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Reference:
Baraka Vito, Delgado Ratto Christopher, Nag Sidsel, Ishengoma Deus S., Madebe Rashid A., Mavoko Hypolite Muhindo, Nabasumba Carolyn, Lutumba Pascal, Alifrangis Michael, Van geertruyden Jean-Pierre.- Different origin and dispersal of sulfadoxine-resistant Plasmodium falciparum haplotypes betw een Eastern Africa and Democratic Republic of Congo
International journal of antimicrobial agents - ISSN 0924-8579 - 49:4(2017), p. 456-464
Full text (Publisher's DOI): https://doi.org/10.1016/J.IJANTIMICAG.2016.12.007
To cite this reference: http://hdl.handle.net/10067/1443090151162165141

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Different origin and dispersal of sulfadoxine-resistant *Plasmodium falciparum* haplotypes between Eastern Africa and Democratic Republic of Congo

Vito Baraka [a,b,1,*], Christopher Delgado-Ratto [b,1], Sidsel Nag [c,d], Deus S. Ishengoma [a], Rashid A. Madebe [a], Hypolite Muhindo Mavoko [b,e], Carolyn Nabasumba [b,f], Pascal Lutumba [e], Michael Alifrangis [c,d], Jean-Pierre Van Geertruyden [b]

[a] *National Institute for Medical Research, Tanga Research Centre, P.O. Box 5004, Tanga, United Republic of Tanzania*

[b] *Global Health Institute, University of Antwerp, Antwerp, Belgium*

[c] *Centre for Medical Parasitology at the Department of Immunology and Microbiology, University of Copenhagen, Copenhagen, Denmark*

[d] *Department of Infectious Diseases, National University Hospital (Rigshospitalet), Copenhagen, Denmark*

[e] *Département de Médecine Tropicale, Faculté de Médecine, Université de Kinshasa, B.P. 747 Kin XI, Democratic Republic of the Congo*

[f] *Epicentre Mbarara Research Base, P.O. Box 930 Mbarara, Uganda*

ARTICLE INFO

Article history:

Received 11 June 2016

Accepted 17 December 2016

Keywords:

Plasmodium falciparum

Sulfadoxine

Drug resistance

Pfdhps

Molecular evolution

Microsatellites

* Corresponding author. Tel.: +255 27 2644775 ; fax:+255 27 2642010.

E-mail address: vitobaraka@gmail.com (V. Baraka).

[1] These two authors contributed equally to this work.

ABSTRACT

Sulfadoxine/pyrimethamine (SP) is still used for malaria control in sub-Saharan Africa, however widespread resistance is a major concern. This study aimed to determine the dispersal and origin of sulfadoxine resistance lineages in the Democratic Republic of the Congo compared with East African *Plasmodium falciparum* dihydropteroate synthetase (*Pfdhps*) haplotypes. The analysis involved 264 isolates collected from patients with uncomplicated malaria from Tanzania, Uganda and DR Congo. Isolates were genotyped for *Pfdhps* mutations at codons 436, 437, 540, 581 and 613. Three microsatellite loci (0.8, 4.3 and 7.7 kb) flanking the *Pfdhps* gene were assayed. Evolutionary analysis revealed a shared origin of *Pfdhps* haplotypes in East Africa, with a distinct population clustering in DR Congo. Furthermore, in Tanzania there was an independent distinct origin of *Pfdhps* S**GE**GA resistant haplotype. In Uganda and Tanzania, gene flow patterns contribute to the dispersal and shared origin of parasites carrying double- and triple-mutant *Pfdhps* haplotypes associated with poor outcomes of intermittent preventive treatment during pregnancy using SP (IPTp-SP). However, the origins of the *Pfdhps* haplotypes in DR Congo and Eastern Africa sites are different. The genetic structure demonstrated a divergent and distinct population cluster predominated by single-mutant *Pfdhps* haplotypes at the DR Congo site. This reflects the limited dispersal of double- and triple-mutant *Pfdhps* haplotypes in DR Congo. This study highlights the current genetic structure and dispersal of high-grade *Pfdhps* resistant haplotypes, which is important to guide implementation of SP in malaria chemoprevention strategies in the region.

1. Introduction

The widespread emergence of *Plasmodium falciparum* resistance to antimalarial drugs is a major public health concern threatening malaria control efforts. Sulfadoxine/pyrimethamine (SP) and amodiaquine were adopted to replace chloroquine as the first-line treatment against uncomplicated malaria following widespread resistance to chloroquine [1]. However, a few years after the adoption of SP, escalating treatment failure rates indicated that SP resistance was spreading. This prompted policy changes to adopt artemisinin-based combination therapies (ACTs) as the first-line treatment in malaria-endemic countries in Africa.

SP is still recommended by the World Health Organization (WHO) in sub-Saharan Africa for intermittent preventive treatment during pregnancy (IPTp-SP) to protect women and to improve fetal outcomes against the consequences of malaria infection. It is also used in seasonal malaria chemoprevention (SMC) strategies in combination with amodiaquine in areas prone to high seasonal malaria transmission across the Sahel subregion as well as in intermittent preventive treatment in infants (IPTi-SP) in areas of moderate to high transmission [2,3]. In some countries (including the Democratic Republic of the Congo), SP is used in combination with artemisinin for the treatment of uncomplicated malaria [4]. However, due to widespread SP resistance, concerns have been raised about whether this strategy is still effective [5,6].

Pyrimethamine resistance is conferred by point mutations in the *P. falciparum* dihydrofolate reductase (*Pfdhfr)* gene leading to substitutions at codons N51I, C59R and S108N [7]. Sulfadoxine resistance is mediated by substitutions in the *P.*

falciparum dihydropteroate synthetase (Pfdhps) at codons S/A436F, A437G, K540E, A581G and A613S/T [8,9]. Evidence suggests that SP resistance tends to increase as a result of stepwise accumulation of single nucleotide polymorphisms (SNPs) in the *Pfdhps–Pfdhfr* genes [10]. Interestingly, parasite isolates from East Africa were shown to harbour *Pfdhps* haplotypes with double mutations (at codons A437**G** and K540**E**) leading to the S**GE**AA haplotype, whereas in Western African countries the mutations are mainly limited to a single A437**G** mutation [11,12]. Recently, in East Africa, expansion of *Pfdhps* mutation at codons A581**G** and K540**E** has worsened SP resistance leading to the emergence of the triple-mutant haplotype (S**GEG**A) [13]. *Pfdhfr–Pfdhps* haplotypes confers high-grade resistance associated with poor outcomes of IPTp-SP [6,14]. Recently, a *Pfdhps* sextuple haplotype, defined as a combination of the triple *Pfdhfr* and triple *Pfdhps* mutations, was associated with reduced birth weight [6]. In addition, a recent meta-analysis estimated that if the prevalence of *Pfdhps* 581**G** is >10.1%, then IPTp-SP is no longer protective against low birth weight and alternative strategies should be considered [15]. In the East African region where the levels of S**GE**AA and S**GEG**A haplotypes are expanding, the effectiveness of IPTp-SP in controlling parasite growth and improving pregnancy outcomes is deteriorating [6,14,15]. In contrast, in West Africa, the majority of haplotypes are either *Pfdhps* wild-type or a single A437**G** (S**G**KAA) or S436**A** (**A**AKAA) mutation, with limited occurrence of the K540**E** and A581**G** mutants, and thus IPTp-SP retains its effectiveness [11]. These observations underscore the need for monitoring the distribution of parasite populations in order to mitigate the dispersal of resistant haplotypes in the region.

Studies from Southeast Asia revealed multiple and limited origins of *Pfdhps* mutant alleles [16,17], and analysis of the *Pfdhps* mutant alleles in malaria parasites from East and West Africa demonstrated that resistance emerged independently in multiple sites in Africa [17]. After segregating the *Pfdhps* triple mutant S**GEG** haplotype lineages by country, the *Pfdhps* haplotypes seemed of local origin [18]. In DR Congo, Taylor et al. [19] demonstrated genetically distinct resistant *Pfdhps* haplotypes between Eastern and Western provinces. However, owing to the escalating levels of *Pfdhps* A581**G** and K540**E** mutations associated with poor IPTp outcomes in Eastern Africa, there is a need to define the genetic structure and dispersal of *Pfdhps* haplotypes in the Central African corridor and their relatedness to the Eastern African lineages. Therefore, this study determined the origin and dispersal of sulfadoxine-resistant lineages in Central Africa compared with Eastern Africa *Pfdhps* haplotypes.

2. Methods

2.1. Sample collection

Samples for this study were collected between 2012 and 2014 in Uganda (Kihurura) and DR Congo (Lisungi-Kinshasa) as part of the QuinACT clinical trial [20]. Samples from Tanzania were collected in 2014 in Muheza District, Tanga Region, as part of a study to assess the efficacy and safety of artemether/lumefantrine versus dihydroartemisinin/piperaquine for the treatment of uncomplicated malaria (Fig. 1). Children aged 6 months to 10 years with uncomplicated falciparum malaria were enrolled. Fingerprick blood samples were collected on Whatman® 3 mm filter papers

(Whatman plc., Maidstone, UK) and were stored at room temperature until further use.

2.2. DNA extraction

Parasite DNA isolation was performed using a QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Extracted DNA was stored at –20 °C until further use for PCR amplification.

2.3. PCR and sequence-specific oligonucleotide probes enzyme-linked immunosorbent assay (SSOP-ELISA)

Plasmodium falciparum DNA extracts were amplified by nested PCR and the products were analysed for detection of *Pfdhps* SNPs at codons 436, 437, 540, 581 and 613 using SSOP-ELISA as described previously [21].

2.4. Microsatellite (MS) genotyping

MS genotyping was performed using neutral *Pfdhps* microsatellites located on chromosome 8 at 0.8, 4.3 and 7.7 kb downstream from codon 437. Semi-nested PCR was used to amplify the microsatellites as previously described by Roper et al. [22]. The sizes of the MS PCR products were determined using the GeneScan™ 500 LIZ size standard on an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). The microsatellites were scored using GeneMapper® software (Applied Biosystems). The MS haplotypes were generated by combining alleles detected in each of the three MS loci (0.8, 4.3 and 7.7 kb) analysed. Polyclonal infections and

samples that turned out negative at one or more of the MS loci were excluded from the analysis.

2.5. Data analysis

2.5.1. Pfdhps microsatellite and single nucleotide polymorphism haplotypes

The *Pfdhps* MS haplotypes were defined as the unique combination of alleles of the three MS loci (0.8, 4.3 and 7.7 kb). Only samples with available allelic MS data for the three loci, excluding mixed infections, were considered for further analysis. Isolates were classified by the *Pfdhps* SNPs at positions 436, 437, 540, 581 and 613. The proportions of SNPs and MS haplotypes have been plotted by country (Figure 2).

2.5.2. Genetic diversity

The genetic polymorphisms of *Pfdhps* microsatellites were compared among countries and were determined by calculating: the total number of alleles and private alleles (alleles unique to a single country) using GenAlEx v.6.5 [23,24]; the allelic richness using Fstat v.2.9.3 [25]; and the expected heterozygosity (H_e) using GenAlEx, where $H_e = [n/(n-1)]\ [1-\Sigma pi^2]$ and n represent the number of isolates sampled and pi is the frequency of the ith allele.

2.5.3. Genetic differentiation among countries and Pfdhps single nucleotide polymorphism haplotypes

Partitioning of the genetic variation was determined through the Analysis of Molecular Variance (AMOVA) implemented in GenAlEx using MS allelic data. The AMOVA was performed by grouping the samples by country and later by SNP haplotypes. The genetic distances (PHI_{PT}) were calculated for the overall and pairwise analysis to define the genetic differentiation between the countries and SNP haplotypes. Probabilities for the AMOVA indexes were calculated based on 9999 permutations. The Principal Coordinate Analysis (PCoA) was used to find and plot the major patterns related to the origin of *Pfdhps* SNPs using genetic and geographic individual-by-individual distance matrices obtained by GenAlEx.

2.5.4. Population structure of the Pfdhps haplotypes

A Markov chain Monte Carlo (MCMC) procedure implemented in STRUCTURE v.2.3.3 was used to assign individual isolates from all countries to *K* populations based on MS allele data. One to ten populations (*K)* were assessed in STRUCTURE assigning 10 runs each with 50 000 iteration steps followed by 150 000 MCMC burn in a period under the admixture model. The most likely *K* was defined according to Evanno et al. [26] by calculating the rate of change of *K* (Δ*K*) using STRUCTURE HARVESTER v.0.6.94 [27]. Genetic clustering was also assessed through an AMOVA-based *K*-means clustering method implemented in the software GENODIVE v.2.0b23 [28,29]. This method combines the *K*-means clustering, which divides the number of individuals into an a priori assigned number of populations (*K*) in such a way that the within-population diversity is minimised and the between-population diversity is maximised. To determine the optimal clustering, the pseudo-F statistic

was calculated using the simulated annealing with 150 000 steps as convergence method and the number of algorithm repeats set to 50. *Rho*, a statistic equivalent to *Fst* or PHI_{PT}, was reported by GENODIVE as an index of genetic differentiation [28].

2.5.5. Genetic relatedness among microsatellite and single nucleotide polymorphism haplotypes

The phylogenetic relationship between haplotypes within and between countries was computed and plotted using PHYLOViZ v.1.0 [30], which implements the eBURST v.3 algorithm [31]. The plots produced by PHYLOViZ were used to identify SNP haplotypes that shared the same MS haplotype ancestor with explicit information on the geographic origin.

3. Results

3.1. Frequency and pattern of Pfdhps single nucleotide polymorphism haplotypes

A total of 264 samples were analysed for *Pfdhps* SNPs at codons 436, 437, 540, 581 and 613. Of these, 140 samples could be constructed into haplotypes, as the rest were mixed infections at one or more of the *Pfdhps* SNPs; DR Congo, *n* = 37; Tanzania, *n* = 41; and Uganda, *n* = 62. In total, six *Pfdhps* SNP haplotypes were detected: SAKAA (wild-type); S**G**KAA, A**G**KAA and SA**E**AA (single mutants); and S**GE**AA and SA**EG**A (double mutants); and S**GEG**A (triple mutant) (Fig. 2). The pattern varied, with single-mutant *Pfdhps* SNP haplotypes being predominantly detected in parasite populations from DR Congo (75.7%; *n* = 28/37), whilst the *Pfdhps* S**GE**AA (double-mutant) was commonly dispersed in Tanzania (75.6%; *n* = 31/41) and Uganda (87.1%; *n* = 54/62) (Fig. 2). The frequencies of highly-resistant

triple mutant (S**GEG**A) were 8.1% (*n* = 3/37), 12.9% (*n* = 8/62) and 14.6% (*n* = 6/41) in DR Congo, Uganda and Tanzania, respectively.

3.2. Comparing Pfdhps haplotype genetic diversity

The extent of genetic diversity in the parasite population was estimated by calculating the expected heterozygosity (*He*) for the flanking MS in single, double and triple *Pfdhps* mutants with a sample size of *n* ≥ 10. The MS diversity was only analysed for the single mutant S**G**KAA (*n* = 25), the double mutant S**GE**AA (*n* = 87) and the triple mutant S**GEG**A (*n* = 15) *Pfdhps* haplotypes. In most of the MS loci, the number of *Pfdhps* SNPs was inversely correlated with the level of MS polymorphisms: isolates carrying the single mutant S**G**KAA were the most polymorphic at position –7.7 kb (H_e = 0.86) and –4.3 kb (H_e = 0.08), followed by double and triple mutants (S**GE**AA and S**GEG**A). For all three SNP haplotypes, the lowest MS polymorphism was detected at position –4.3 kb (H_e = 0–0.08) (Supplementary Fig. S1).

3.3. Genetic differentiation of the Pfdhps haplotypes in DR Congo, Uganda and Tanzania

The AMOVA analysis-based MS allelic data indicated that the overall genetic variation of the parasites almost equally contributed to the diversity within the countries (49%) and between the countries (51%) (Table 1). Moreover, significant genetic differentiation was found among parasites when they were grouped by country of origin (PHI_{PT} = 0.49; $P \leq 0.0001$). When the parasites were grouped by SNP haplotype, 36% of the total variability was contributed by parasites carrying the same SNP haplotype (Table 1).

3.4. Genetic relatedness of Pfdhps haplotypes between DR Congo, Uganda and Tanzania

Further analysis of the MS allelic data revealed that the single mutant S**G**KAA haplotype only found in DR Congo was highly unrelated based on genetic distance to the other haplotypes found in Tanzania and Uganda (PHI_{PT} = 0.58–0.75, $P < 0.0001$) (Table 2). Double mutants from Tanzania were more similar to the double and triple-mutant parasites from Uganda (PHI_{PT} = 0.08–0.10) compared with the triple mutants from Tanzania (Tanzanian double vs. triple mutant, PHI_{PT} = 0.31, P = 0.01). In contrast, all parasites circulating in Uganda appear to have the same origin (double vs. triple PHI_{PT} = 0.01, P = 0.38) (Table 2).

3.5. Principal Coordinate Analysis (PCoA) and genetic clustering of the Pfdhps haplotypes

Using the data with full MS and *Pfdhps* SNP results (n = 131), PCoA analysis revealed two main distinct clusters of parasite expressing double-mutant (S**GE**AA) and triple-mutant haplotypes (S**GEG**A): one in Tanzania and one in Uganda (Fig. 3). Moreover, the cluster of the S**G**KAA and S**GE**AA parasites from DR Congo was distant from the parasite populations from Uganda and Tanzania. The data suggest distinct divergence of S**G**KAA and S**GE**AA haplotypes from DR Congo *versus* Uganda and Tanzania with limited gene flow between the populations.

3.6. Origin of resistant Pfdhps haplotypes

The lineages were defined on the basis of a shared allele size at the MS loci 0.8, 4.3 and 7.7 kb (*n* = 131) (Fig. 4). Of the 30 MS alleles from DR Congo, single mutant S**G**KAA/SA**E**AA exhibited more diversity associated with 10 different MS haplotypes (H3–H12) compared with the other sites (Fig. 4; Supplementary Table S1). Interestingly, haplotype H11(117-104-141) that was predominantly linked to single mutant haplotypes was only found in DR Congo and was unrelated to lineages in Uganda or Tanzania. Other MS haplotypes linked to a single-mutant haplotype were found at low frequency (Fig. 4).

The haplotype H15(131-104-107) predominantly associated with S**GE**AA was found in the isolates from Tanzania and Uganda, which also exhibited low diversity by having mainly the H15 haplotype linked to S**GE**AA in both Tanzania (55%) and Uganda (78%). In contrast, none of the MS haplotypes associated with S**GE**AA in Tanzania or Uganda were detected in the few S**GE**AA samples from DR Congo, indicating different ancestry and limited gene flow between the regions (Fig. 4). Thus, the Tanzania and Uganda MS alleles associated with S**GE**AA appear to have a common origin with a relatively low diversity of the MS haplotypes compared with DR Congo.

In Tanzania and Uganda, the triple *Pfdhps* mutant S**GEG**A alleles shared MS haplotype H15(131-104-107). Among the S**GEG**A haplotypes in Tanzania, we found an independent origin of MS haplotype H21 that was not observed in Uganda and DR Congo isolates. In DR Congo, the S**GEG**A haplotype was quite rare (*n* = 1) and this one sample showed a unique MS haplotype, H1(117-104-104). These results

suggest that the *Pfdhps* S**GEG**A haplotype from Tanzania and Uganda shared the same lineage, whilst there is an additional and independent occurrence of the highly resistant S**GEG**A haplotype confined only to parasite isolates from Tanzania regarding the H21 haplotype.

3.7. Inferred population structure of the Pfdhps microsatellite haplotypes

STRUCTURE v.2.3.3 was used to infer the genetic structure of the *Pfdhps* MS haplotypes (n = 131) with full *Pfdhps* MS and SNP haplotypes results. The most probable number of clusters was defined using STRUCTURE was K = 2 and K = 3. STRUCTURE analysis suggested the same lineages of mutant *Pfdhps* haplotypes in Tanzania and Uganda with a separate cluster in DR Congo (Fig. 5). The K-means clustering analysis confirmed the presence of two clusters with high genetic differentiation among them (*Rho* = 0.69). This result showed a parasite population with two different origins: from DR Congo and from Tanzania–Uganda.

Median-joining network analysis of the relationships among these MS haplotypes (Fig. 6) confirmed the clustering patterns of the haplotypes. The analysis reveals the clustering pattern of double S**GE**AA and triple-mutant S**GEG**A haplotypes from Tanzania and Uganda (Fig. 6A). Contrastingly, the network of single mutant S**G**KAA reveals that parasite populations from DR Congo harbour diverse MS lineages. The network analysis suggests a shared lineage of evolved mutant *Pfdhps* haplotypes in Tanzania and Uganda, whilst the lineages from DR Congo could have a different origin unrelated to the East African lineages. The different clustering of the single-mutant haplotypes (S**G**KAA) from the double- and triple-mutant (S**GE**AA and

S**GEG**A) haplotypes suggested different genetic lineages of *Pfdhps* haplotypes in East and Central Africa.

4. Discussion

Molecular analysis of *Pfdhps* revealed a high presence of double (S**GE**AA) and triple (S**GEG**A) mutant haplotypes predominantly in the East African region (Tanzania and Uganda), whilst in DR Congo the single *Pfdhps* mutants were predominant. In contrast, the frequency of the *Pfdhps* S**GEG**A observed was somewhat low compared with recent studies in the same area of Northern Eastern Tanzania [32]. None the less, the level of the *Pfdhps* triple mutation correlated with the increasing level of *Pfdhps* A581**G** mutation in the region, which is regarded as a focus of resistance, and in particular earlier reports of SP resistance were documented [12]. At present, SP resistance is widespread and heterogeneous in East Africa and in some areas it is close to fixation [6,33,34]. It is evident that the levels of the *Pfdhps* mutant haplotypes associated with poor IPTp-SP outcomes are escalating, particularly in the East African region where SP resistance is at an alarming level [6,35,36]. These findings corroborate previous findings that resistant *Pfdhps* haplotypes are circulating in Tanzania, Kenya and Uganda and are dispersing to other countries in the region presumably due to maintained drug pressure [32,37]. Also, we previously demonstrated that the pattern of S**GE**AA and S**GEG**A haplotypes in Tanzania appears to be vastly heterogeneous and high (ca. 50%) in the northeastern regions [32]. In DR Congo and the neighbouring Congo-Brazzaville, it is mainly the wild-type and single-mutant S**G**KAA haplotype that predominates, with a low frequency of double- and triple-mutant *Pfdhps* haplotypes, suggesting the predominance of single *Pfdhps* mutants in Central Africa [19,38].

The observed differences in the distribution of double and triple *Pfdhps* mutant haplotypes reflect the differences in SP effectiveness in West-Central Africa compared with East Africa. Whilst SP retains its efficacy in Western Africa, in Eastern Africa it is seriously compromised due to increased frequencies of *Pfdhps* K540**E** and A581**G** [39]. However, these dynamics could change as the political and socioeconomic interactions in the region increase movements of refugees and minority groups such as peacekeeping soldiers as recently documented [40]. It is important for drug sellers in informal drug outlets and accredited drug dispensing outlets to adhere to national guidelines and to limit casual access to SP for the treatment of uncomplicated malaria. This will reduce the sustained drug pressure on parasite populations in the region.

The expected heterozygosit*y* was high among the single *Pfdhps* S**G**KAA isolates. In contrast, the heterozygosity values among S**GE**AA and S**GEG**A isolates were low as expected, corroborating previous studies [17,22]. This could be a result of continuing high selection pressure due to sustained high SP usage [34,41,42]. Also, the decline in malaria prevalence could have reduced the sexual recombination events resulting in low diversity within the parasite population in East Africa. The low genetic diversity and high resistance levels suggest a limited likelihood of sexual recombination events and therefore it is less likely that re-emergence of SP-susceptible strains will expand. In this scenario, fixation of sulfadoxine resistance could further exacerbate resistance, rendering SP unsuitable for IPT strategies.

These data suggest shared lineages of the double *Pfdhps* S**GE**AA haplotypes from Tanzania and Uganda, but also regarding the S**GEG**A haplotype from Uganda that all were associated with MS haplotype H15(131-104-107). Similarly, the same resistant lineages were demonstrated by previous studies in other neighbouring countries in the region linked with double *Pfdhps* S**GE** [17]. Considering K540**E** and A581**G** as a proxy for quintuple haplotype, as well as the evidence presented here, it is clear that the widespread of the H15 haplotype presents a concern for utilisation of SP in malaria control. The H21(131-104-125) haplotype linked to S**GEG**A was limited to Tanzania, suggesting a distinct occurrence of the S**GEG**A haplotype as also observed in a recent study performed in the same region [13]. In contrast, the *Pfdhps* haplotypes from DR Congo demonstrated distinct and divergent *Pfdhps* double-mutant haplotypes and consistent with the previous observation that sulfadoxine-resistant *P. falciparum* emerged independently at multiple sites in Africa and that the molecular basis for sulfadoxine resistance is different in East and Central African regions [17]. The haplotypes in Central Africa, mostly S**G**KAA associated with H11(117-104-141), appears more diverse and comparable with Western Africa where there is high *Pfdhps* single-mutant haplotypes (S**G**KAA and A**G**KAA) and a different pattern of double haplotypes as documented in previous studies [16,17].

The data also present a different dispersal of high-level resistant double- and triple-mutant S**GE**AA and S**GEG**A haplotypes in East Africa compared with Central Africa. These findings suggest that parasite lineages from Tanzania and Uganda were associated with S**GE**AA and S**GEG**A as elucidated in the genetic STRUCTURE analysis (Fig. 5), median-joining network analysis (Fig. 6) and PCoA (Fig. 3). The

evidence presented by the PCoA and Network analyses suggests the independent origin of *Pfdhps* S**GEG**A (triple mutant) lineage circulating in Tanzania. The occurrence of the lineage suggests the indigenous emergence of S**GEG**A in Tanzania where the spread of A581**G** is evident. These findings corroborate a previous study that also supported the independent lineage of *Pfdhps* S**GEG**A in East Africa [13,18]. Also, the current data strongly suggest the relatedness of parasites from Tanzania and Uganda in contrast to observed parasite population clustering in DR Congo, implying limited gene flow between DR Congo and East Africa countries. The S**G**KAA mutant strains from the DR Congo appear to be clustered, whilst in Tanzania and Uganda they share multiple lineages harbouring S**GE**AA and S**GEG**A in line with studies in DR Congo where similar results were reported [19,43]. The results suggest existing gene flow between Tanzania and Uganda parasite populations presumably due to improved transportation and human migration patterns and highlight the importance of co-ordinated control policies across the region. The current findings mirror the evidence on the presence of human migration networks at local and national levels that could facilitate the dispersal of malaria parasites and drug resistance within and between countries in Southeast Africa [44,45]. A report elucidated high levels of infection movements in the East Africa region, which corroborate our finding that there is limited evidence of intercountry infection movement that is likely to occur between the sites in Eastern Africa and DR Congo. In addition, a separate report demonstrated that short movements between different transmission settings in East Africa contribute to local malaria transmission in the region [45]. However, these human migration patterns are constantly affected by several factors including political stability, climatic factors, trade, transport and infrastructure [46].

This study had several shortcomings. The statistical power was limited due to the fact that only the pure MS haplotypes and *Pfdhps* haplotypes were analysed. However, given the similarity of the MS haplotypes, we believe the observed results do represent the parasite lineages between the sites.

5. Conclusion

In Uganda and Tanzania, gene flow patterns contribute the dispersal and shared the origin of parasites carrying double- and triple-mutant *Pfdhps* haplotypes associated with poor IPTp-SP outcomes. However, the origins of the *Pfdhps* haplotypes in DR Congo and Eastern Africa sites are different. The genetic structure demonstrated a divergent and distinct population cluster predominated by single-mutant *Pfdhps* haplotypes in the DR Congo site. This reflects the limited dispersal of double- and triple-mutant *Pfdhps* haplotypes in DR Congo. This study highlights the current genetic structure and dispersal of high-grade *Pfdhps* resistant haplotypes, which is important to guide the implementation of SP in malaria chemoprevention strategies in the region.

Acknowledgments: The study participants are thanked for agreeing to take part in this study. NIMR Tanga (Tanzania) staff involved in the WB project are thanked for their role in field sample collection. This work would not have been possible without the medical and study team involved in the QuinACT trial at Lisungi Health Centre (DR Congo) and Kihuru Kazo Health Centre (Uganda). Ulla Abildtrup at the Centre for Medical Parasitology (Copenhagen, Denmark) is thanked for technical support during the laboratory analyses. Gwen Lemey and Jan Vervoort (University of

Antwerp, Antwerp, Belgium) and Claire Alberte Charasse (Centre for Medical Parasitology, Denmark) are thanked for their logistical support.

Funding: Data collection in Tanzania was supported by the World Bank through the Ministry of Health. FWO [grant reference G.078.11], Belgian Technical Cooperation [grant reference FEE/06/12] and EDCTP [grant reference MS.2010.10800.004] supported the field sample and data collection in DR Congo and Uganda. The project was supported by the VLIR South Initiative project [code ZAIN2014Z168]. VB is fully supported by a VLIR-ICP PhD scholarship (Belgium).

Competing interests: None declared.

Ethical approval: The studies were approved by the Ethical Committee of the University of Antwerp (Antwerp, Belgium) [reference UA A11-02], the Uganda National Council for Science and Technology (Uganda) [reference HS 1108] and the Ethics Committee of the School of Public Health, University of Kinshasa (Kinshasa, DR Congo) [reference ESP/CE/012B/2012] and the Medical Research Coordination Committee of the National Institute for Medical Research (NIMR) (Tanzania). Studies were conducted according to the Declaration of Helsinki and Good Clinical Practice guidelines. Informed consent was obtained from all individual patients or their legally acceptable representatives.

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Fig. 1. Location of the study sites and major cities within the Democratic Republic of the Congo, Uganda and Tanzania (adapted from Google Maps).

Fig. 2. Distribution of *Plasmodium falciparum* dihydropteroate synthetase (*Pfdhps*) single nucleotide polymorphism (SNP) haplotypes collected from DR Congo , Tanzania and Uganda sites. SNP haplotypes were defined by amino acid residues at positions 436, 437, 540, 581 and 613.

Fig. 3. Principal Coordinate Analysis (PCoA) of the *Plasmodium falciparum* dihydropteroate synthetase (*Pfdhps*) microsatellite (MS) haplotypes in DR Congo, Tanzania and Uganda. The three-dimensional plot (*x*-axis represents PC1, *y*-axis PC2 and *z*-axis PC3) displays the genetic distances among haplotypes, which are coloured according to the assignment to a specific *Pfdhps* single nucleotide polymorphism (SNP) haplotype and country. Double mutants from Uganda and Tanzania formed distinct clusters that overlapped each other, whilst those isolated from DR Congo formed a discrete cluster with minimal overlap. The three co-ordinate axes collectively represent 71.4% of the total variation.

Fig. 4. Microsatellite (MS) haplotypes linked to wild-type (SAKAA), single mutants (S**G**KAA/SA**E**AA), double mutants (S**GE**AA) and triple mutants (S**GEG**A) in DR Congo, Tanzania and Uganda. The *x*-axis indicates the MS haplotypes determined by the combination of allele sizes at loci 0.8 kb, followed by 4.3 kb and 7.7 kb, for samples for which full haplotypes could be determined. The association of specific MS haplotypes with different *Plasmodium falciparum* dihydropteroate synthetase (*Pfdhps*) single nucleotide polymorphisms (SNPs) is observed from the percentage

(%) of each haplotype shown in the individual charts. The *y*-axis represents the number of alleles associated with each MS haplotype in percentage (%) of alleles sampled in the respective country.

Fig. 5. STRUCTURE analysis of the *Plasmodium falciparum* lineages with *P. falciparum* dihydropteroate synthetase (*Pfdhps*) microsatellite (MS) haplotypes. The *x*-axis represents individual isolates sorted according to their reported ancestries. Individual isolates are represented by a single vertical line broken into *K* coloured segments proportional to each of the inferred clusters.

Fig. 6. Median-joining network analysis of *Plasmodium falciparum* samples collected in DR Congo, Tanzania and Uganda. The genetic relationships among 131 parasites were constructed using three microsatellite (MS) loci flanking *P. falciparum* dihydropteroate synthetase (*Pfdhps*). The size of the circle represents the exponential number of isolates harbouring a particular haplotype. (A) Distinct lineages by country rendered in different colours; and (B) clustering of subpopulation presented by unique *Pfdhps* haplotypes at amino acid codons 436, 437, 540, 581 and 613 of the *Pfdhps* gene (mutant amino acids in bold and underlined). Only samples with full genotyping results were included.

Figure 1

Figure 2

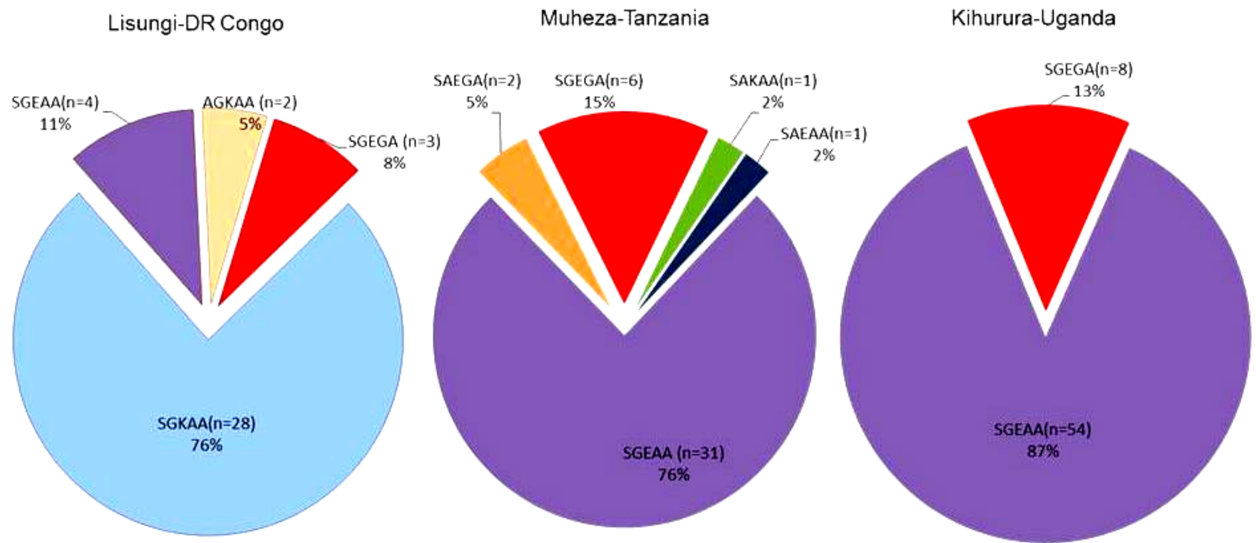


Figure 3

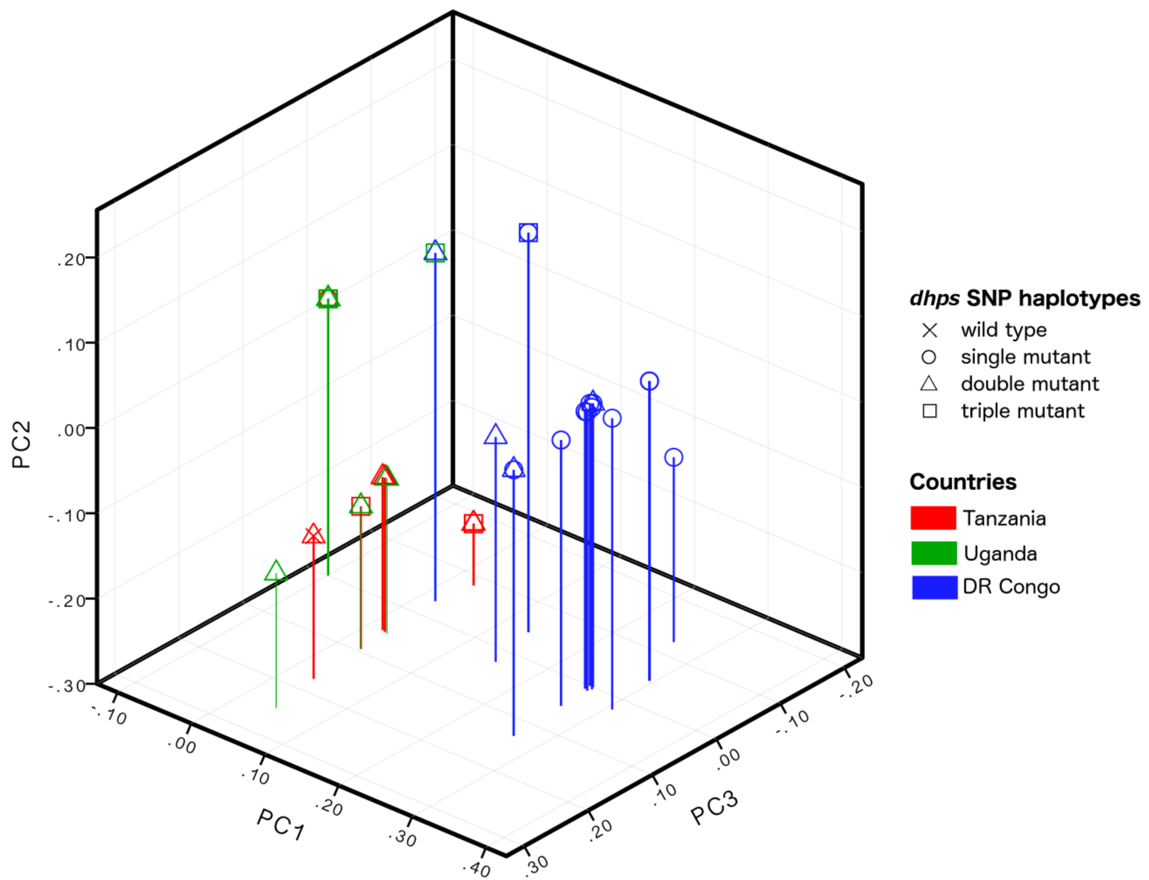


Figure 4

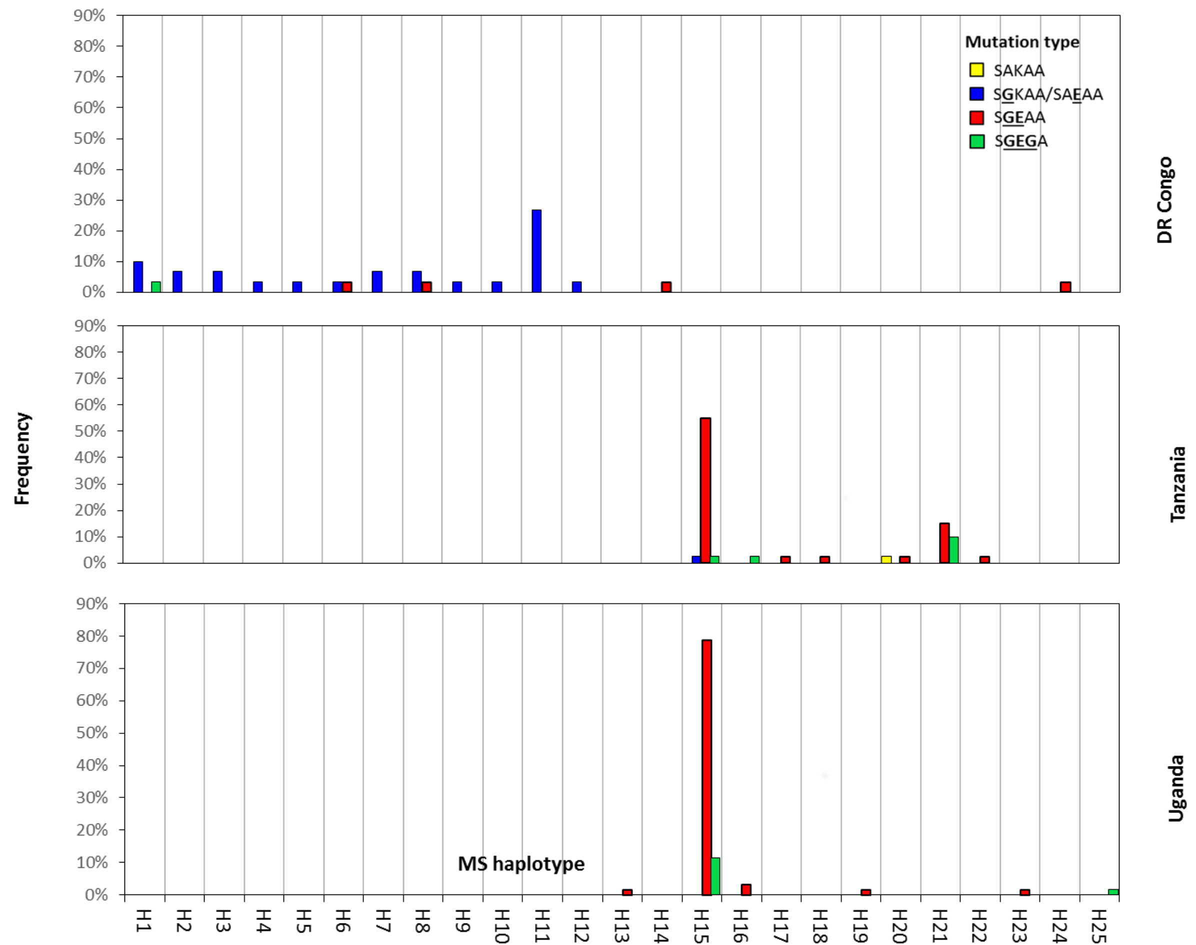


Figure 5

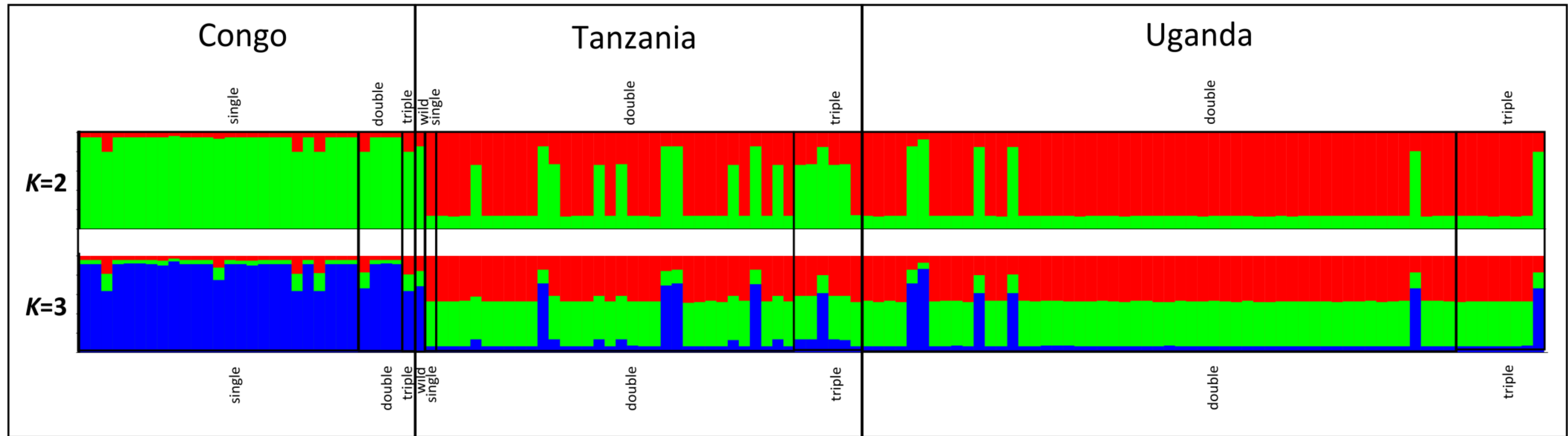


Figure 6

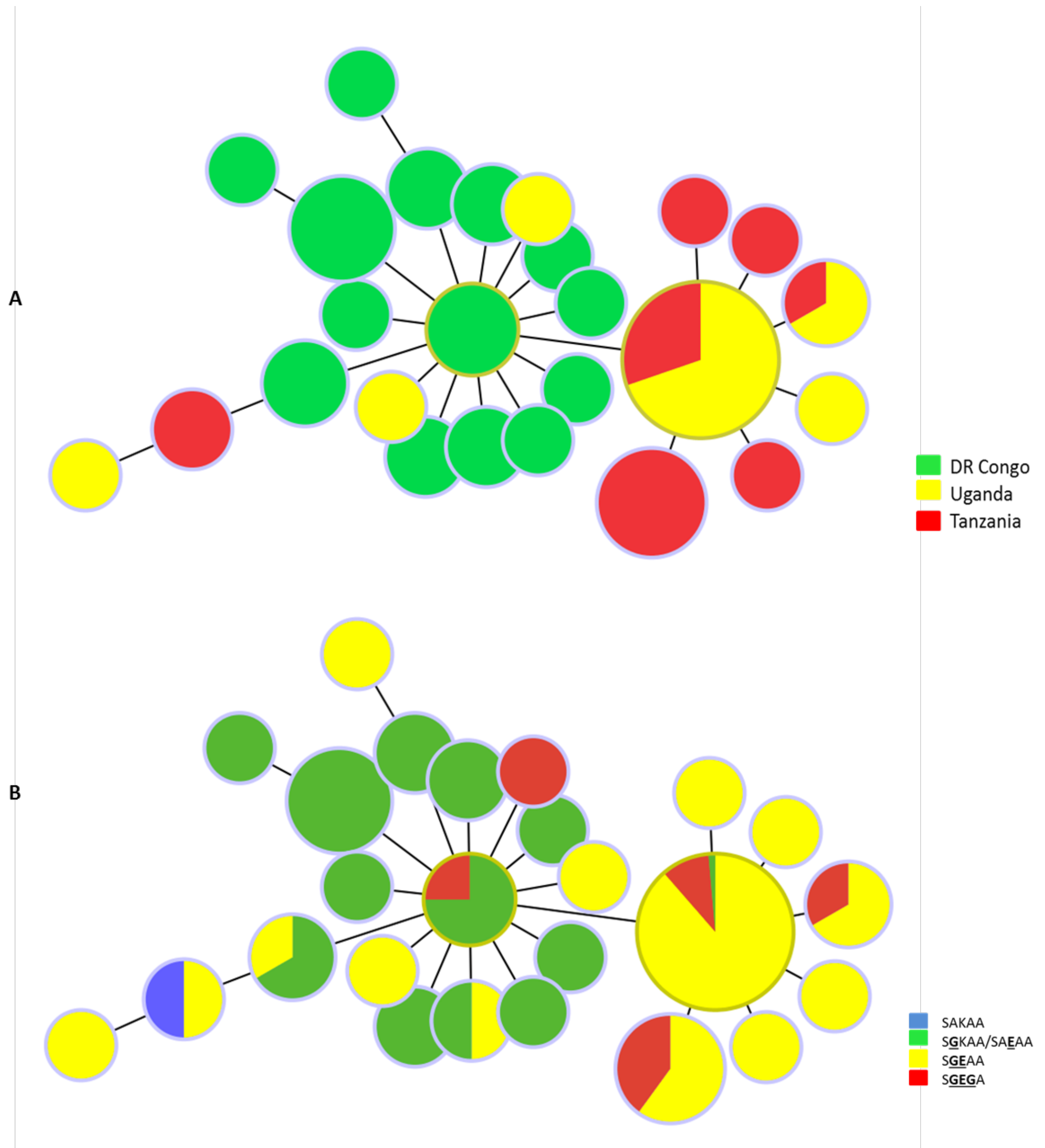


Table 1

Analysis of Molecular Variance (AMOVA) showing the partitioning of genetic variation of *Plasmodium falciparum* dihydropteroate synthetase (*Pfdhps*) single nucleotide polymorphism (SNP) haplotypes within and between Uganda, Tanzania and DR Congo study sites

Grouping	Source	DF	SS	MSq	Est. var.	PTV%	PHI_{PT}	*P*-value [a]
Geographic origin	Among sites	2	70.27	35.14	0.40	49%	0.49	≤0.0001
	Within sites	261	108.44	0.42	0.42	51%		
SNP haplotypes	Among SNP haplotypes	2	26.44	13.22	0.44	64%	0.64	≤0.0001
	Within SNP haplotypes	119	29.40	0.25	0.25	36%		

DF, degrees of freedom; SS, sum of squares; MSq, mean squares; Est. var., estimate of variance; PTV%, percentage of total variation; PHI_{PT}, estimate of the proportion of the genetic variance presented by sites and between the *Pfdhps* haplotypes.

[a] *P*-value is based on 9999 permutations.

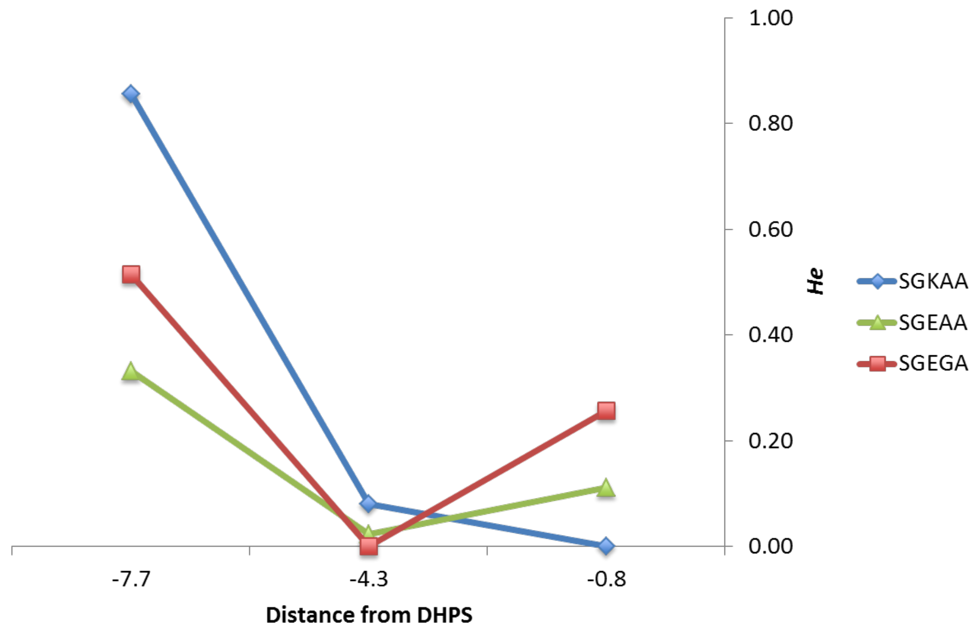
The *Pfdhps* haplotypes were grouped into single, double and triple based on amino acids mutation at codons 436, 437, 540, 581 and 613.

Table 2

Pairwise PHI_{PT} values among the observed *Plasmodium falciparum* dihydropteroate synthetase (*Pfdhps*) haplotypes among the sites in DR Congo, Uganda and Tanzania

Country–haplotype	DR Congo–S**G**KAA	Tanzania–S**GE**AA	Uganda–S**GE**AA	Tanzania–S**GEG**A	Uganda–S**GEG**A
DR Congo–S**G**KAA	–	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Tanzania–S**GE**AA	0.64	–	**<0.003**	**0.01**	0.10
Uganda–S**GE**AA	0.75	0.10	–	**<0.001**	0.38
Tanzania–S**GEG**A	0.58	0.31	0.66	–	**<0.003**
Uganda–S**GEG**A	0.62	0.08	0.01	0.58	–

The *Pfdhps* haplotypes are defined by the amino acids at codons 436, 437, 540, 581 and 613 (mutant amino acids underlined and bold). The calculated PHI_{PT} values are shown below the diagonal. Haplotype groups with less than five samples were not considered for the pairwise PHI_{PT} calculations. The probability based on 9999 permutations is shown above the diagonal. Significant values are shown in bold determined at $P < 0.05$.



Supplementary Fig. S1. Expected heterozygosity (H_e) at flanking loci 0.8, 4.3 and 7.7 kb grouped by single nucleotide polymorphism (SNP) haplotype. The microsatellite (MS) diversity from all three countries found in the single mutant S**G**KAA (*n* = 25), double mutant S**GE**AA (*n* = 87) and triple mutant S**GEG**A (*n* = 15) *Plasmodium falciparum* dihydropteroate synthetase (*Pfdhps*) haplotypes are presented. H_e was calculated for SNP haplotypes with observation $n \geq 10$.

Supplementary Table S1

Plasmodium falciparum dihydropteroate synthetase (*Pfdhps*) microsatellite (MS) haplotypes classified by site and *Pfdhps* single nucleotide polymorphism (SNP) haplotypes

***Pfdhps* MS haplotype**	***Pfdhps* MS allele**			**MS haplotypes by site**			***Pfdhps* SNP haplotypes**						
	0.8 kb	4.3 kb	7.7 kb	DR Congo	Tanzania	Uganda	SAKAA	S**G**KAA	SA**E**AA	S**GE**AA	SA**EG**A	S**GEG**A	Total
H1	117	104	107	4	0	0	0	3	0	0	0	1	4
H2	117	104	109	2	0	0	0	2	0	0	0	0	2
H3	117	104	111	2	0	0	0	2	0	0	0	0	2
H4	117	104	113	1	0	0	0	1	0	0	0	0	1
H5	117	104	115	1	0	0	0	1	0	0	0	0	1
H6	117	104	117	2	0	0	0	1	0	1	0	0	2
H7	117	104	119	2	0	0	0	2	0	0	0	0	2
H8	117	104	121	3	0	0	0	2	0	1	0	0	3
H9	117	104	125	1	0	0	0	1	0	0	0	0	1
H10	117	104	129	1	0	0	0	1	0	0	0	0	1
H11	117	104	141	8	0	0	0	8	0	0	0	0	8
H12	117	106	141	1	0	0	0	1	0	0	0	0	1
H13	123	104	107	0	0	1	0	0	0	1	0	0	1
H14	125	104	107	1	0	0	0	0	0	1	0	0	1
H15	131	104	107	0	24	55	0	0	1	69	1	8	79
H16	131	104	111	0	1	2	0	0	0	2	0	1	3
H17	131	104	113	0	1	0	0	0	0	1	0	0	1
H18	131	104	115	0	1	0	0	0	0	1	0	0	1
H19	131	104	119	0	0	1	0	0	0	1	0	0	1
H20	131	104	121	0	2	0	1	0	0	1	0	0	2
H21	131	104	125	0	10	0	0	0	0	5	1	4	10
H22	131	104	131	0	1	0	0	0	0	1	0	0	1
H23	131	106	121	0	0	1	0	0	0	1	0	0	1
H24	133	104	109	1	0	0	0	0	0	1	0	0	1
H25	136	104	107	0	0	1	0	0	0	0	0	1	1
Total							1	25	1	87	2	15	131