdhfr-ts* and *pfdhps* genes. Samples without a predominant genotype or samples with missing data were excluded from expected heterozygosity calculations. Statistical significance was determined via permutation. Diversity ratios were calculated for $H_{urban-low}/H_{rural-high}$, $H_{urban-low}/H_{rural-moderate}$, and $H_{rural-moderate}/H_{rural-high}$. Calculations for He, standard deviation and permutations were conducted in R [38].

D. RESULTS

Of the genotyped samples, complete *pfdhfr-ts* haplotypes were assembled for 549 samples from the urban-low setting, 726 samples from the rural-moderate setting, and 660 samples from the rural-high setting. (Figure V.1A). Of the genotyped samples, complete *pfdhps* haplotypes were assembled for 546 from the urban-low setting, 733 from the rural-moderate setting, and 558 samples from the rural-high setting (Figure V.1B). No difference in the prevalence of the highly resistant DHFR 51I/59R/108N or DHPS 437G/540E was found between the three transmission settings. Haplotype prevalence of the DHFR 51I/59R/108N haplotype was >95% for all transmission settings. The prevalence of the highly resistant DHPS 437G/540E was also >95% for all three sites. A greater prevalence of DHFR 59R/108N was found in the rural-moderate setting, relative to the rural-high setting (Yate's corrected p=0.020), and was borderline different from the urban-low setting (p=0.067). A single, sensitive parasite infection was identified in the rural-moderate setting.

Due to similar prevalence of highly resistant SP-haplotypes between the three sites, only subsets of samples were subjected to microsatellite analyses. Of the subset, complete microsatellite haplotypes were generated for rural-moderate setting (n=15 *pfdhfr-ts*), (n=21 *pfdhps*), rural-high setting (n=10, *pfdhfr-ts*), (n=18, *pfdhps*), and urban-low setting (n=15 *pfdhfr-ts*) and (n=20 *pfdhps*). Average H_e at unlinked microsatellites was 0.898. The proportion of samples scored as polyclonal based on unlinked microsatellites did not differ significantly between settings (p=0.611). None of the settings were consistently higher or lower in H_e relative to each other (Figure V.2). Significant changes in H_e, were seen between the rural-moderate setting, and the rural-high setting and urban-low setting ($p<0.001$) though no differences between rural-high setting and urban-low setting were found. Analysis of flanking microsatellites indicated the presence of a core haplotype flanking *pfdhfr-ts* and *pfdhps* of Southeast Asian decent in all three settings (Supplementary Figure VII.3). In addition we observed microsatellite haplotypes flanking *pfdhps* found in the urban-low and rural-high settings that were not present in the rural-moderate, and microsatellite haplotypes found in the rural-moderate setting not found in the other two locations (Figure V.3).

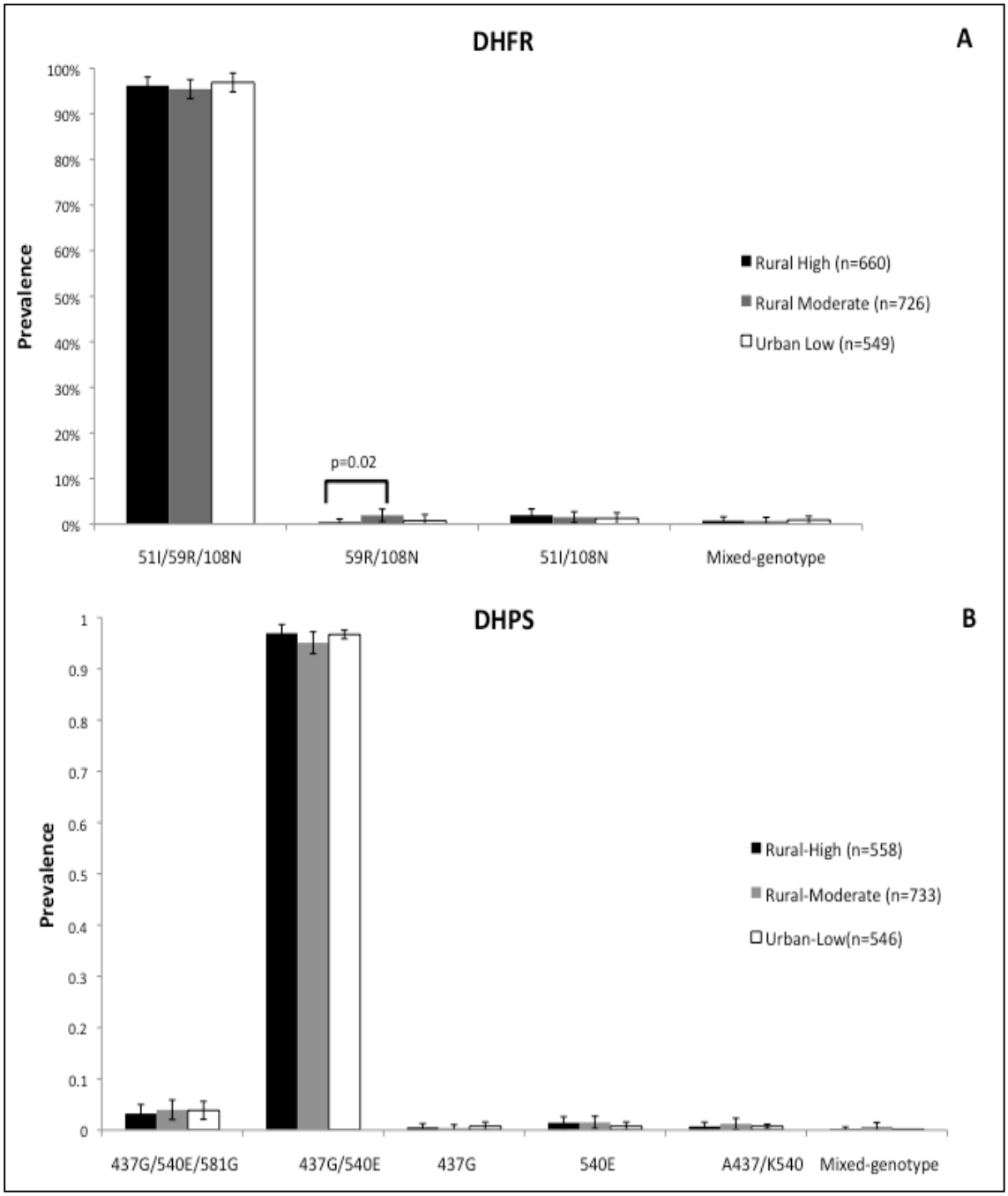


Figure V.1: Effect of transmission setting on SP resistant haplotype prevalence. Haplotype prevalence at DHFR (A) and DHPS (B) in three transmission settings within Malawi. Rural-high (Chikwawa), urban-low (Ndirande), and the rural-moderate (Thyolo) setting, Prevalence is calculated as the percentage of individuals with a given haplotype. Error bars represent 95% CI.

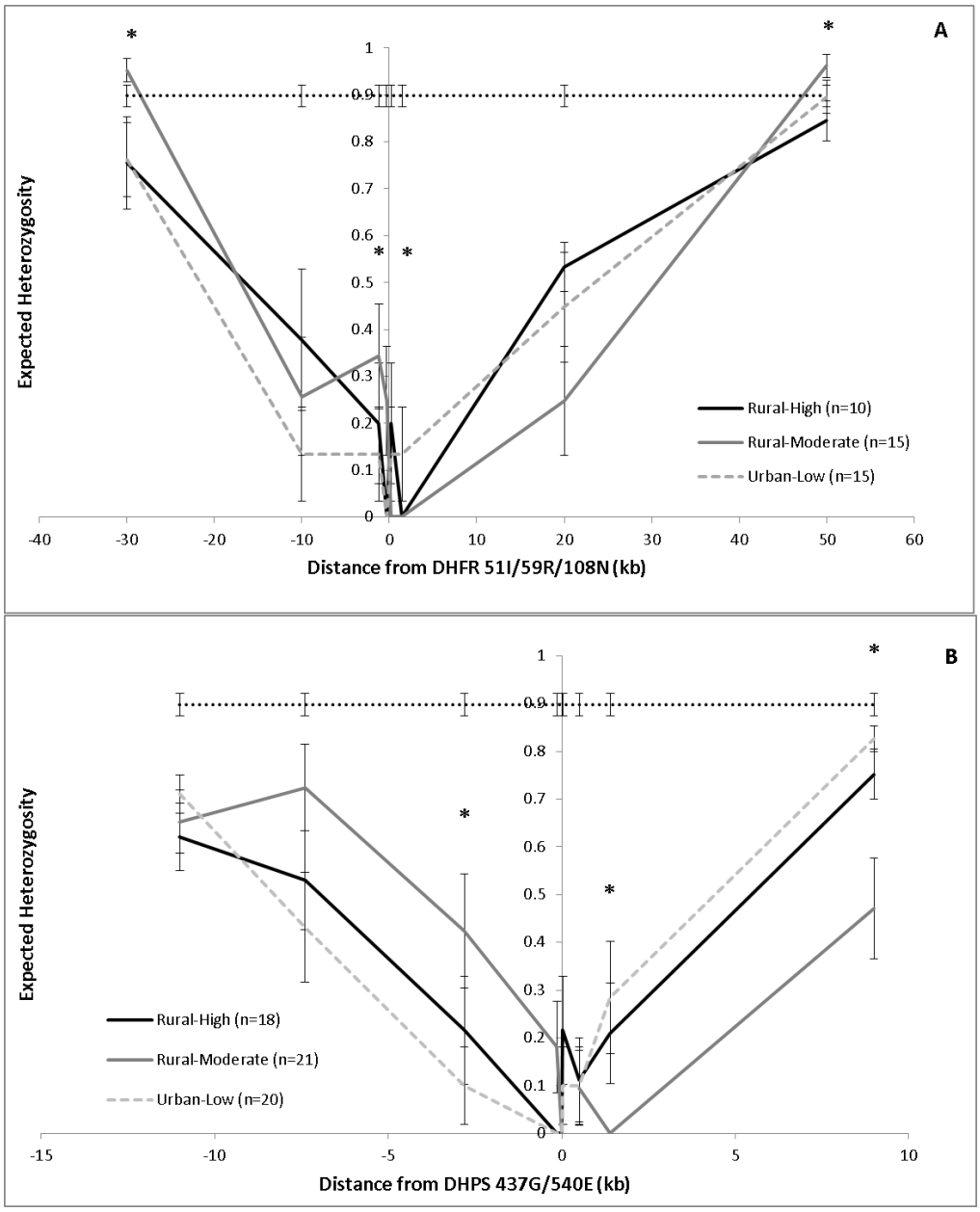


Figure V.2: Effect of transmission setting on SP resistant haplotype selective sweep characteristics. Expected heterozygosity in microsatellite loci flanking DHFR 51I/59R/108N(A) and DHPS 437G/540E(B). Samples with missing data excluded. Error bars represent +/- 1 standard deviation. Dashed lines represent genomic level, average H_e, based on unlinked loci. Alpha <0.05 * indicates significant difference between two transmission settings based on permutation.

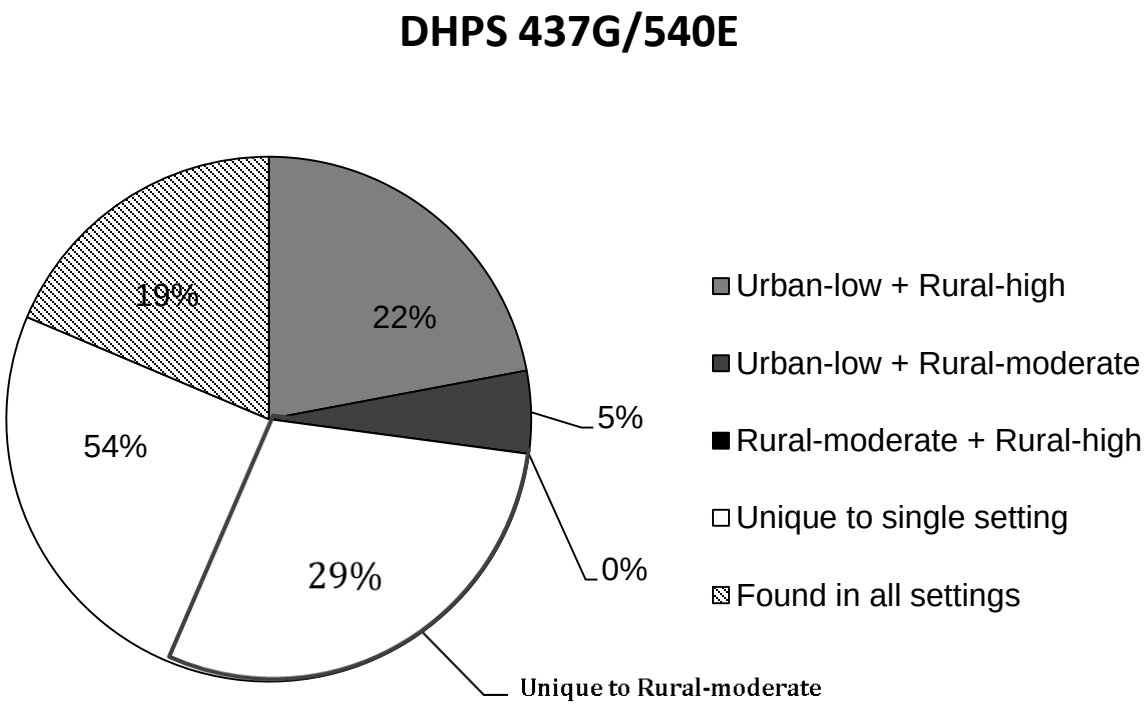


Figure V.3: Proportion of unique vs common microsatellite haplotypes between transmission settings. Proportion of microsatellite haplotypes found between transmission settings.

E. DISCUSSION

This study estimates the effect of local differences in transmission on the prevalence of SP-resistant haplotypes and characteristics of associated selective sweeps. Our research indicates that the local differences in transmission intensity are not sufficient to affect a difference in prevalence of the DHFR 51I/59R/108N and DHPS 437G/540E haplotypes.

The similarity in the proportion of samples scored as polyclonal between transmission settings may indicate similarity in transmission level not evident in percent parasitemia. Recent EIRs were unavailable for any of the settings, and we therefore used percent parasetemia as a surrogate estimation of transmission intensity. We would have expected to find higher proportions of polyclonal samples in the higher transmission settings (given the background level of genetic diversity in the population) if percent parasitemia was an accurate estimation of transmission intensity in our data set. This discrepancy could indicate that percent parasitemia is not a good surrogate for transmission intensity in our sample set. Our estimation of transmission intensity was based on average percent parasitemia from a community-based, district-wide cross-sectional survey, while our data set was based on a facility-based survey. The cross sectional data provides a single estimate of percent parasitemia and may not reflect historical differences in transmission intensity between the sites. It is possible that individuals reporting to the district health centers included in the data set may have been from more similarly intense transmission settings than their district wide average percent parasitemia value would suggest. It is also possible that the almost 3 fold difference in

percent parasitemia between these settings is not sufficient to produce a measurable difference in proportion of polyclonality, haplotype prevalence or sweep characteristics.

While we do not show a clinically relevant effect of local transmission intensity, as estimated by percent parasitemia, on SP haplotype prevalence, this study provides evidence of the fitness of DHFR 51I/59R/108N and DHPS 437G/540E Southeast Asian core haplotype. Parasites carrying these SP-resistant genotypes maintain high prevalence, across diverse transmission settings, even in the absence of strong SP drug pressure.

VI. GENERAL DISCUSSION

A. Study Summary

The expansion of drug resistant malaria poses a significant public health threat in the developing world. The repeated origin and expansion of drug-resistant strains from Southeast Asia to Africa threatens to repeat itself with the emergence of artemisinin resistance on the Thai-Cambodian border [3]. The emergence and expansion of artemisinin resistance in Southeast Asia has renewed interest in the use of previously abandoned drugs for the safe and effective treatment of uncomplicated malaria. This dissertation research estimated the effect of drug pressure and transmission setting on *P.falciparum* population dynamics and signatures of selection within the parasite genome. This research revealed the persistence of the highly SP-resistant DHFR 51I/59R/108N and DHPS 437G/540E mutants in Malawi, five years after the removal of SP as the first line treatment of uncomplicated malaria; as well as the emergence and rise of the DHPS 437G/540E/581G. The persistence of the highly SP-resistant haplotypes was demonstrated to be the result of little to no selection against the haplotypes in the absence of strong SP pressure. This research has also demonstrated that regional differences in transmission settings were insufficient to effect differences in the prevalence of SP-resistant haplotypes. Overall, this research provides vital clinical information on the state of SP-resistance in Malawi, and suggests continued SP-resistance across diverse transmission settings, under current epidemiological conditions. In addition, it highlights the need for continued molecular monitoring of the DHPS 581G allele, as this haplotype has been associated with reduced SP-IPTp efficacy.

B. Effect of drug pressure on SP-resistant haplotype prevalence and sweep characteristics, in Malawi

In keeping with our hypothesis, this study observed the continued selection for highly SP-resistant haplotypes under sustained SP drug pressure. However, contrary to the observations in the case of chloroquine resistance there was persistence of highly SP-resistant parasites five years after SP removal as the first-line treatment for uncomplicated malaria. Our findings differ from those in Peru and Kenya, which showed reduction in the prevalence of highly resistant haplotypes under reduced SP pressure. [19,54] In the case of the Peruvian study, the decline was seen in DHFR 51I/108N/164L and DHPS 437G/540E/581G haplotypes, which may indicate that the presence of the 164L and 581G alleles, on the Peruvian genetic background, bears a greater fitness cost than the Malawian DHFR 51I/59R/108N and DHPS 437G/540E/581G. This suggests that not all 'triple' mutants bear similar fitness costs, and that change in resistant haplotype prevalence is more a result of the specific haplotype than removal of drug pressure. The same study found an increase in 108N allele in Peru, suggesting that this allele may bear little or no fitness cost in the absence of strong SP drug pressure; a finding supported by our research. It is also possible that the South American *pfdhfr-ts* and *pfdhps* resistance polymorphisms (genetically distinct from the Southeast Asian *pfdhfr*-ts and *pfdhps* haplotypes) arose upon a genetic background that bears an additional fitness cost, in comparison with the South American drug sensitive parasites. In the case of the Kenyan study the persistence of SP resistance in Malawi, and decline of SP resistance in other Kenya may be, in part, to the near fixation of the DHFR 51I/59R/108N and DHPS

437G/540E haplotypes in Malawi, as compared to other locations. In Malawi and both Kenyan sites the prevalence of the DHFR 108N, and DHPS 437G and 540E alleles was at or near fixation prior to SP reduction. In Kombewa Kenya (the sites that saw the greatest decline in SP-resistance), the DHFR 51I/59R/108N and DHPS 437G/540E haplotypes were non-existent, or at lower prevalence than our study; allowing for a more rapid re-emergence of SP sensitive parasites in these locations. At both Kenyan sites, the decrease in highly resistant haplotype prevalence was mostly due to reduction in the prevalence of DHFR 51I and DHFR 59R codons, with only minor changes in the DHFR 108N allele, again suggesting little to no fitness cost of the 108N allele. However, in the Kenyan study, methodological differences in reporting change in allele frequency rather than haplotype prevalence and haplotype definition make direct comparison of the results difficult. Targeted entomological interventions in the Kenyan study, leading to decreased transmission and malaria prevalence overall, were implemented at the same time of SP pressure reductions, and may have had a confounding impact on haplotype prevalence in the region, by reducing effective population size leading to population bottlenecks. The emergence of compensatory mutations on the DHFR triple or DHPS double mutant genetic background could also account for the persistence of these haplotypes under reduced drug pressure, however, no data yet exists to support the emergence of compensatory mutations in Malawi.

While we predicted a decline in resistant haplotypes in the absence of strong SP pressure, the decline of DHFR double and DHPS single mutant haplotypes shown in our study does not suggest that the DHFR double and DHPS single mutants bear a greater fitness cost relative to drug sensitive parasites. Without an observed increase in sensitive

haplotypes, the decline of less resistant haplotypes may be attributed to the removal of less frequent haplotypes in favor of the triple haplotype or due to genetic drift.

This dissertation goes on to reveal an earlier date for the emergence and expansion of the DHPS 437G/540E/581G haplotype in Malawi, than was previously reported [55]. The increase in DHPS 437G/540E/581G haplotype could threaten the efficacy of ongoing SP-IPTp treatment in the nation, as the presence of 581G allele is associated with SP-IPTp failure.[53]. While no decline in SP-IPTp efficacy was found by Taylor *et al.* despite >95% prevalence of the DHFR 51I/59R/108N and DHPS 437G/540E/581G mutant, the prevalence of the DHPS 437G/540E/581G was still found at less than <10% prevalence[55]. If the prevalence of this DHPS 437G/540E/581G continues to rise, the efficacy of SP-IPTp treatment in Malawi may suffer. Minja *et al.*, found the presence of the 581G allele in sextuple-mutants to be associated with lower birth weight following SP-IPTp treatment, as compared to DHFR 51I/59R/108N+DHPS 437G/540E alone[56]. The ascendency of this sextuple mutant, under low SP drug pressure may threaten utility of SP-IPTp treatments throughout the region.[56].

Another important contribution of this dissertation to the body genetic epidemiological data, is the observed decline in the prevalence of polyclonal infections (infections with two or more different parasite clones) without a reduction in genetic diversity. These data suggest a reduction in the absolute amount of malaria in Malawi in accordance with clinical observations.[57] In the absence of SP selective pressure, the reduction in the overall amount of malaria, may cause a disproportionate effect of drift to remove less prevalent SP-sensitive haplotypes, thus fixating the highly resistant

haplotype and providing a genetic background necessary for continued selection for the sextuple-mutant DHFR 51I/59R/108N and DHPS 437G/540E/581G.

The finding of continued high rates of SP resistant parasites despite cessation of SP use for the treatment of malaria could also be due in part to antifolate drug pressure that continues from other sources. Use of the antibiotic sulfamethoxazole-trimethoprim as prophylaxis in people living with HIV and low level SP pressure through SP-IPTp exposure have been raised as possible sources of continued selection for SP-resistance mutations, as they both act upon the same folate enzymes DHFR and DHPS. However, there is no evidence sulfamethoxazole-trimethoprim has any impact on SP-resistant allele prevalence, or that SP resistance impacts sulfamethoxazole-trimethoprim efficacy [49,50].

Microsatellite analysis performed in this dissertation successfully identified the presence of selective sweeps flanking the DHFR 51I/59R/108N and DHPS 437G/540E mutant haplotypes. The asymmetrical nature of changes in sweep characteristics, and/or lack of change in sweep characteristics, provides evidence of little to no selective pressure against the DHFR 51I/59R/108N and DHPS 437G/540E mutant haplotypes, in the absence of strong SP pressure. Changes in sweep characteristics, between time periods, was most likely due to individual differences in mutation rate at each microsatellite marker, rather than selection for less resistant haplotypes allowing for recombination and sweep degradation. Unfortunately, predictions of sweep degradation and a return of SP sensitivity cannot be developed based on these data. However, in this case the absence of evidence of a strong fitness cost is in and of itself a significant finding to inform public health strategies. These findings cannot rule out a role of allele

fixation or sulfamethoxazole-trimethoprim pressure in the maintenance of DHFR 51I/59R/108N and DHPS 437G/540E haplotype prevalence, though these findings do suggest a limited role, of these two factors.

C. Effect of transmission setting on SP-resistant haplotype prevalence and sweep characteristics, in Malawi

Little to no effect of transmission setting was observed on haplotype prevalence or sweep characteristics. Despite large differences in the observed percent parasitemia between the rural-high and urban low settings, no difference in haplotype prevalence or sweep characteristics was observed. Statistically significant differences between rural-moderate and rural-high settings are possibly a statistical artifact due to large samples size. This study also indicated strong similarity in sweep characteristics between rural-high and urban-low settings, counter to what would be expected under the assumption that higher transmission setting would result in greater genetic diversity. These findings may indicate a shared parasite population between the two locations. The divergence of some sweep characteristics in the rural-moderate setting from the other two settings, further suggests that regional demographic events may act to effect sweep characteristics, without a significant effect on haplotype prevalence. Such events may have been the migration of SP-resistant haplotypes of diverse genetic background to the rural-moderate setting (perhaps from neighboring Mozambique), or historical differences in the degree of selective pressure. While the prevalence of the DHFR 51I/59R/108N and DHPS 437G/540E mutant haplotypes were both >95% in 2012, samples from previous time periods in the rural-moderate's evolutionary history were unavailable. These data provide

intriguing evidence of regional difference in population dynamics within a relatively small geographic region.

D. Study Limitations

The high incidence of malaria in Malawi results in a large proportion of polyclonal infections (~60%-80%). These polyclonal infections complicate statistical analyses, which were developed to assess allelic diversity with each sample representing a single individual organism. Statistical analyses require the designation of a predominant haplotype to satisfy the requirements of the statistical methodology. The inherent limitation of this method is the disregarding of rare haplotypes (those that never appear in a predominant clone infection) from calculations of H_e. While we suspect haplotypes that appear at such low prevalence are not biologically relevant to clinical infections, these rare haplotypes may signal the re-emergence or emergence of clinically relevant haplotypes that had not yet have risen to a detectable level, or that are not efficiently detected by the particular assay. In this study, however, the number of rare haplotypes that may have been discounted and the effect their elimination may have on the calculations of H_e, would not affect the calculation of haplotype prevalence. As far as clinical outcome is concerned, resistant haplotype prevalence in the patient population is a more useful metric than resistant haplotype prevalence in the parasite population.

Another limitation of the statistical analyses was the issue of missing data in microsatellite analysis. Amplification of all microsatellite markers was not obtained for each sample. Estimation of H_e is done on a maker by marker basis. The inclusion of missing data using permutation would have resulted in a different population size for

each marker (for between year/site comparisons) by averaging out the number of missing samples in the randomized data sets as compared to the empirical set. There was the concern that this may affect the validity of the significance findings of this analysis. To avoid this issue and simplify the analysis, samples with missing data were excluded across all microsatellite analyses. Hale *et al.*, have shown that it is possible to accurately estimate allele frequencies using microsatellites with small sample sizes. [47] Sweeps were constructed including all samples with missing data points, and while H_e was higher at several markers, overall changes in sweeps characteristic between time periods and settings was not affected by the exclusion of missing data.

While, H_e does not follow a standard sampling distribution larger sample sizes are generally more desirable than small sample sizes. Exclusion of missing data and polyclonal samples resulted in relatively small sample numbers for the three transmission settings. Given the lack of difference in SP-resistant haplotype prevalence the total number of samples subjected to microsatellite analysis was reduced from ~600, to 92 samples for the rural-moderate and rural-high settings (urban-low having been run for all 968 samples). As with the selective sweeps flanking DHFR and DHPS under differing drug pressure, sweeps were constructed with and without missing data to ensure that the exclusion of missing samples did not bias the overall conclusions of the study. Sweep characteristics remained similar, and showed no trend of increased or decreased H_e in one setting as compared to another setting.

For this dissertation I chose to analyze haplotype prevalence rather than haplotype frequency. Frequency is the proportion of parasites with a given haplotype, whereas prevalence reflects the proportion of infected humans with the given haplotype. Until

very recently (July 2014), methods to accurately estimate the multiplicity of infection (number of distinct parasite clones within an infection) in order to estimate haplotype frequency (based on population level estimates of multiplicity of infection) was unavailable. While these new methods allow for a more accurate estimation of resistance allele dynamics and would lead to larger samples sizes for statistical analyses they too have their limitations. Both parameters are dependent upon the sensitivity of the assay to detect minor alleles within an infection. Therefore, an estimation of haplotype frequency may also miss minor alleles within an infection and similarly bias the analysis to the more prevalent resistance haplotypes. Given the low prevalence of mixed genotype infections, and no change in the proportion of polyclonal infections between transition and low SP-use periods, such an analysis would not be expected to change the clinical relevance of our findings. Estimation of multiplicity of infection can also be used to estimate transmission intensity. Future collaboration seek to estimate the multiplicity of infection in the urban-low, rural-moderate, and rural-high settings and compare these estimations to percent parasitemia as an estimation of transmission intensity

While this study found no effect of transmission setting on haplotype prevalence or sweep characteristics, these findings are based on the presumption that the community-based, cross-sectional study estimate of average percent parasitemia was a good surrogate for transmission intensity in our data set. Recent entomological inoculation rates (EIR) are preferable measures of transmission intensity, but were unavailable for all settings. If percent parasitemia (as estimated in the cross-sectional study) were a good surrogate for transmission intensity we would have expected to see higher proportions of polyclonal samples in the rural-high setting (as compare to other settings) given the ~90% genetic

diversity observed in unlinked markers. The failure of percent parasitemia to predict proportion of polyclonal infections could indicate that percent parasetemia is not a good surrogate of transmission intensity in the region, or it may indicate that the samples collected for this study were not representative of district wide transmission intensity. In the community-based, cross-sectional study average percent parasetemia was taken from several sites within each district, while the facility based data used to calculate haplotype prevalence and sweep characteristics was collected at only the district health center. It is possible that the individuals reporting to the district health centers came from more similarly intense transmission settings within their district than the district average percent parasitemia would suggest. It is also possible that our assumption that a 2.8 fold difference is percent parasetemia between setting could impact genetic diversity in an observable way was incorrect. Much higher differences in transmission may be needed to observe the effect of transmission setting on allele dynamics. Lastly, the estimate of percent parasetemia represents a single year and does not reflect any historical changes in transmission intensity, as the result of regional control programs could have resulted in similarly high levels of SP resistance that were unaffected by later reduction in transmission intensity. As previously mentioned, the recently developed method by Schnider *et al.* provides an estimate of transmission intensity based on estimation of multiplicity of infection via unlinked markers, and re-analysis of these data may be performed when the tool becomes available[48].

E. Research Implications and future directions

This study is the first to report the persistence of highly SP-resistant DHFR 51I/59R/108N and DHPS 437G/540E haplotypes in the absence of strong SP pressure. When coupled with the microsatellite analysis, these findings indicate that SP will continue to be an ineffective treatment for uncomplicated malaria in Malawi, and other drugs, such as chloroquine, should be considered in the face of ACT-resistance. Evidence of the expansion of the 581G allele will impact molecular monitoring efforts in the region as its continued expansion may threaten SP-IPTp utility [56,58]. Given Laufer *et al.*'s 2006 finding of renewed chloroquine clinical efficacy, the use of chloroquine for IPTp prophylaxis should be considered should the expansion of the DHPS 437G/540E/581G haplotype reduce clinical efficacy [5]. Future studies of SP-IPTp efficacy and malaria in pregnancy outcomes should consider 581G allele prevalence in the region and in infections to further monitor the expansion of the DHPS 437G/540E/581G haplotype.

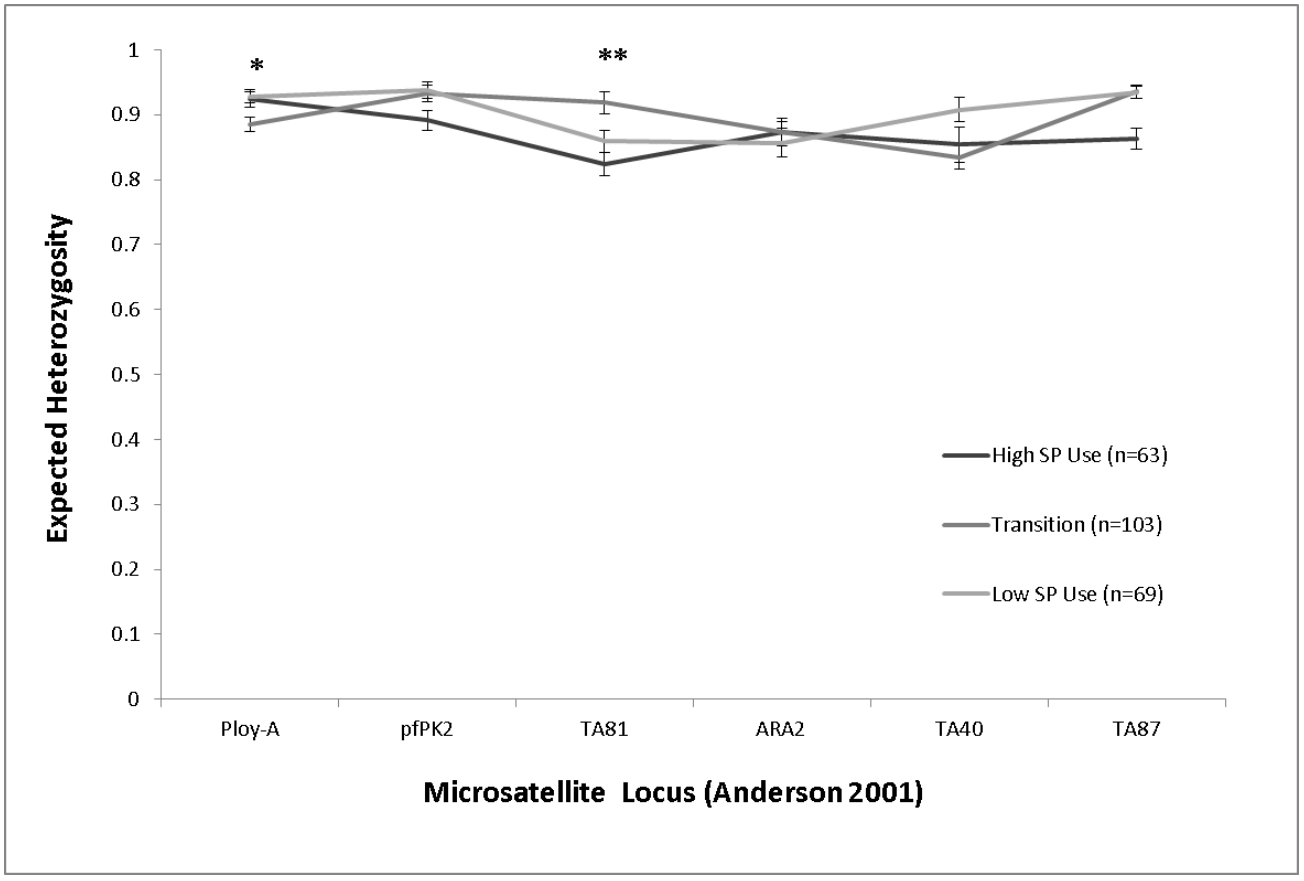
The lack of difference in haplotype prevalence or sweep characteristics between different transmission settings will better inform study site selection for future epidemiological studies. This research presents evidence that after nearly two decades of high SP resistance, differences in regional transmission settings do not impact the prevalence of clinically relevant haplotypes. Investigators working in epidemiologically similar settings can reasonably assume similarity in haplotype prevalence and sweep characteristics between different transmission settings within the same geographical region. Future studies will seek to incorporate the findings of Schneider *et al*. to ascertain a within-data-set multiplicity of infection and get a more accurate estimate of transmission intensity.

This research study provides evidence of the power of molecular monitoring to inform epidemiological studies and public health policy. The combination of clinically relevant haplotype prevalence and microsatellite analysis, in the context of varying drug pressure and transmission setting, can be used to inform predictive models of SP resistant haplotype population dynamics. Not only will this study inform current drug policy, but it warns of a potential threat to SP-IPTp. If this warning is heeded, and the pressure favoring the 581G allele is identified, control and elimination strategies can be developed to prevent the spread of the 581G allele, and preserve SP-IPTp utility. Overall this dissertation provides important genetic epidemiological insight that can be used to inform public health policy both in the region and worldwide.

VII. APPENDIX

A. Supplementary figures

1. Supplementary Figure VII.1: Expected heterozygosity at unlinked neutral microsatellites. Samples with missing data were excluded. Error bars represent +/- 1 standard deviation. Dashed lines represent genomic level, average $H_{e,}$ based on unlinked loci. * indicates significant difference between two time periods. ** Indicates significant difference between all three time periods, based on permutation. P-values 0.05 were considered significant.



2. Supplementary Figure VII.2 Haplotype map of DHFR 51I/59R/108N and DHPS 437G/540E under varying drug pressure

DHPS 437G/540E Microsatellite Haplotype								
ID	-11	-7.4	-2.8	-0.1	0.03	0.5	1.4	9
HB3	217	175	192	132	139	141	268	101
V1S	217	175	180	130	125	147	268	109
3D7	217	157	190	134	149	147	246	111
Alam (2010)	220	ND	189	132	141	145	250	102*
Mta (2011)	ND	ND	189	132	ND	ND	ND	ND
High1	217	151	189	132	139	143	246	101
High2	217	173	189	132	139	143	246	101
High3	217	161	180	132	143	141	246	101
High4	217	173	189	132	139	143	246	101
High5	213	151	189	132	139	143	246	101
High6	217	151	189	132	139	143	246	101
High7	213	151	189	132	139	143	246	101
High8	217	151	189	132	139	143	248	101
High9	217	173	189	132	139	143	246	101
High10	217	151	189	132	139	143	246	103
High11	217	151	189	132	139	143	246	103
High12	219	151	189	132	139	143	244	107
High13	217	151	189	132	139	143	248	103
High14	217	173	189	132	139	143	248	103
High15	217	151	189	132	139	143	246	101
High16	219	151	189	132	139	143	246	101
High17	217	173	189	132	141	143	246	103
High18	213	151	189	132	139	143	246	101
High19	213	151	189	132	139	143	246	101
High20	217	173	189	132	139	143	246	101
High21	219	151	189	132	139	143	246	103
High22	213	151	189	132	139	143	246	101
High23	213	151	189	132	139	143	248	101
High24	217	177	189	132	139	143	246	101
High25	213	151	189	132	139	143	246	101
High26	213	151	189	132	139	143	246	101
High27	213	151	189	132	139	143	248	107
High28	217	173	189	132	139	143	248	101
High29	217	173	189	132	143	143	252	101
High30	217	151	189	132	139	143	246	103
High31	213	151	189	132	139	143	246	101
High32	213	151	189	132	139	139	248	101
High33	217	173	189	132	139	141	246	101
High34	219	151	189	132	139	143	246	103
High35	213	151	189	132	139	143	246	101
High36	219	151	189	132	139	143	248	103
High37	217	151	189	132	139	143	246	103
High38	217	173	189	130	139	143	246	101
High39	217	157	202	130	139	143	246	101
High40	217	165	190	130	139	143	246	109

DHPS 437G/540E Microsatellite Haplotype								
ID	-11	-7.4	-2.8	-0.1	0.03	0.5	1.4	9
HB3	217	175	192	132	139	141	268	101
V1S	217	175	180	130	125	147	268	109
3D7	217	157	190	134	149	147	246	111
Alam (2010	220	ND	189	132	141	145	250	102*
Mta (2011)	ND	ND	189	132	ND	ND	ND	ND
Trans1	219	151	189	132	139	143	246	101
Trans2	213	151	189	132	139	143	246	101
Trans3	213	151	189	132	139	143	246	101
Trans4	213	151	189	132	139	143	246	101
Trans5	217	173	189	132	139	143	246	111
Trans6	217	161	189	132	139	143	246	101
Trans7	219	151	189	132	139	143	250	103
Trans8	219	151	189	132	139	143	246	103
Trans9	213	151	189	132	139	143	246	101
Trans10	219	151	189	132	139	143	246	103
Trans11	213	151	189	132	139	143	246	101
Trans12	213	151	189	132	139	143	246	101
Trans13	213	151	189	132	139	143	246	95
Trans14	217	165	189	132	139	143	246	101
Trans15	213	151	189	132	139	143	246	101
Trans16	217	151	189	132	139	143	246	101
Trans17	217	173	189	132	139	143	246	93
Trans18	219	151	189	132	139	143	246	103
Trans19	213	151	189	132	139	143	246	101
Trans20	217	151	189	132	139	143	246	101
Trans21	217	173	189	132	139	143	246	135
Trans22	213	151	189	132	139	143	248	103
Trans23	213	151	189	132	139	143	246	101
Trans24	219	151	189	132	139	143	246	103
Trans25	217	173	189	132	139	143	246	101
Trans26	219	151	189	132	139	143	246	101
Trans27	217	161	192	132	139	143	246	103
Trans28	217	175	189	132	139	143	246	101
Trans29	219	161	189	132	139	143	250	115
Trans30	213	151	189	132	139	143	246	101
Trans31	219	151	189	132	139	143	246	103
Trans32	217	151	189	132	139	143	246	101
Trans33	217	173	189	132	139	143	246	101
Trans34	213	151	189	132	139	143	246	101
Trans35	219	151	189	132	139	143	246	109
Trans36	217	165	180	132	139	143	256	105
Trans37	219	151	189	132	139	145	246	103
Trans38	213	151	189	132	139	143	246	101
Trans39	219	151	189	132	139	143	246	101
Trans40	217	167	180	132	123	141	244	101
Trans41	217	173	189	132	139	143	246	101
Trans42	213	151	189	132	139	143	246	101
Trans43	217	173	189	132	139	143	246	105
Trans44	217	151	189	132	139	143	246	107
Trans45	213	151	189	132	139	143	246	101
Trans46	213	151	189	132	139	143	246	101
Trans47	219	151	189	132	139	143	246	101
Trans48	213	151	189	132	139	143	254	115
Trans49	217	165	189	132	139	143	254	105
Trans50	219	161	189	132	139	143	252	115
Trans51	219	149	189	132	139	143	246	99
Trans52	217	175	189	132	139	143	246	101
Trans53	217	151	189	132	139	143	246	101
Trans54	217	173	189	132	139	143	248	103
Trans55	217	173	189	132	139	143	246	101
Trans56	217	165	189	132	139	143	246	101
Trans57	217	161	192	132	139	143	248	103
Trans58	213	151	189	132	139	143	246	107
Trans59	213	151	189	132	131	141	234	97
Trans60	213	151	189	132	139	143	246	103
Trans61	217	157	202	130	139	143	246	107
Trans62	213	151	189	132	139	143	246	101
Trans63	217	173	189	132	139	143	246	105
Trans64	219	167	189	132	139	143	246	101
Trans65	213	151	189	132	139	143	248	101
Trans66	217	173	189	132	139	143	252	101
Trans67	213	151	189	132	139	143	240	111
Trans68	217	151	189	132	139	143	246	101
Trans69	217	165	189	132	139	143	246	101
Trans70	213	151	189	132	139	143	248	101
Trans71	213	151	189	132	139	143	246	101
Trans72	217	151	189	132	139	143	246	101
Trans73	217	175	189	132	139	143	246	101
Trans74	213	151	189	132	139	143	246	101
Trans75	213	151	189	132	139	143	246	101
Trans76	213	151	189	132	139	143	248	101
Trans77	213	151	189	132	139	143	246	101
Trans78	217	165	176	132	139	139	248	105
Trans79	219	151	189	132	139	143	246	103
Trans80	213	151	189	132	139	143	246	101
Trans81	217	173	189	132	139	143	246	101
Trans82	217	173	189	132	137	141	248	101
Trans83	213	151	189	132	137	143	246	105
Trans84	217	173	189	132	139	143	246	101

DHPS 437G/540E Microsatellite Haplotype								
ID	-11	-7.4	-2.8	-0.1	0.03	0.5	1.4	9
HB3	217	175	192	132	139	141	268	101
V1S	217	175	180	130	125	147	268	109
3D7	217	157	190	134	149	147	246	111
Alam (2010	220	ND	189	132	141	145	250	102*
Mta (2011)	ND	ND	189	132	ND	ND	ND	ND
Low1	219	149	190	132	139	145	246	101
Low2	213	149	189	132	139	143	246	105
Low3	213	149	189	132	141	143	246	99
Low4	213	151	189	132	139	143	246	99
Low5	217	167	189	132	139	143	246	99
Low6	217	173	190	132	139	143	246	99
Low7	217	149	189	132	139	143	246	101
Low8	217	165	189	132	139	143	246	105
Low9	219	151	189	132	139	143	246	103
Low10	217	149	189	132	139	143	246	103
Low11	219	173	180	130	139	143	246	97
Low12	213	149	189	132	139	143	246	101
Low13	213	149	189	132	131	139	248	93
Low14	217	169	192	132	139	143	246	99
Low15	219	161	189	132	139	143	246	99
Low16	219	149	189	132	139	143	246	103
Low17	217	173	186	132	139	143	246	99
Low18	217	173	182	130	139	143	246	99
Low19	219	149	189	132	139	143	246	103
Low20	213	149	189	132	139	143	246	99
Low21	219	149	189	132	139	143	246	101
Low22	219	149	189	132	139	143	246	99
Low23	219	149	189	132	139	143	246	101
Low24	221	163	189	132	139	143	246	99
Low25	217	169	189	132	139	143	246	113
Low26	219	151	189	132	139	143	246	103
Low27	219	173	192	130	139	143	246	103
Low28	213	149	189	132	139	143	246	101
Low29	213	149	189	132	139	143	246	99
Low30	219	151	189	132	139	143	246	103
Low31	219	151	189	132	139	143	246	101
Low32	221	173	189	132	139	143	246	101
Low33	217	173	189	132	139	143	246	101
Low34	213	149	189	132	139	143	246	101
Low35	213	149	189	132	139	143	246	99
Low36	219	149	189	132	139	143	246	103
Low37	213	149	189	132	139	143	246	101
Low38	213	151	189	132	139	143	246	99
Low39	219	171	189	132	139	143	246	105
Low40	213	151	189	132	139	143	246	101
Low41	213	149	189	132	139	143	246	99
Low42	225	161	196	130	125	143	250	99
Low43	213	149	189	132	139	143	246	99
Low44	219	149	189	132	139	143	246	109
Low45	213	149	189	132	139	143	246	101
Low46	217	167	189	132	139	143	246	95
Low47	217	173	189	132	139	143	246	99
Low48	219	173	180	130	139	143	246	97
Low49	213	149	189	132	139	143	246	103
Low50	219	149	189	132	139	143	248	97
Low51	213	149	189	132	139	143	246	101
Low52	219	149	189	132	139	143	246	101
Low53	219	149	189	132	139	143	246	103
Low54	217	165	189	132	139	143	246	99
Low55	213	151	189	132	139	143	248	99
Low56	217	173	189	132	139	143	246	103
Low57	219	149	189	132	139	143	246	101
Low58	217	149	189	132	139	143	246	99
Low59	219	173	189	132	139	143	242	99
Low60	219	149	189	132	139	143	246	103
Low61	219	149	189	132	139	143	246	99
Low62	219	151	189	132	139	145	246	103
Low63	219	149	189	132	139	143	246	101
Low64	217	149	189	132	139	143	246	101
Low65	213	149	189	132	139	143	246	101
Low66	217	173	189	132	139	143	252	101
Low67	217	165	189	132	139	143	246	103
Low68	217	173	189	132	139	143	246	99
Low69	219	149	189	132	139	143	246	103
Low70	219	165	194	132	133	143	254	105
Low71	219	149	189	132	139	143	246	103
Low72	219	149	189	132	139	143	246	103
Low73	217	165	189	132	139	143	246	101
Low74	219	149	189	132	139	143	246	101
Low75	217	173	189	132	139	143	246	99

DHFR 51I/59R/108N Microsatellite Haplotypes											
ID	-30	-10	-4.5	-3.8	-1.2	-0.03	0.02	0.52	1.48	20	50
HB3	ND	255	199	202	220	121	166	111	197	280	ND
3D7	146	259	195	196	210	101	170	103	197	280	151
V1S	150	255	197	190	210	105	170	103	195	280	153
Trans1	154	255	197	190	210	107	170	103	195	290	145
Trans2	158	255	197	190	210	105	170	103	195	280	145
Trans3	158	255	197	190	210	105	170	103	195	280	153
Trans4	146	255	197	190	210	105	170	103	195	280	159
Trans5	141	255	197	190	210	105	170	103	195	280	137
Trans6	158	255	197	190	210	105	170	103	195	280	140
Trans7	142	259	197	190	212	105	170	103	195	276	149
Trans8	158	255	197	190	210	105	170	103	195	280	139
Trans9	158	255	197	190	214	105	170	103	195	280	149
Trans10	154	255	197	192	210	105	170	103	195	280	143
Trans11	158	255	197	190	210	105	170	103	195	280	137
Trans12	158	255	197	190	210	105	170	105	195	280	149
Trans13	158	255	197	190	210	105	170	103	195	280	133
Trans14	158	255	197	190	210	105	170	103	195	280	151
Trans15	158	255	197	190	210	105	170	103	195	280	159
Trans16	142	255	197	190	210	105	170	103	195	280	161
Trans17	158	255	197	190	210	105	170	103	195	280	161
Trans18	158	255	197	190	210	105	170	103	195	280	143
Trans19	158	255	197	190	210	105	170	103	195	280	149
Trans20	144	255	197	190	210	105	170	103	195	276	137
Trans21	158	255	197	190	210	105	170	103	195	280	144
Trans22	158	255	197	190	210	105	170	103	195	280	155
Trans23	146	251	197	190	210	105	170	103	195	280	160
Trans24	154	255	197	190	210	105	170	103	195	278	149
Trans25	158	259	197	190	210	107	170	103	195	280	137
Trans26	144	255	197	190	210	105	170	103	195	280	145
Trans27	146	255	197	190	210	105	170	103	195	280	149
Trans28	150	255	197	190	210	105	170	103	195	280	151
Trans29	158	255	197	190	210	105	170	103	195	280	153
Trans30	158	255	197	190	210	105	170	103	195	280	145
Trans31	158	255	197	190	210	105	170	103	195	280	147
Trans32	144	255	197	190	212	105	170	103	195	280	143
Trans33	150	255	197	190	210	105	170	105	195	280	149
Trans34	158	255	197	190	210	105	170	103	195	280	153
Trans35	154	255	197	190	210	105	170	103	197	280	143
Trans36	142	255	193	190	210	105	172	105	195	280	159
Trans37	158	255	197	190	210	105	170	103	195	280	151
Trans38	146	255	197	190	210	105	170	103	195	278	136
Trans39	144	255	197	190	210	105	170	103	195	280	153
Trans40	138	257	197	190	216	105	170	103	195	280	143
Trans41	162	255	197	190	210	105	170	103	195	280	147
Trans42	146	257	197	190	210	105	170	103	195	280	159
Trans43	158	255	197	190	210	105	170	103	195	264	149
Trans44	158	255	197	190	210	105	170	103	195	280	155
Trans45	142	255	197	190	210	105	170	103	195	280	159
Trans46	146	255	197	190	212	105	170	103	189	278	155
Trans47	146	255	197	190	210	105	170	103	195	278	159
Trans48	154	255	197	190	210	105	154	93	195	280	159
Trans49	158	255	197	190	210	105	170	103	195	280	149
Trans50	158	255	197	190	210	105	170	103	195	276	149
Trans51	156	255	197	190	210	105	170	103	195	280	143
Trans52	148	249	197	190	210	105	170	103	195	280	149
Trans53	150	255	197	190	210	105	170	103	195	280	130
Trans54	158	255	197	190	210	105	170	103	195	280	136
Trans55	158	259	197	190	210	105	170	103	195	280	159
Trans56	158	255	197	190	210	105	170	103	195	280	151
Trans57	158	255	197	190	210	105	172	103	195	280	159

DHFR 51I/59R/108N Microsatellite Haplotypes											
ID	-30	-10	-4.5	-3.8	-1.2	-0.03	0.02	0.52	1.48	20	50
HB3	ND	255	199	202	220	121	166	111	197	280	ND
3D7	146	259	195	196	210	101	170	103	197	280	151
V1S	150	255	197	190	210	105	170	103	195	280	153
High1	NA	255	197	190	210	105	170	103	195	278	NA
High2	NA	255	197	190	210	105	170	103	195	280	NA
High3	NA	255	197	190	210	105	170	103	195	280	NA
High4	NA	255	197	190	212	105	170	103	195	258	NA
High5	NA	255	197	190	210	105	170	103	195	280	NA
High6	NA	255	197	190	210	105	170	103	195	280	NA
High7	NA	255	197	190	210	105	170	103	195	284	NA
High8	NA	255	197	190	210	105	170	103	195	280	NA
High9	NA	255	197	190	210	105	170	103	195	280	NA
High10	NA	255	197	190	210	105	170	103	195	258	NA
High11	NA	255	197	190	210	105	170	103	195	278	NA
High12	NA	255	197	190	210	105	170	103	195	280	NA
High13	NA	255	197	190	210	103	168	103	195	278	NA
High14	NA	255	197	190	210	105	170	103	195	276	NA
High15	NA	255	197	190	210	105	170	103	195	280	NA
High16	NA	255	197	190	210	105	170	103	195	280	NA
High17	NA	253	197	190	210	105	154	91	195	280	NA
High18	NA	255	197	190	210	105	170	103	195	280	NA
High19	NA	255	197	190	210	103	170	103	195	278	NA
High20	NA	255	197	190	210	105	170	103	195	280	NA
High21	NA	255	197	190	210	105	170	103	195	280	NA
High22	NA	253	197	190	210	105	170	103	195	280	NA
High23	NA	255	197	190	210	105	170	103	195	258	NA
High24	NA	255	197	190	210	105	170	103	195	276	NA
High25	NA	251	197	190	210	105	170	103	195	280	NA
High26	NA	255	197	190	210	105	170	103	195	280	NA
High27	NA	257	197	190	210	105	170	103	195	280	NA
High28	NA	257	197	190	210	105	170	103	195	280	NA
High29	NA	255	197	190	210	105	170	103	195	278	NA
High30	NA	255	197	190	210	105	170	109	195	278	NA
High31	NA	255	197	190	210	105	170	103	195	280	NA
High32	NA	255	197	190	210	105	170	103	195	280	NA
High33	NA	255	197	190	210	105	170	103	195	280	NA
High34	NA	255	197	190	210	105	170	103	195	280	NA
High35	NA	253	199	184	210	105	170	103	195	280	NA
High36	NA	255	197	190	210	105	170	103	195	278	NA
High37	NA	255	197	190	210	105	170	103	195	280	NA
High38	NA	255	197	190	210	105	170	103	195	270	NA
High39	NA	255	197	190	210	105	170	103	195	280	NA
High40	NA	255	197	190	210	105	170	103	195	278	NA
High41	NA	255	197	190	210	105	170	103	195	278	NA
High42	NA	255	197	190	210	105	168	103	195	280	NA
High43	NA	255	197	190	210	105	170	103	195	280	NA
High44	NA	255	197	190	210	105	170	103	195	280	NA
High45	NA	255	197	190	210	105	170	103	195	280	NA
High46	NA	255	197	190	210	105	170	103	195	280	NA
High47	NA	255	197	190	210	105	170	103	195	280	NA
High48	NA	255	197	190	210	105	170	103	195	280	NA
High49	NA	255	197	190	210	105	170	103	195	280	NA
High50	NA	255	197	190	210	105	168	103	195	278	NA
High51	NA	255	197	190	210	105	170	103	195	280	NA
High52	NA	255	197	190	210	105	170	103	195	280	NA
High53	NA	255	197	190	210	105	170	103	195	278	NA
High54	NA	255	197	190	210	105	154	103	195	280	NA
High55	NA	255	197	190	210	105	170	103	195	280	NA
High56	NA	255	197	190	210	105	170	103	195	280	NA
High57	NA	255	197	190	210	105	170	103	195	278	NA
High58	NA	255	197	190	210	105	170	103	195	280	NA
High59	NA	255	197	190	210	105	170	103	195	280	NA
High60	NA	255	197	190	210	105	170	103	195	278	NA

DHFR 51I/59R/108N Microsatellite Haplotypes											
ID	-30	-10	-4.5	-3.8	-1.2	-0.03	0.02	0.52	1.48	20	50
HB3	ND	255	199	202	220	121	166	111	197	280	ND
3D7	146	259	195	196	210	101	170	103	197	280	151
V1S	150	255	197	190	210	105	170	103	195	280	153
Low1	146	255	197	190	210	105	170	101	195	300	147
Low2	152	253	201	190	210	105	154	93	195	280	149
Low3	159	255	197	190	214	105	170	103	195	280	147
Low4	142	255	197	190	210	105	170	103	195	280	137
Low5	159	255	197	194	210	105	170	103	197	280	153
Low6	159	255	197	190	210	105	170	103	195	280	147
Low7	146	255	197	190	210	105	170	103	195	280	147
Low8	159	255	197	190	210	105	170	103	195	280	155
Low9	159	255	197	190	210	105	170	103	195	278	149
Low10	157	255	197	190	210	105	170	105	195	278	149
Low11	159	255	197	190	210	105	170	103	195	278	157
Low12	158	255	197	190	210	105	170	103	195	280	164
Low13	146	255	197	190	210	105	170	103	195	280	151
Low14	158	255	197	190	210	105	170	103	189	280	151
Low15	159	255	197	190	210	105	170	103	195	280	153
Low16	159	255	197	190	210	105	170	103	195	284	151
Low17	161	255	197	190	210	105	170	103	195	278	153
Low18	159	247	191	192	210	105	170	103	195	280	151
Low19	159	255	197	190	210	105	170	103	195	278	155
Low20	148	259	197	190	210	105	170	103	195	278	149
Low21	156	255	197	190	212	105	170	103	195	280	160
Low22	144	255	197	190	208	105	170	103	195	280	159
Low23	159	255	197	190	210	105	170	103	195	280	166
Low24	167	255	197	190	210	105	170	103	195	280	151
Low25	146	255	197	190	210	105	170	103	195	280	164
Low26	159	255	197	190	210	105	170	103	195	280	143
Low27	159	255	197	190	210	105	170	103	195	280	151
Low28	159	255	197	190	210	105	170	103	195	280	160
Low29	142	255	197	190	210	105	170	103	195	280	163
Low30	159	255	197	190	210	105	170	103	195	280	155
Low31	146	257	197	190	210	105	170	103	195	280	143
Low32	144	255	197	190	210	105	170	103	195	280	151
Low33	144	255	197	190	210	105	170	103	195	278	147
Low34	144	255	197	190	210	105	170	103	195	280	153
Low35	144	255	197	190	210	105	170	91	201	280	153
Low36	144	255	197	190	212	105	170	103	193	278	143
Low37	142	255	197	190	210	105	170	103	195	280	137
Low38	158	255	197	190	210	105	170	103	195	280	159
Low39	146	255	197	190	210	105	170	103	195	280	141
Low40	159	255	197	190	210	105	170	103	195	280	141
Low41	159	255	197	190	210	105	170	103	195	280	157
Low42	159	255	219	190	210	105	170	103	195	280	165
Low43	159	257	197	190	210	105	170	103	195	280	151
Low44	159	255	197	190	210	105	170	103	195	280	147
Low45	165	255	197	190	210	105	170	103	195	280	141
Low46	159	255	197	190	210	105	170	103	195	280	139
Low47	148	255	197	190	210	105	170	103	195	280	159
Low48	167	255	197	190	210	105	170	103	195	280	149
Low49	144	253	207	194	192	105	154	91	195	280	141
Low50	159	255	197	190	210	105	170	103	195	280	135
Low51	159	255	197	190	210	105	170	103	195	280	155
Low52	154	255	197	190	210	105	170	103	195	280	149
Low53	148	255	197	190	210	105	170	103	195	280	159
Low54	156	255	197	190	214	105	170	103	195	280	153
Low55	152	255	197	190	210	105	170	103	195	280	143
Low56	154	255	197	190	210	105	170	103	195	280	159
Low57	158	255	197	190	210	105	170	103	195	280	155
Low58	159	259	197	190	210	109	170	103	195	280	147
Low59	159	255	197	190	210	105	170	103	195	280	145
Low60	159	255	197	190	210	105	170	103	195	280	159
Low61	148	255	197	190	212	105	170	103	195	278	147
Low62	138	255	197	190	210	105	170	103	195	280	147
Low63	158	255	197	190	210	105	170	103	195	280	149
Low64	148	259	207	196	210	105	170	103	195	278	155
Low65	146	255	197	190	210	105	170	103	195	280	139
Low66	159	255	197	190	210	105	170	103	195	278	149
Low67	159	255	197	190	210	105	170	103	195	280	149
Low68	158	255	197	190	210	105	170	103	195	280	145
Low69	158	255	197	190	210	105	170	103	195	280	151
Low70	150	255	197	190	210	105	170	103	195	278	149
Low71	150	255	197	190	210	105	170	103	195	280	147
Low72	159	255	197	190	210	107	170	103	195	280	147
Low73	163	255	197	190	210	105	170	103	195	280	141
Low74	146	255	197	190	210	105	170	103	195	290	143

3. Supplementary Figure VII.3: Haplotype maps of DHFR and DHPS microsatellite haplotypes. Haplotype maps of DHFR and DHPS microsatellite haplotypes. Reference strains V1S, 3D7 and HB3. “C”=Chikwawa, rural-high, “T”=Thyolo, rural-moderate, and “N”=Ndirande, urban-low.

DHFR	-30	-10	-1.2	-0.3	0.2	1.48	20	50
HB3	ND	255	220	121	166	197	280	ND
3D7	146	259	210	101	170	197	280	151
V1S	150	255	210	105	170	195	280	153
C1	144	255	210	105	170	195	280	160
C2	152	255	210	105	170	195	280	147
C3	158	255	210	105	170	195	276	149
C4	146	261	210	105	170	195	280	153
C5	159	255	210	105	170	195	280	149
C6	158	255	210	105	170	195	276	149
C7	158	255	210	105	154	195	276	151
C8	158	255	210	105	170	195	276	151
C9	146	257	208	105	170	195	280	151
C10	158	255	210	105	170	195	280	153
T1	158	255	212	105	170	195	280	147
T2	152	255	210	105	170	195	280	149
T3	152	255	210	107	170	195	278	153
T4	156	255	210	105	170	195	280	145
T5	163	255	210	105	170	195	280	170
T6	140	255	210	105	170	195	280	159
T7	144	255	210	105	170	195	280	158
T8	159	255	210	105	170	195	280	160
T9	158	247	212	105	170	195	280	160
T10	144	255	210	107	170	195	280	162
T11	158	255	210	105	170	195	280	149
T12	142	257	212	105	170	195	280	168
T13	148	255	210	105	170	195	280	166
T14	150	255	210	105	170	195	278	160
T15	154	255	210	105	170	195	280	155
N1	146	255	210	105	170	195	300	147
N2	152	253	210	105	154	195	280	149
N3	159	255	214	105	170	195	280	147
N4	142	255	210	105	170	195	280	137
N5	159	255	210	105	170	197	280	153
N6	159	255	210	105	170	195	280	147
N7	146	255	210	105	170	195	280	147
N8	159	255	210	105	170	195	280	155
N9	159	255	210	105	170	195	278	149
N10	157	255	210	105	170	195	278	149
N11	159	255	210	105	170	195	278	157
N12	158	255	210	105	170	195	280	164
N13	146	255	210	105	170	195	280	151
N14	158	255	210	105	170	195	280	151
N15	159	255	210	105	170	195	280	153

DHPS	-11	-7.4	-2.8	-0.1	0.03	0.5	1.4	9
HB3	217	175	192	132	139	141	268	101
V1S	217	175	180	130	125	147	268	109
3D7	217	157	190	134	149	147	246	111
Alam (201	220	ND	189	132	141	145	250	102
Mta (2011	ND	ND	189	132	ND	ND	ND	ND
C1	217	173	188	132	139	143	248	103
C2	219	175	188	132	139	143	246	101
C3	219	151	188	132	139	143	246	103
C4	219	151	188	132	139	143	246	103
C5	219	151	188	132	139	143	246	101
C6	219	151	188	132	139	141	246	103
C7	213	151	188	132	139	143	246	105
C8	213	151	188	132	139	143	246	101
C9	217	173	188	132	141	143	248	99
C10	213	173	188	132	139	143	246	101
C11	219	151	188	132	139	143	246	101
C12	219	151	188	132	139	143	246	103
C13	213	151	188	132	139	143	246	99
C14	219	151	188	132	139	143	246	103
C15	219	151	188	132	135	143	246	109
C16	217	173	186	132	139	143	246	109
C17	219	151	188	132	139	143	246	103
C18	213	177	192	132	139	143	246	101
T1	217	151	188	132	139	143	246	99
T2	219	155	184	132	139	143	246	99
T3	217	169	188	132	139	143	246	101
T4	213	151	188	132	139	143	246	99
T5	217	165	188	132	139	143	246	105
T6	217	173	188	132	139	143	246	99
T7	219	151	188	132	139	143	246	99
T8	219	151	188	132	139	143	246	99
T9	213	151	188	132	139	143	246	99
T10	219	151	188	132	139	143	246	101
T11	217	161	190	130	139	143	246	99
T12	217	163	188	132	139	143	246	99
T13	217	151	188	132	139	143	246	99
T14	219	179	190	130	139	143	246	99
T15	217	151	188	132	139	141	246	99
T16	219	151	180	132	139	143	246	101
T17	213	151	188	132	139	143	246	99
T18	223	161	188	132	139	143	246	99
T19	217	155	178	132	139	143	246	111
T20	217	151	188	132	139	143	246	101
T21	217	173	188	132	139	143	246	99
N1	217	173	188	132	139	143	252	101
N2	213	151	188	132	139	143	246	103
N3	213	151	188	132	131	139	248	93
N4	217	173	190	132	139	143	246	99
N5	213	151	188	132	139	143	246	101
N6	211	151	188	132	139	143	246	103
N7	217	165	188	132	139	143	246	103
N8	219	151	188	132	139	143	246	103
N9	219	151	188	132	139	143	246	99
N10	219	151	188	132	139	143	246	101
N11	219	151	188	132	139	143	246	101
N12	213	151	188	132	139	143	246	101
N13	219	151	188	132	139	143	246	99
N14	213	151	188	132	139	143	246	105
N15	213	151	188	132	139	143	246	99
N16	219	151	188	132	139	143	246	103
N17	213	151	188	132	139	143	246	105
N18	213	151	188	132	139	143	246	99
N19	217	173	188	132	139	143	246	105
N20	219	163	188	132	139	143	258	107

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