



RESEARCH ARTICLE

Micro-epidemiological structuring of *Plasmodium falciparum* parasite populations in regions with varying transmission intensities in Africa. [version 1; referees: 4 approved]

Irene Omedo ¹, Polycarp Mogeni ¹, Teun Bousema^{2,3}, Kirk Rockett ⁴, Alfred Amambua-Ngwa⁵, Isabella Oyier¹, Jennifer C. Stevenson^{3,6}, Amrish Y. Baidjoe², Etienne P. de Villiers ^{1,7,8}, Greg Fegan ¹, Amanda Ross ⁹, Christina Hubbard⁴, Anne Jeffreys⁴, Thomas N. Williams^{1,10}, Dominic Kwiatkowski^{4,11}, Philip Bejon^{1,12}

¹KEMRI-Wellcome Trust Research Programme, Centre for Geographic Medicine Research-Coast, Kilifi, Kenya

²Department of Medical Microbiology, Radboud University Nijmegen Medical Centre, Radboud Institute for Molecular Life Sciences, Nijmegen, Netherlands

³London School of Hygiene and Tropical Medicine, London, UK

⁴Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford, UK

⁵Medical Research Council Unit, Fajara, Gambia

⁶Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland, USA

⁷Centre for Tropical Medicine and Global Health, Nuffield Department of Medicine Research Building, University of Oxford, Oxford, UK

⁸Department of Public Health, Pwani University, Kilifi, Kenya

⁹Department of Epidemiology and Public Health, Swiss Tropical and Public Health Institute, Basel, Switzerland

¹⁰Department of Medicine, South Kensington Campus, Imperial College London, London, UK

¹¹Wellcome Trust Sanger Institute, Wellcome Genome Campus, Hinxton, Cambridge, UK

¹²Centre for Clinical Vaccinology and Tropical Medicine, University of Oxford, Oxford, UK

v1 First published: 14 Feb 2017, 2:10 (doi: [10.12688/wellcomeopenres.10784.1](https://doi.org/10.12688/wellcomeopenres.10784.1))
Latest published: 14 Feb 2017, 2:10 (doi: [10.12688/wellcomeopenres.10784.1](https://doi.org/10.12688/wellcomeopenres.10784.1))

Abstract

Background: The first models of malaria transmission assumed a completely mixed and homogeneous population of parasites. Recent models include spatial heterogeneity and variably mixed populations. However, there are few empiric estimates of parasite mixing with which to parametrize such models.

Methods: Here we genotype 276 single nucleotide polymorphisms (SNPs) in 5199 *P. falciparum* isolates from two Kenyan sites and one Gambian site to determine the spatio-temporal extent of parasite mixing, and use Principal Component Analysis (PCA) and linear regression to examine the relationship between genetic relatedness and relatedness in space and time for parasite pairs.

Results: We show that there are no discrete geographically restricted parasite sub-populations, but instead we see a diffuse spatio-temporal structure to parasite genotypes. Genetic relatedness of sample pairs is predicted by relatedness in space and time.

Open Peer Review

Referee Status:

	Invited Referees			
	1	2	3	4
version 1				
published 14 Feb 2017	report	report	report	report
<hr/>				
1	Michel Tibayrenc , Institut de Recherche pour le Développement (IRD), France			
2	Cristian Koepfli , University of California, Irvine, USA			
3	Christopher Delgado-Ratto , University of Antwerp, Belgium			

Conclusions: Our findings suggest that targeted malaria control will benefit the surrounding community, but unfortunately also that emerging drug resistance will spread rapidly through the population.

4 **Liwang Cui**, Pennsylvania State University, USA

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Corresponding author: Irene Omedo (iomedo@kemri-wellcome.org)

Competing interests: No competing interests were disclosed.

How to cite this article: Omedo I, Mogeni P, Bousema T *et al.* **Micro-epidemiological structuring of *Plasmodium falciparum* parasite populations in regions with varying transmission intensities in Africa. [version 1; referees: 4 approved]** Wellcome Open Research 2017, 2:10 (doi: [10.12688/wellcomeopenres.10784.1](https://doi.org/10.12688/wellcomeopenres.10784.1))

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Grant information: Sample collection at the Rachuonyo South site was supported by the Bill and Melinda Gates Foundation, under the Malaria Transmission Consortium, Grant No.45114 and the Grand Challenge Grant No. OPP1024438. Thomas N. Williams is funded by the Wellcome Trust, grant number 091758. Philip Bejon, Polycarp Mogeni and Irene Omedo are funded by the UK Medical Research Council (MRC) and the UK Department for International Development (DFID) under the MRC/DFID Concordat agreement. Polycarp Mogeni is funded by the Gottfried und Julia Bangerter-Rhyner Stiftung and the Novartis Foundation for Medical Biological Research project 13A13. Sample collection in Kilifi was supported by core funding from the Wellcome Trust to the Kenya Programme.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

First published: 14 Feb 2017, 2:10 (doi: [10.12688/wellcomeopenres.10784.1](https://doi.org/10.12688/wellcomeopenres.10784.1))

Introduction

The earliest models of malaria transmission assumed a completely mixed and homogenous parasite population^{1,2}. However, malaria transmission is highly heterogeneous, and follows the Pareto principle where 80% of infections occur in only about 20% of the population³. Consequently, there is increasing interest in models allowing for spatial heterogeneity and variably mixed populations of parasites^{4–7}. There are now several epidemiological studies describing spatial heterogeneity of malaria on varying geographical scales^{8–19}. This heterogeneity is characterized by infection hotspots which usually persist even after transmission has been reduced in surrounding areas^{9,11,20–25}, and thus act as reservoirs of infection^{21,26}. Achieving any meaningful reduction in transmission in regions containing malaria hotspots will require a scale up of control activities, including repeated mass administration of Artemisinin Combination Therapy (ACT) drugs, increased coverage of long lasting insecticide treated nets (LLINs) and intensive indoor residual spraying (IRS). These measures are very costly and may not be realistic for universal coverage in most of the resource-poor endemic countries. Thus, targeted control may be more important, and is likely to be required to eliminate malaria^{3,21,27,28}.

Mathematical models show that targeting hotspots may reduce transmission in surrounding areas^{11,22}. These models, however, assume that hotspots are stable and that mosquito mixing in the community is homogeneous²². Studies have shown that certain species of mosquitoes exhibit some level of site fidelity, where they return to the same homesteads to feed²⁹. If such behaviour is the norm with very little mixing, then this would greatly reduce the community-wide impact of targeted interventions, and interventions would be beneficial only to individuals within the targeted region. If, however, transmission networks operate freely over large geographical areas, then these interventions would likely have an impact beyond the targeted region. Furthermore, parasite evolution takes place in a micro-epidemiological context and the spread of drug resistance or new antigenic variants through the population will also be critically dependent on the degree of mixing of parasite populations.

Few studies currently provide empiric evidence on the mixing of parasites over space and time, yet this evidence is important as parasite mixing is likely to affect the outcome of targeted control interventions²³. The community-wide impact of targeted control has not been studied extensively, although early controlled trials showed that bed nets were effective at reducing child morbidity and mortality associated with malaria, in villages or communities randomised to the intervention in The Gambia³⁰ and Kilifi³¹. More recent studies have shown that the use of bed nets in a village randomized to intervention in Asembo, western Kenya, also protected individuals just outside the intervention village who were themselves not using bed nets³². A cluster-randomized controlled trial on the impact of targeting integrated control measures to hotspots showed temporally limited effect on reducing transmission in areas surrounding the targeted hotspots²³. In order to inform future targeted control strategies more precise empiric data on parasite mixing is required.

We hypothesized that by genotyping parasites with fine-scale temporal and spatial data we would be able to determine fine-scale structure to the population and infer the degree of parasite mixing over small geographical areas which are likely to be the focus of targeted malaria control programs^{23,27}. We used SNP genotyping of *Plasmodium falciparum* field isolates from three African sites and analysed the genetic relatedness among parasites within individual sites, in order to determine the level of parasite mixing on micro-epidemiological scales in each population. Principal Component Analysis (PCA) was used to detect parasite subpopulations in each site, and tests of spatial autocorrelation including Moran's *I* and spatial scan statistics were used to test for autocorrelation among parasite genotypes. The analyses were carried out at different spatial scales ranging from intensive within-village surveillance through to county-wide surveillance.

Materials and methods

Study sites

P. falciparum infected blood samples were collected from individuals at three sites in two African countries: Kombo coastal districts of The Gambia on the West African coast; Kilifi, Kenya on the East African coast, and Rachuonyo South District in the Western Kenyan highlands. The Gambia has a subtropical climate with a single rainy season between the months of June and October^{33,34}, while Kenya has two rainy seasons, experiencing short rains between October and December and long rains between April and August³⁵. In all three sites, *P. falciparum* is the main causative agent of malaria^{22,33,35} and transmission occurs almost exclusively during and immediately after the rainy seasons^{34,36}. The common vectors in The Gambia are *Anopheles gambiae* s.s., *Anopheles arabiensis* and *Anopheles melas*³⁷, while the common vectors in the Kenyan coast have historically been *A. gambiae* s.s. and *A. funestus*, but a recent shift to *A. arabiensis* and *A. merus* has been detected along the coast³⁸. In Rachuonyo South district, the main vectors transmitting malaria are *A. gambiae* s.l. and *A. funestus*³⁹. Temporal trends show declining malaria transmission in The Gambia and Coastal Kenya^{17,33,34,40}, although not in Western Kenya⁴¹. Asymptomatic parasite prevalence is lowest in The Gambia at 8.7%⁴², intermediate in Kilifi at 14%⁴³ and slightly higher in Rachuonyo South at 16%⁴⁴.

Ethics statement

Ethical approval for this study was obtained from Kenya Medical Research Institute (KEMRI) Ethical Review Committee (under SSC No. 2239). Written informed consent was obtained from parents/guardians of the study participants. The study methods were carried out in accordance with the approved guidelines.

Sample collection, DNA extraction and Genotyping

5199 *P. falciparum* infected blood samples were collected during hospital admissions and community surveys over a 14-year period from 1998 to 2011. The Gambian samples were collected at Fajara and Brikama health facilities from children aged 8 months to 16 years who were living in the Kombo coastal districts and who were part of a clinical malaria study in 2007–2008³³. The Kilifi samples came from children aged 1 to 6 years who had been recruited into a

phase 2b randomized trial looking at the efficacy of the Candidate Malaria Vaccines FP9 ME-TRAP (multiple epitope–thrombospondin-related adhesion protein) and MVA ME-TRAP in 2005⁴⁵, as well as clinical malaria studies looking at antibody responses to Merozoite Surface Protein 2 (MSP2) among individuals 3 weeks to 85 years old⁴⁶; the effect of declining transmission on mortality and morbidity in children up to 14 years old⁴⁰ and definitions of clinical malaria endpoints⁴⁷. The Rachuonyo south samples were collected during a community survey conducted in 2011 as part of a trial looking at the impact of hotspot targeted control interventions on reducing malaria transmission in the wider community²². Prior to genotyping, DNA was extracted from these samples using either ABI prism 6100 Nucleic Acid prepstation (Applied Biosystems, Waltham, Massachusetts, USA) or Chelex Extraction.

276 SNPs in 177 genes were typed in the three parasite populations (Dataset 1⁶⁶). The SNPs were selected from a panel of 384 SNPs previously designed for a study on population structure of *P. falciparum* parasites from Africa, Southeast Asia and Oceania⁴⁸ and were chosen based on three criteria:

- a) polymorphic among three of the most studied and well characterized *P. falciparum* strains (3D7, HB3 and IT).
- b) uniformly distributed across the parasite genome.
- c) ease of typing on the sequenom platform.

Genes typed included antigen-encoding, housekeeping and hypothetical genes. 52 and 9 SNPs were typed in the antigen-encoding parasite ligands Erythrocyte Binding Antigen 175 (EBA-175) and Apical Membrane Antigen 1 (AMA-1), respectively. In the Kilifi parasite population, between 158 and 226 SNPs were typed in each sample, while in The Gambia and Rachuonyo south populations, 131 and 111 SNPs were typed in 143 and 2744 samples, respectively. Genotyping was done on the Sequenom MassARRAY iPLEX platform, which allows multiplexing of up to 40 SNPs in a single reaction well and differentiates alleles based on variations in their mass⁴⁹. Locus specific PCR and iPLEX extension primers were designed with the sequenom MassARRAY designer software (Version 3.1) using 3D7 as the reference genome (PlasmoDB release 9.0) (Dataset 2⁶⁷). A multiplexed PCR reaction was performed by pooling locus-specific primers, and un-incorporated dNTPs were dephosphorylated enzymatically using shrimp alkaline phosphatase. Extension primers binding immediately adjacent to the SNP site of interest were then extended by a single nucleotide base, using mass-modified dideoxynucleotides. The extended products were resin cleaned to remove excess salts and the mass of the different alleles determined using MALDI-TOF mass spectrometry.

Sample and SNP cut-off selection criteria

Genotype data was aggregated to determine genotyping success rates for individual samples and SNPs. Samples where >40% of SNP typing failed were excluded from analysis, and among the remaining samples, SNP typing for which >30% of samples failed were further excluded from analysis. The criteria for successful SNP

typing were based on the SNP intensity values (r) and allelic intensity ratios (θ). Alleles were called as successful if they were above an intensity cut-off value ranging between 0.5 and 1.0, set depending on the performance of the individual SNP assay, and were classified as failed if they were below this cut-off. For those SNPs that were above the cut-off, allelic intensity ratios ranging between 0 and 1 were used to classify them as homozygous or heterozygous. Theta values nearing 0 and 1 indicate different homozygous alleles, while intermediate values indicate heterozygous SNPs, representing mixed parasite populations. Where mixed parasite populations were identified, we took the majority SNP calls at each position to indicate the dominant genotype.

Statistical analyses

All statistical analyses of genotype data were conducted in R statistical software (version 3.0.2)⁵⁰ except for the spatial scan statistics which were computed using SaTScan software (version 9.3)⁵¹. Analyses were carried out separately for each parasite population, except for the Fixation index (FST) analyses which by definition involve the comparison of populations and so were carried out between samples in the different sites.

In each population, genotype data for all samples was aggregated and analysed collectively. Separate analyses were also carried out for subsets of SNPs typed in EBA 175 and AMA1. In the Kilifi population, we ran additional analyses for samples collected from community surveys (asymptomatic infections) and hospital admissions (symptomatic infections).

Calculating pairwise time, distance and SNP differences. Analysis was carried out separately for each of the three sites. Each parasite was compared to every other parasite in that site (i.e. a pairwise analysis), noting the time, distance and SNP differences between the parasite pair (Dataset 3–Dataset 5^{68–70}). We took half the lower limit of detection of temporal and spatial differences for parasite pairs collected on the same day and/or at the same location. Parasite pairs collected on the same day were assigned a difference of 0.5 days. For older samples in Kilifi (i.e. collected prior to 2004) where location was known to a 5 km accuracy, pairs collected at the same location were assigned a difference of 2.5km. We had precise geospatial co-ordinates for recent samples in Kilifi (i.e. collected after 2004) as well as all samples from The Gambia and Rachuonyo South, so parasite pairs in these three groups collected from the same location were assigned a difference of 0.02km.

SNP differences were computed by comparing genotype data for parasite pairs within each population and counting the number of SNPs between them. Missing SNP data for each parasite was replaced with the major allele in the respective population, after excluding SNP typing where >30% of assays failed as described above.

Population genetics analyses. Minor allele frequencies were computed for SNPs in each population. Principal components analysis (PCA) was performed using singular value decomposition on a covariance matrix of pairwise SNP differences between

parasites in individual populations. To detect inter-population genetic differentiation and within-population genetic diversity, we restricted analysis to 33 SNPs that had been successfully typed in all three populations.

Spatial autocorrelation. Moran's I was calculated using geographical coordinates to specify location and scores for the first 3 principal components to specify associated attribute values. Moran's I was computed at distance classes of 1 km, 2 km and 5 km, using 100 bootstrap resampling steps to determine statistical significance.

Spatial scan statistics were calculated using SaTScan software. Analysis was purely spatial using a normal probability distribution model on continuous variables. During the analysis, a scanning window moves over the geographical space and computes observed and expected principal component values for different locations and window sizes. The locations with the greatest ratios of observed to expected values were noted as clusters and their statistical significance was determined using random permutations to account for multiple comparisons.

Raster analysis. To identify possible spatial barriers to parasite movement and mixing over short distances, each study area was divided into pixels of varying sizes which were then scored with 1 or 0, based on whether or not a straight line linking any two parasites crossed their boundaries. These pixels were then used as independent variables in a multivariable linear regression analysis that had the number of SNP differences as the dependent variable. Significance of the coefficient estimates were determined using non-parametric bootstrapping with 100 resampling steps.

To test for correlations between transmission intensity and population genetics at fine scale, each pixel was assigned the mean of the PC scores and either Malaria Positive Fraction (for Kilifi data) or asymptomatic parasite prevalence by PCR (for Rachuonyo) for all samples found within that pixel. The correlation between PC score and MPF or between PC score and parasite prevalence was tested by Spearman's rank ordered correlation coefficient.

Dataset 1. Information on the 276 SNPs genotyped in 177 genes in *P. falciparum* parasite populations from The Gambia, Kilifi and Rachuonyo South

<http://dx.doi.org/10.6084/m9.figshare.4640707>

The columns contain the following information: study_location, site of sample collection; sample_id, unique sample identifier; gene_symbol, gene name (if available); chr_valid, chromosome; coord_valid= base position of SNP on chromosome; sequence_code, SNP name; assay_code, name of assay; rsnumber, unique SNP identifier in dbSNP; reference_allele, 3D7 reference allele, alternative_allele, alternative allele; single_letter_code, IUPAC code for SNPs; result, genotype call after processing; allele1, IUPAC code for allele 1; allele2, IUPAC code for allele 2; allele_ratio1, proportion of allele 1; allele_ratio2, proportion of allele 2; pass_fail, coding of SNP based on availability of valid genotype (pass) or lack of a valid genotype (fail). Geospatial data for homestead location is considered sensitive data and therefore cannot be made open access. However, it can be accessed through a request to our data governance committee, using the email address mmunene@uat/newsite.

Dataset 2. Sequenom assay design information

<http://dx.doi.org/10.6084/m9.figshare.4640719>

Data includes the locus and IPLEX specific primers used in the sequenom reaction to amplify and type the SNPs of interest. Gene product, gene product name; Gene_symbol, gene name; Chromosome, chromosome location of gene; SNP position on chromosome, SNP site; reference_allele, 3D7 reference allele; alternative_allele, alternative allele; sequence, 3D7 reference sequence spanning the SNP site; first_pcrp, first PCR primer sequence; second_pcrp, second PCR primer sequence; extension_primer, IPLEX extension primer sequence; extension1_call, IPLEX primer with extended SNP; extension1_mass, Mass of the extended IPLEX primer; extension1_sequence, sequence of extended IPLEX primer; extension2_call= IPLEX primer with alternative extended allele; extension2_mass, Mass of the extended IPLEX primer with alternative allele; extension2_sequence, sequence of extended IPLEX primer with alternative allele.

Dataset 3. SNP, distance and time differences between *P. falciparum* parasite pairs in The Gambia population

<http://dx.doi.org/10.6084/m9.figshare.4640722>

Differences were computed for all parasite pairwise comparisons. Sample_id and sample_id_x are unique sample identifiers; snps represent the number of snp differences between parasite pairs; km_distance represents geographical distance, in kilometres, between parasite pairs; time_diff represents the temporal distance, in days, between parasite pairs.

Dataset 4. SNP, distance and time differences between *P. falciparum* parasite pairs in the Kilifi population

<http://dx.doi.org/10.6084/m9.figshare.4640725>

Differences were computed for all parasite pairwise comparisons. Sample_id and sample_id_x are unique sample identifiers; snps represent the number of snp differences between parasite pairs; km_distance represents geographical distance, in kilometres, between parasite pairs; time_diff represents the temporal distance, in days, between parasite pairs.

Dataset 5. SNP and distance differences between *P. falciparum* parasite pairs in the Rachuonyo South population

<http://dx.doi.org/10.6084/m9.figshare.4640728>

Differences were computed for all parasite pairwise comparisons. Sample_id and sample_id_x are unique sample identifiers; snps represent the number of snp differences between parasite pairs; km_distance represents geographical distance, in kilometres, between parasite pairs.

Results

Study populations

5199 *P. falciparum* parasite isolates were collected from the Kombo coastal districts in The Gambia, and Kilifi County and Rachuonyo South district in Kenya (Figure 1) between 1998 and 2011. 107, 177 and 82 SNPs were successfully genotyped in 133, 1602, and 1034 parasite isolates from The Gambia, Kilifi and Rachuonyo South district, respectively (Table 1). 26, 57 and 49 SNPs were present at frequencies of 5% and above in The Gambia, Kilifi and Rachuonyo, respectively. In each of the populations, there was a positive correlation between SNP assay performance and parasite density.

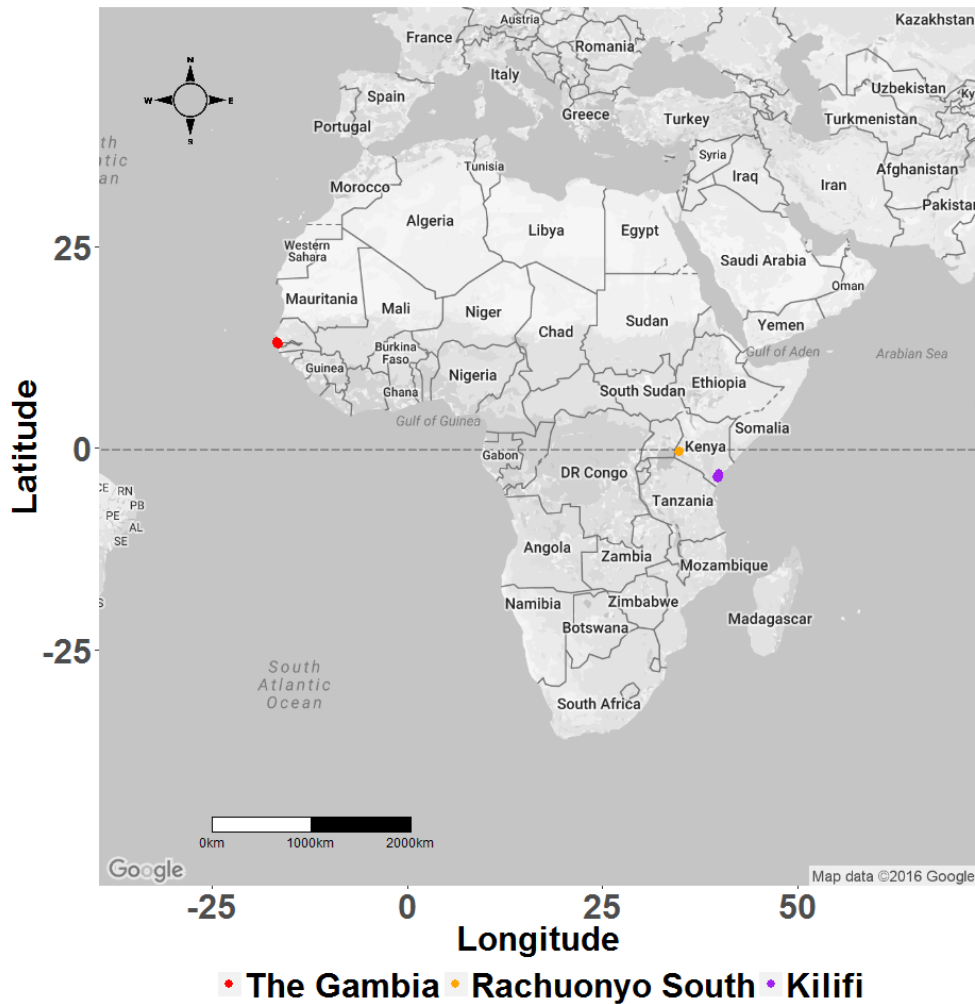


Figure 1. Map of Africa showing the three study sites. The study was conducted on *P. falciparum* samples collected in The Gambia, West Africa and Rachuonyo South District and Kilifi County in Kenya, East Africa.

Table 1. Summary of information on *P. falciparum* infected blood samples collected from The Gambia, Kilifi and Rachuonyo South study sites.

Study site	Contributing study	Study period	Average parasite density	Samples genotyped	Samples analysed	SNPs genotyped	SNPs analysed
The Gambia (Kombo Coastal Districts)	Clinical malaria study	Sep '07 – Dec '08	406,093	143	133	131	107
Kilifi	Community surveys	Feb – Oct '05	4562	748	195	240	177
Kilifi	Clinical malaria surveys	Jul '98 – Apr '10	352,428	1564	1407	240	177
Rachuonyo South	Community surveys	2011	NA	2744	1034	111	82

In each study site, we found similar results when we analysed the EBA 175 and AMA1 SNP subsets separately, and when we analysed the aggregated SNP data. Only the results of the aggregated analyses are presented here. In the Kilifi population, results were similar between the community surveys and hospital admissions. Here we present the results of the combined analyses of these data subsets.

Parasite genetic diversity and population differentiation

Weir and Cockerham's fixation index (F_{ST}) estimates showed that the level of differentiation amongst the three populations was 0.046, comparable with results of other studies of African populations^{52,53}. Pairwise population analysis gave F_{ST} values of 0.041 between Kilifi and Rachuonyo South, 0.078 between The Gambia and Kilifi and 0.108 between The Gambia and Rachuonyo South, showing the greatest genetic differentiation between The Gambia and Rachuonyo South parasite populations.

Analysis of within-population genetic diversity (π), based on a set of 33 SNPs that had been typed in samples from all three populations, showed that parasites in Rachuonyo South had the highest genetic diversity with an average of 3.384 (95% CI: 3.380 – 3.388) SNP differences per parasite pair. Those in The Gambia had the lowest SNP differences per parasite pair at an average of 2.867 (95% CI: 2.836 – 2.898) SNPs, while Kilifi had intermediate genetic diversity at 3.229 (95% CI: 3.226 – 3.231) SNP differences per parasite pair.

Principal Component Analysis (PCA) was carried out separately for each population using the 107, 177 and 82 SNPs that were successfully typed in The Gambian, Kilifi and Rachuonyo South parasite populations. Cumulatively, the first three principal components accounted for 36.1% (PC1=18.4%, PC2=10.4%, PC3=7.3%) of

the variability seen in The Gambia, 13.2% (PC1=5.1%, PC2=4.4%, PC3=3.7%) of the variability seen in Kilifi and 12.7% (PC1=4.4%, PC2=4.3%, PC3=4%) of the variability seen in Rachuonyo South. We were unable to resolve parasite populations into distinct sub-populations using principal component analysis (Figure 2 and Figure 3, Supplementary Figure 1).

Global and local spatial autocorrelation analysis

Having not seen sub-populations by PCA alone, we then included spatial analyses to test for spatial structure to the principal component values. Moran's I analysis for spatial autocorrelation showed slight positive correlations for parasites that were statistically significant for at least one principal component at 2 km and below in The Gambia, 5 km and below in Kilifi, and 1 km and below in Rachuonyo South (Figure 4).

Spatial scan statistics using SaTScan identified statistically significant ($p \leq 0.01$) clusters of different sizes in Kilifi and Rachuonyo South parasite populations. In Kilifi, one cluster with a radius of 1.54 km ($p=0.01$) was detected, while in Rachuonyo South, a smaller cluster of genetically distinct parasites was detected with a radius of 0.5 km ($p=0.001$). No clusters were detected in The Gambian population.

Spatio-temporal variations in genetic differences between parasite isolates

We examined the effect of distance and time separating parasite pairs on genetic relatedness to determine the spatial extent and rate of parasite mixing. We used linear regression models where the number of SNP differences between parasite pairs was an outcome predicted by the distance between parasite pairs and the time between parasite pairs. Time was not included for the

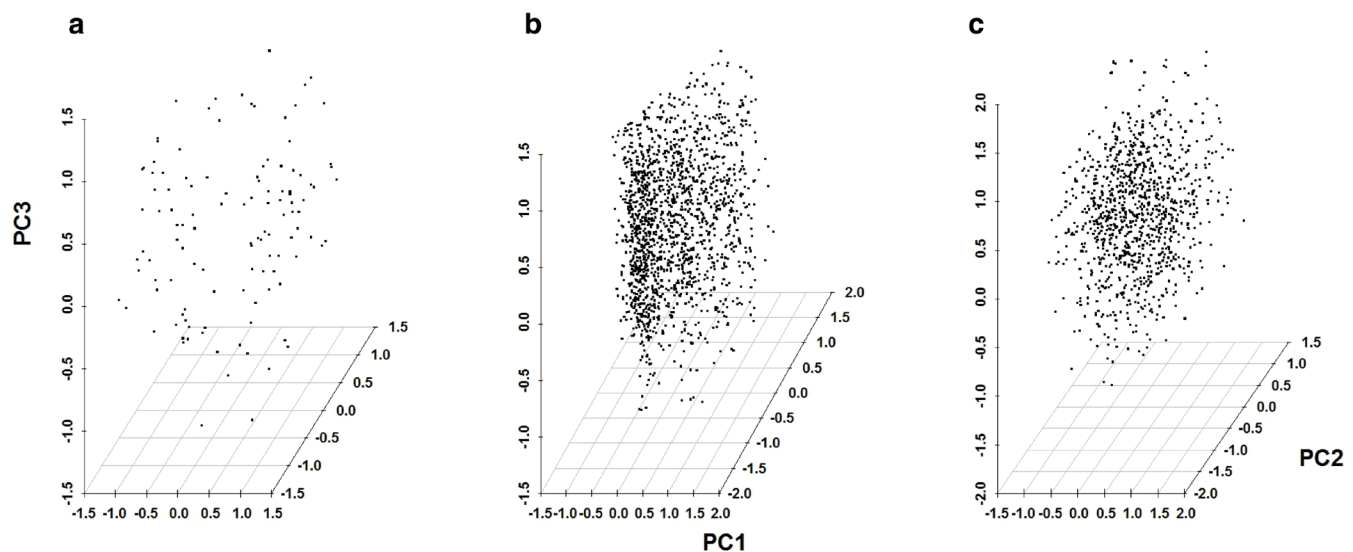


Figure 2. Plots of Principal Component Analysis scores for *P. falciparum* parasite populations in the study sites. Each point represents one of 133 parasites in The Gambia (a), 1602 parasites in Kilifi (b) and 1034 parasites in Rachuonyo South (c). Genetic structuring was not observed for any of the parasite populations based on these three principal components. Cumulatively, the first three principle components accounted for 36.1% (PC1=18.4%, PC2=10.4%, PC3=7.3%), 13.2% (PC1=5.1%, PC2=4.4%, PC3=3.7%) and 12.7% (PC1=4.4%, PC2=4.3%, PC3=4%) of the variability seen in The Gambia, Kilifi and Rachuonyo South populations, respectively.

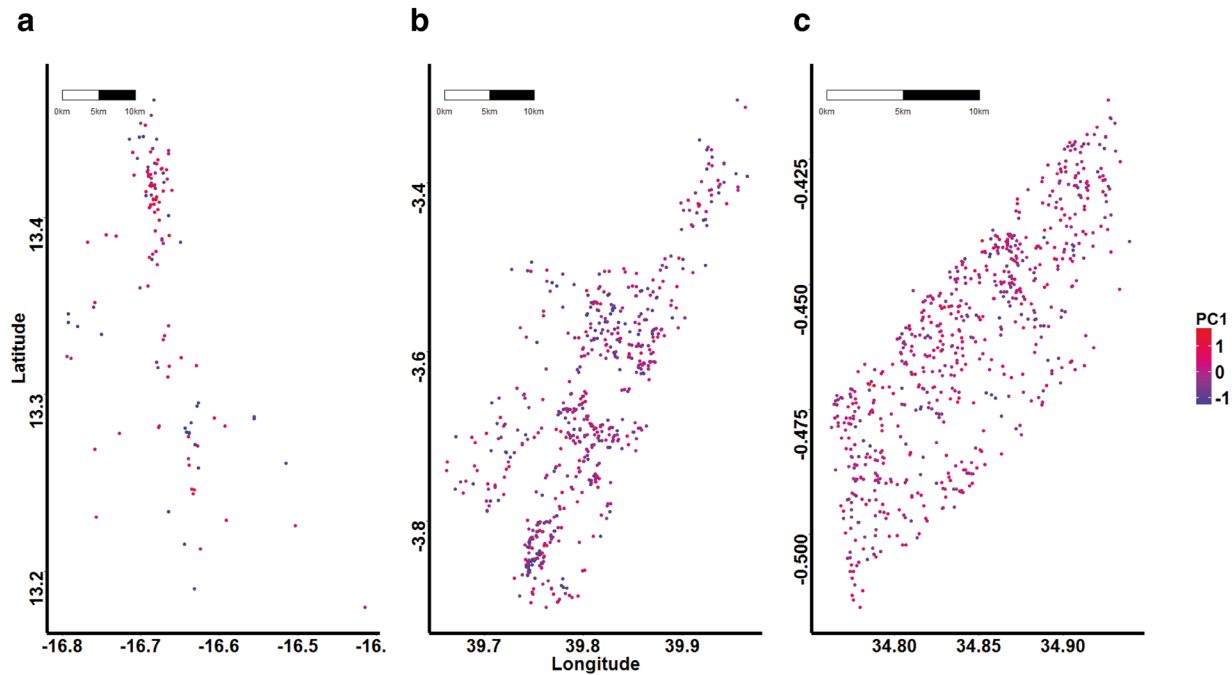


Figure 3. Geographic distribution of *P. falciparum* parasite genotypes based on scores for the first principal component. Each point represents the location of an individual parasite isolate and the colour shading represents distinct genotypes for parasites in (a) The Gambia, (b) Kilifi and (c) Rachuonyo South study sites.

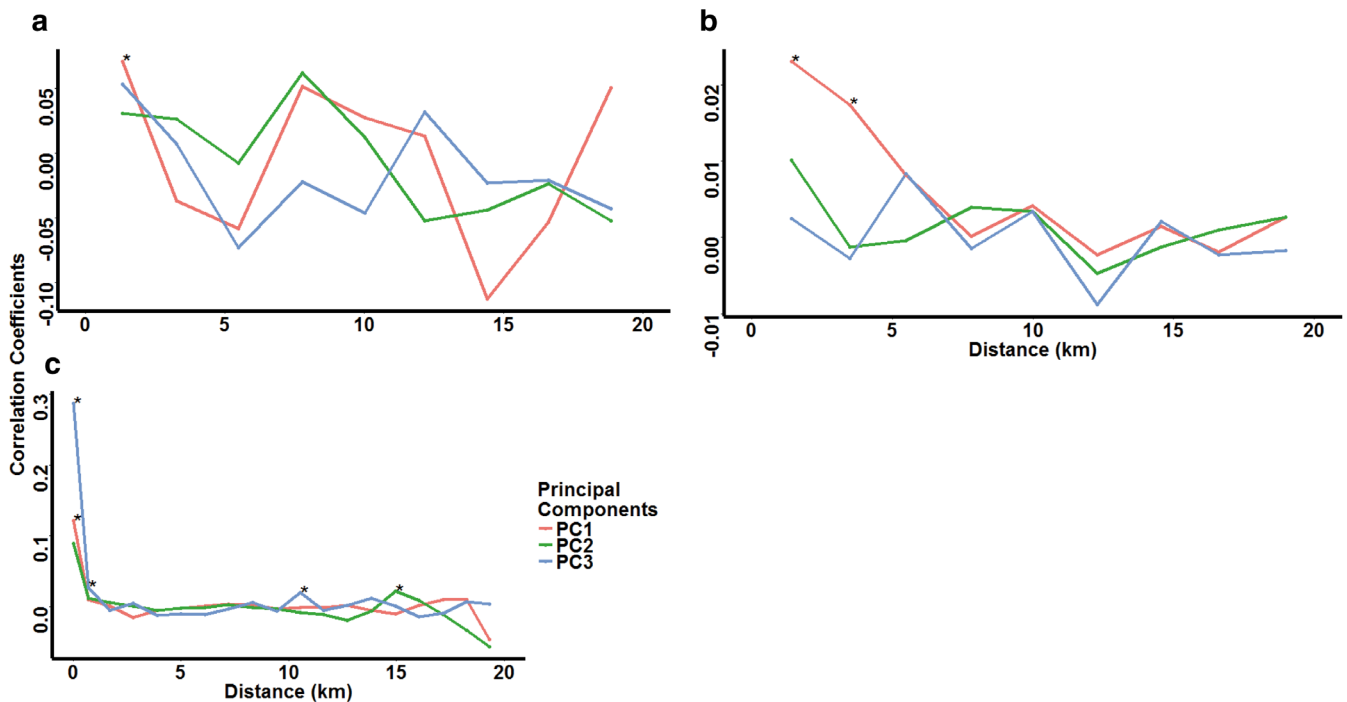


Figure 4. Moran's *I* spatial autocorrelation analysis for the first three principal components. Coefficients were computed at distance classes of 2 km for (a) The Gambia and (b) Kilifi, and 1 km for (c) Rachuonyo South parasite populations. Asterisks indicate distances at which parasites have significant ($p < 0.01$) autocorrelations. In The Gambia and Kilifi populations, only a few samples were collected from the same location, so Moran's *I* was not computed at this distance (0 km).

Rachuonyo South population as the samples were collected in a single cross-sectional survey taken over a few days. Across all three datasets, distance was independently associated with increasing variation in genotype, i.e. the further apart in space any two parasites were, the greater the number of SNP differences between them. In The Gambia and Kilifi populations, time was also shown to be associated with increasing variation in genotype, with parasite pairs collected further apart in time having greater number of genetic differences. Additionally, in The Gambia and Kilifi populations, time interacted antagonistically with distance to attenuate the effect of distance on genotype relatedness (Figure 5). This means that the genetic differences between any two parasites increased with distance, but at a decreasing rate when time between these samples increased. We observed that in The Gambian population, parasites acquired SNP differences over distance at a slower rate than in the Kilifi and Rachuonyo populations.

Bootstrapping the analyses (to take into account the linked nature of pairwise observations) gave statistically significant effects

of distance, time and the interaction between distance and time (Table 2).

Identification of geographical barriers to parasite movement

We conducted raster analysis by pixels to examine a) the spatial relationship between distinct parasite genotypes as represented by the principal component analysis and either malaria positive fraction (MPF) data (in Kilifi) or PCR positive data (in Rachuonyo South) and b) the presence of possible spatial barriers to parasite movement that would act as factors. The analysis of principal components did not show any consistent or statistically strong associations with markers of transmission intensity (i.e. malaria positive fraction and prevalence of asymptomatic parasitaemia by PCR) (Supplementary Figure 2).

Bootstrapping the multivariable linear regression analysis of pairwise comparisons of samples for SNP differences using 189, 703 and 340 pixels for The Gambia, Kilifi and Rachuonyo South,

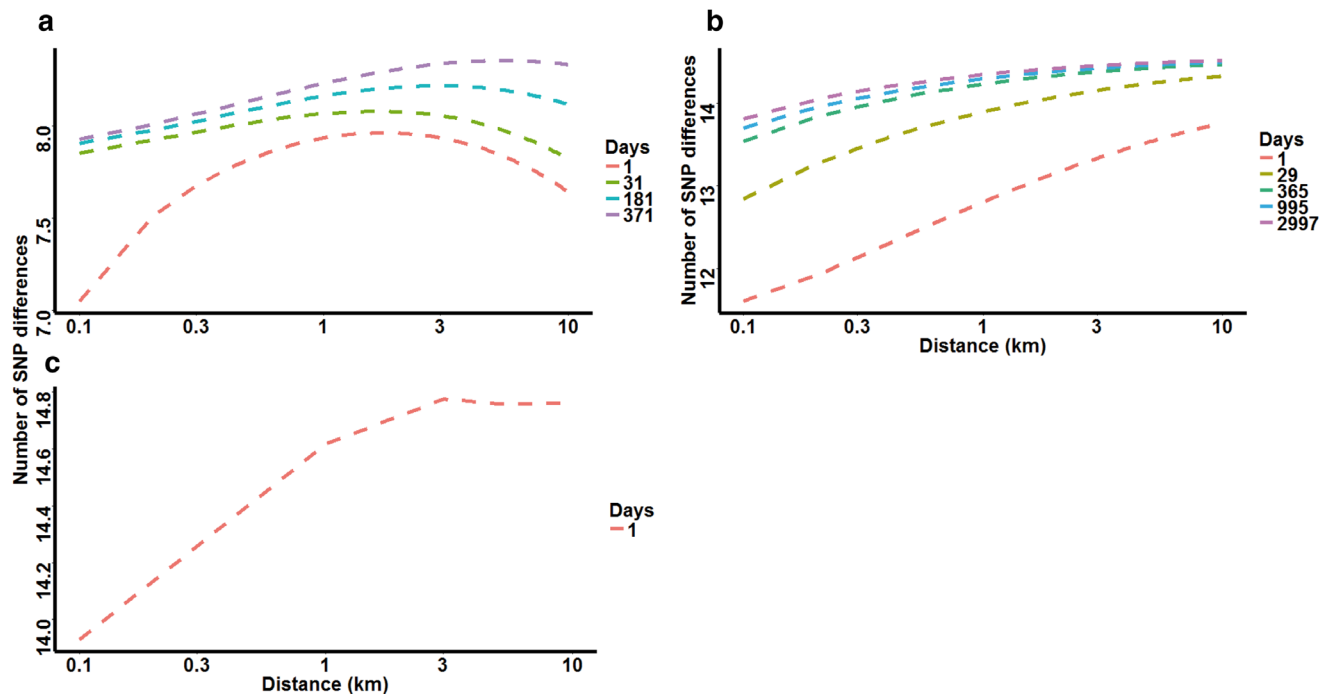


Figure 5. Effects of time-distance interaction on the number of SNP differences between parasite pairs. Dashed lines represent time intervals separating parasite pairs in (a) The Gambia, (b) Kilifi and (c) Rachuonyo South study sites.

Table 2. 95% bootstrap confidence intervals for the linear effects of time, distance and the interaction of time and distance on changes in SNP differences between parasite pairs.

	Time (days)	Distance (km)	Time-Distance interaction
The Gambia	-0.005 - -0.001 (p=0.004)	0.086 - 0.723 (p<0.001)	-0.0003 - -0.002 (p=0.003)
Kilifi	0.190 - 0.647 (p<0.001)	0.297 - 1.363 (p=0.001)	-0.453 - -0.072 (p=0.003)
Rachuonyo South	-	0.0104 - 0.275 (p=0.018)	-

respectively, showed that the majority of pixels were not significant influences on SNP differences (Supplementary Figure 3). The few pixels that were significant ($p < 0.05$) were non-significant after applying Bonferroni correction to account for multiple testing. Furthermore the distribution of p values was uniform for each dataset (mean p value ~ 0.5 in each population).

Discussion

As malaria transmission declines, targeted control at the micro-epidemiological scale is likely to be important in eliminating malaria in any remaining transmission foci. The effectiveness of such targeted measures will depend on the extent of parasite mixing in and around these foci²³. In the current analysis, we did not identify any population structure by simple inspection of the Principal components derived from SNP genotyping in The Gambia, Kilifi and Rachuonyo South (Figure 2 and Figure 3), indicative of a parasite population that is well mixed. However we did not conclude that there was no structure to the population, only that we could not identify it in the absence of spatial data. We therefore went on to analyse the genotype data using spatio-temporal data, and identified spatial autocorrelation using Moran's I in all three populations, with statistical significance ($p < 0.01$) for the first principal component in The Gambia and Kilifi and the third principal component in Rachuonyo South (Figure 4). Overall, the consistent pattern observed in the Moran's I analyses was that of spatial autocorrelation at close proximity (i.e. at a range of a few km), and little or no auto-correlation at larger distances. The auto-correlation was modest in effect size but statistically significant with p values ranging from 0.01 to 0.001 at < 1 km. However, using scan statistics we identified only two specific clusters of parasites, one in Kilifi and another in Rachuonyo South. The limited evidence of specific local clusters of parasite populations in the face of evidence of spatial auto-correlation over the whole study site implies that there is a high degree of mixing among parasites within the study sites, leading to limited clustering of parasites into genetically distinct sub-populations.

We further looked at the effect of time, distance and time-distance interaction on the variation in SNP differences between parasite pairs within individual study sites. We found that time and distance were independently associated with increasing variation between parasite genotypes (i.e. the further apart in time or space two parasites were, the greater the genetic differences observed between them). However, in the case of The Gambia and Kilifi populations where we had longitudinal data, time was shown to interact antagonistically with distance, with an increase in time reducing the variations in genetic differences between parasites as distance between the parasites increased (Figure 5). This implies that distance between samples was no longer predictive of genetic variation when there were longer time periods between samples, indicating that, given enough time, even parasites that are separated by large distances would get a chance to interact and recombine, especially if they are not geographically isolated.

Lack of genetic structuring of parasite populations observed in this study is indicative of a population that is well mixed. This observation of a highly mixing parasite population is in agreement

with results of similar studies using microsatellites^{52,54,55}, immune selected genes^{56,57} and SNPs⁵⁸. However, other studies have shown population structure when looking at the same population^{48,59–61}, although these analyses were carried out on larger geographical scales than those analysed here.

On an international level, for example, some studies have been able to distinguish between Senegalese and Thai parasite isolates using a 24-SNP barcode⁶², and another study using 4 SNPs out of a set of 384 SNPs was able to resolve East and West African parasites⁴⁸, showing that parasite populations can be resolved on a large geographical scale. A study in Senegal was also able to identify population structure among parasites using a 24 SNP barcode, despite a high level of similarity among the parasites analysed⁶³. It is possible that more detailed genotyping using a larger number of markers, for instance by whole genome sequencing, would start to identify mutations that are private to particular sub-populations at a finer geographical scale, although the degree of mixing observed here suggests that discrete populations are unlikely.

We identified spatial autocorrelation among parasites in the different study areas. However, most of these correlations were found over short distances, pointing to the existence of parasite sub-populations over small spatial scales. This indicates the presence of clusters of genetically distinct parasites at micro-epidemiological scales within the study sites. Previous studies have identified parasite sub-populations based on clustering of serological responses to the important antigen *Plasmodium falciparum* Erythrocyte Membrane Protein 1 (*PfEMP1*) in children in Kilifi⁶⁴, supporting our observations of parasite sub-populations at this site. In Papua New Guinea, sub-populations of parasites have also been identified at a micro-epidemiological scale using *PfEMP1*⁶⁵, indicating that this may be a good marker for population differentiation at the micro-epidemiological level.

Studies on hotspots of symptomatic malaria infection have identified hotspots or clusters of infections down to the level of individual homesteads in Kilifi⁹. The lack of consistent correlations between parasite genotypes and infection prevalence shown through raster analysis of pixels in this study (Supplementary Figure 2) indicate that infections within higher incidence areas are likely not caused by distinct parasite sub-populations. Instead, such infections are likely caused by parasites that are well mixed within the general population. Our inability to detect barriers to parasite movement over short distances indicates that parasites move freely within the study areas, and the spatial extent of such parasites may be limited only by the ecology and dispersal range of mosquito vectors. Furthermore, recent examination of the epidemiology of hotspots shows that they occur at the full range of spatial scales, with a pattern of spatial auto-correlation that does not show a discontinuity at any scale (i.e. a smooth semi-variogram)⁹. This further argues against the existence of discrete “units” of transmission with sub-populations of parasites.

This has implications for public health interventions that may target transmission hotspots. If hotspots consist of distinct parasite populations that do not mix with parasite populations in the wider parasite

community, the impact of hotspot-targeted interventions beyond the hotspot boundaries can be expected to be limited. If parasites mix freely, as suggested by our data, the impact of hotspot-targeted interventions may affect community-wide malaria transmission. This assumes that hotspots can be detected, are stable in time²⁰ and the spread of parasite populations indeed primarily occurs from hotspots to the surrounding community²³.

This study had some limitations. First, the number of SNPs typed was relatively small, and this would have limited our power to detect genetic structuring among the highly similar parasite populations, especially in The Gambia. Detecting structuring in highly similar parasite populations may require either a much larger panel of SNPs or the use of more informative SNPs, as shown in the study by Campino *et al*, 2011⁴⁸. However, despite the small SNP panel used in this study, we were still able to detect population structuring on a micro-epidemiological scale. Our analysis suggests that this structure was a uniform spatial and temporal auto-correlation rather than driven by discrete clusters of parasites at specific locations. Despite the limitations of our SNP typing and sample size we can therefore conclude that any specific clustering is less prominent as a feature than the auto-correlations in space and time that we can detect.

A second limitation is that we conducted our study in only two sites in Kenya, and one site in the Gambia. It may be premature to generalize our results more widely and an analysis of more sites will be required to make confident generalizations. On the other hand the three sites selected do demonstrate differing transmission intensities typical of many endemic Sub Saharan African countries, and this was reflected in the level of genetic diversity observed in the populations. Furthermore, our findings are consistent across all three sites. Nevertheless, patterns of parasite mixing may differ between populations based on distinctive features such as geographic isolation and patterns of human movement. Further data are required to make more general conclusions.

In conclusion, we have shown that *Plasmodium falciparum* parasite populations mix evenly within The Gambia, Kilifi and Rachuonyo South and there appear to be no detectable geographical barriers to parasite movement over short distances within these sites. That said, autocorrelations of genotype were detected at the micro-epidemiological level. We would conclude that control strategies that efficiently target hotspots will likely benefit the wider community outside the hotspots at the District/County level (we are however unable to comment on larger geographical scales), although this is likely to be affected by factors such as the underlying transmission level, heterogeneity of transmission, and patterns of human movement²³. On the other hand, following mass-treatment campaigns we would predict that if residual foci of transmission are retained this will rapidly lead to reinfection of the wider community, and that parasites acquiring mutations conferring drug resistance or immunological escape will be rapidly spread at a micro-epidemiological level.

Data availability

Figshare: Dataset 1: Information on the 276 SNPs genotyped in 177 genes in *P. falciparum* parasite populations from The Gambia, Kilifi and Rachuonyo South, doi: <http://dx.doi.org/10.6084/m9.figshare.46407076>⁶⁶

Figshare: Dataset 2: Sequenom assay design information, doi: <http://dx.doi.org/10.6084/m9.figshare.46407197>⁶⁷

Figshare: Dataset 3: SNP, distance and time differences between *P. falciparum* parasite pairs in The Gambia population, doi: <http://dx.doi.org/10.6084/m9.figshare.46407228>⁶⁸

Figshare: Dataset 4: SNP, distance and time differences between *P. falciparum* parasite pairs in the Kilifi population, doi: <http://dx.doi.org/10.6084/m9.figshare.46407259>⁶⁹

Figshare: Dataset 5: SNP and distance differences between *P. falciparum* parasite pairs in the Rachuonyo South population, doi: <http://dx.doi.org/10.6084/m9.figshare.46407287>⁷⁰

Author contributions

P.B, D.K and T.B conceived and designed the study. T.B, A.A, J.C.S, A.Y.B, T.N.W, K.R, D.K. were involved in sample and/or data collection. C.H, A.J, K.R genotyped the SNPs. I.A.O, P.B, P.M., A.R, E.V, I.O and G.F analysed the data. I.A.O wrote the first draft manuscript. All authors reviewed, edited and approved the final manuscript.

Competing interests

No competing interests were disclosed.

Grant information

Sample collection at the Rachuonyo South site was supported by the Bill and Melinda Gates Foundation, under the Malaria Transmission Consortium, Grant No.45114 and the Grand Challenge Grant No. OPP1024438. Thomas N. Williams is funded by the Wellcome Trust, grant number 091758. Philip Bejon, Polycarp Mogeni and Irene Omedo are funded by the UK Medical Research Council (MRC) and the UK Department for International Development (DFID) under the MRC/DFID Concordat agreement. Polycarp Mogeni is funded by the Gottfried und Julia Bangerter-Rhyner Stiftung and the Novartis Foundation for Medical Biological Research project 13A13. Sample collection in Kilifi was supported by core funding from the Wellcome Trust to the Kenya Programme.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgements

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Supplementary material

Supplementary Figure 1. Geographical distribution of *P. falciparum* parasite genotypes based on scores for the second (PC2) and third (PC3) principal components. Each point represents an individual parasite isolate and the colour shading represents distinct genotypes for parasites in The Gambia (**d** and **g**), Kilifi (**e** and **h**), and Rachuonyo South (**f** and **i**) study sites.

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Supplementary Figure 2. Raster analysis by pixels. This was carried out to determine the spatial relationship between distinct parasite genotypes as represented by principal component analysis and either malaria positive fraction (MPF) or PCR positive fraction (PPF) data. (**a**) and (**b**) show the distribution of scores for the first principal component (PC1) and MPF over a 1 km × 1 km grid area of Kilifi. (**d**) and (**e**) show the distribution of scores for the first principal component and PPF over a 1 km × 1 km grid area of Rachuonyo South. Spearman's correlation coefficients computed to show the relationship between parasite genotypes and either MPF (**c**) or PPF (**f**) showed no strong associations between genotypes and the two markers of transmission.

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Supplementary Figure 3. Raster analysis by pixels to examine the presence of spatial barriers to parasite movement. The pixel plots represent p values of bootstrapped linear regression correlation coefficients and show the significance of different geographical locations in acting as barriers to parasite mixing. Individual grid sizes were of approximately 1 km × 1 km in (**a**) Kilifi and (**c**) The Gambia and 0.5 km × 0.5 km in (**b**) Rachuonyo South. The colour key in each case indicates the range of p values from 0.0001 to 1. Significant p values shown on the plot were non-significant after applying Bonferroni correction to account for multiple testing.

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69. Omedo I, Mogeni P, Bousema T, *et al.*: **Dataset 4: SNP, distance and time differences between *P. falciparum* parasite pairs in the Kilifi population.** *Figshare.* 2017.
[Data Source](#)
70. Omedo I, Mogeni P, Bousema T, *et al.*: **Dataset 5: SNP and distance differences between *P. falciparum* parasite pairs in the Rachuonyo South population.** *Figshare.* 2017.
[Data Source](#)

Open Peer Review

Current Referee Status:**Version 1**

Referee Report 19 June 2017

doi:[10.21956/wellcomeopenres.11628.r23565](https://doi.org/10.21956/wellcomeopenres.11628.r23565)**Liwang Cui**

Department of Entomology, Pennsylvania State University, State College, PA, USA

This study analyzed large sample sets of malaria parasites taken from the western and eastern coasts of Africa (The Gambia and Kenya) and genotyped at 276 SNPs. For two of the sample sets, parasites were collected at different time points, allowing identification of population changes over time and space. Overall, the analysis was sound and results were well explained. The authors also notified the limitations of the study. For example, inclusion of additional parasite samples between these western and eastern sites, and use of more SNP markers would validate whether the conclusions drawn here represent the whole African continent.

Comments:

1. The assumption for comparing the temporally collected samples is that malaria case numbers have been reduced, which might lead to genetic isolation and structuring of parasite populations. It would be great if malaria epidemiology at the beginning and end of sample collection in the sites where samples were collected is clearly stated. It is possible that despite the overall reduction in malaria cases, some of the sites may represent hotspots where malaria epidemiology remained more or less unchanged over the time. As a result, this would make the parasite populations and genetics relatively stable over the time.
2. The inclusion of numerous SNPs for this type of analysis is a nice practice. However, the authors may want to separate those that are clearly under selection (such as EBA175 and AMA1), since these mutations are subject to strong immune selection and will have different evolutionary trajectories as compared to more neutral SNPs.
3. More detailed comparison of the two Kenyan sites might be interesting to see whether gene flow between these sites exists, given that these sites are relatively closely located, yet separated by potential gene flow barriers (such as the rift valley).

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Referee Expertise: Molecular epidemiology

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Referee Report 18 April 2017

doi:[10.21956/wellcomeopenres.11628.r21343](https://doi.org/10.21956/wellcomeopenres.11628.r21343)



Christopher Delgado-Ratto 

Global Health Institute, University of Antwerp, Antwerp, Belgium

This is a study that used SNP genotyping data finely analysed to describe the geographic structuring of *Plasmodium falciparum* parasites at micro-epidemiological level in three regions from Gambia and Kenya.

The authors were not able to compare the parasite populations among the study sites due to the samples were originally obtained for studies with different study designs (differences in sampling time, study population and design). The genetic diversity and clustering may not only be affected by geographic location and time but also by different ways of sampling the data. Say so, I appreciated that the authors focused in the population dynamics within the study sites.

Regarding the hypothesis that exists gene flow within the study sites, gene flow models could be also useful to prove such genetic exchange of parasites. There are various software that may help on this matter, i.e. Migrate-n.

Specific remarks:

Conclusions section:

- This paragraph is not fully justified on basis of the results: “following mass-treatment campaigns we would predict that if residual foci of transmission are retained this will rapidly lead to reinfection of the wider community, and that parasites acquiring mutations conferring drug resistance or immunological escape will be rapidly spread at a micro-epidemiological level.”

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Referee Expertise: Molecular epidemiology

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Referee Report 11 April 2017

doi:[10.21956/wellcomeopenres.11628.r21345](https://doi.org/10.21956/wellcomeopenres.11628.r21345)



Cristian Koepfli 

University of California, Irvine, Irvine, CA, USA

This is a relevant study, assessing the ability to identify small-scale foci of transmission and parasite gene flow to surrounding areas based in SNP-typing. While it is overall clearly presented and well written, more detail, in particular in the results section, would help to better understand the data, and to assess its power.

Specific comments:

Abstract:

1. Please state how many samples and SNP markers were included in the final analysis.
2. I wonder whether “relatedness in space and time” is the correct term, or “distance in space and time” would be more appropriate.

Results:

1. In the part on “Identification of geographical barriers to parasite movement” it would be useful to include the range of prevalence or MPF per pixel analyzed.
The second paragraph of this part is difficult to follow, as the term ‘cluster’ is used consistently, without further indication on what the clusters represent. It would help to include a sentence describing that spatial clusters were analyzed based on the PCA values of all isolates found within the cluster. Thus, clusters of isolates differing from all other isolates were identified. The same is

the case in the discussion. What sizes were the clusters identified, and how many haplotypes were included per cluster?

2. Figure 5: Given that for almost every pair of samples the number of days differs, how were the days for the different curves calculated? I assume each color represents a range, yet only a point estimate is given.

Also, please indicate in brackets for each curve the number of samples included. For example, how many samples were available for the 1-day and 31-days analysis in The Gambia? Could the apparent reduction in SNP difference at 10 km be a chance finding due to limited sample size?

Including the number of SNPs analyzed in each population would further help to interpret the data. E.g. it is interesting that in Rachuonyo South the proportion of different SNPs is approx. twice as high as in the other sites, yet this is only evident when Figure 5 is compared to Table 1.

Would it be possible to include confidence intervals for the 1-day curves in the figure? This would help to understand the power of the data. For example, the statement in the abstract "Genetic relatedness of sample pairs is predicted by relatedness in space and time" suggests that genetic relatedness can be inferred, once the distance by space and time is known. This is however difficult to assess without more detail on the variance of the data.

3. In Table 2, what is the unit of the results showed? I assume it is SNP-difference/day (or SNP-difference/km), with days and distance log-transformed. Please state if/how data was transformed.

Discussion:

1. Paragraph 3 of the discussion could be expanded. At what spatial scales was population structure found in previous studies (as compared to the approx. 50 km range of the present study)? Have any of these studies included relatedness? This information would help to assess the feasibility to identify foci of higher transmission, and to estimate the level of gene flow to surrounding areas in different transmission settings.
2. The number of SNP differences plateaus at approx. 1 km in The Gambia, 3 km in Kilifi, and increases up to 10 km in Rachuonyo South. Are there possible explanations for these differences due to the characteristics of the local parasite populations?

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Referee Report 29 March 2017

doi:[10.21956/wellcomeopenres.11628.r21178](https://doi.org/10.21956/wellcomeopenres.11628.r21178)



Michel Tibayrenc

Maladies Infectieuses et Vecteurs Ecologie, Génétique, Evolution et Contrôle (MIVEGEC), Institut de Recherche pour le Développement (IRD), Montpellier, France

This is a fine population genetic analysis of 3 samples taken in Gambia and Kenya, relying on the typing of 5199 samples by 276 SNPs. I have little to say about this work, which uses sound approaches and yields clear conclusions. A few remarks:

1. How can heterozygous genotypes be detected in haploid populations of the parasite?
2. As noted by the authors themselves, using 276 SNPs is rather limited. Genetic studies dealing with human populations at nowadays routinely rely on 500000 SNPs or more. One main feature of such studies is that microgeographical structures are detected mostly from low frequency variants and rare variants, which of course are undetectable when using a limited set of markers. Moreover, these low frequency and rare variants are supposed to be highly relevant for phenotypic expression, in particular disease susceptibility and are largely responsible for recent and localized evolution in human populations. (see for example Leslie et al. (2015)¹). It is most probable that these patterns exist in parasite populations too. The authors should discuss this point more, since it is probably one of the main avenues of future researches in microbiology.

References

1. Leslie S, Winney B, Hellenthal G, Davison D, Boumertit A, Day T, Hutnik K, Royrvik EC, Cunliffe B, Wellcome Trust Case Control Consortium 2, International Multiple Sclerosis Genetics Consortium, Lawson DJ, Falush D, Freeman C, Pirinen M, Myers S, Robinson M, Donnelly P, Bodmer W: The fine-scale genetic structure of the British population. *Nature*. 2015; **519** (7543): 309-14 [PubMed Abstract](#) | [Publisher Full Text](#)

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.
