Genetic diversity of *Plasmodium falciparum* parasite by microsatellite markers after scale-up of insecticide-treated bed nets in western Kenya

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**Abstract**

**Background:** An initial study of genetic diversity of *Plasmodium falciparum* in Asembo, western Kenya showed that the parasite maintained overall genetic stability 5 years after insecticide-treated bed net (ITN) introduction in 1997. This study investigates further the genetic diversity of *P. falciparum* 10 years after initial ITN introduction in the same study area and compares this with two other neighbouring areas, where ITNs were introduced in 1998 (Gem) and 2004 (Karemo).

**Methods:** From a cross-sectional survey conducted in 2007, 235 smear-positive blood samples collected from children ≤15-year-old in the original study area and two comparison areas were genotyped employing eight neutral microsatellites. Differences in multiple infections, allele frequency, parasite genetic diversity and parasite population structure between the three areas were assessed. Further, molecular data reported previously (1996 and 2001) were compared to the 2007 results in the original study area Asembo.

**Results:** Overall proportion of multiple infections (Mₐ) declined with time in the original study area Asembo (from 95.9% - 2001 to 87.7% - 2007). In the neighbouring areas, Mₐ was lower in the site where ITNs were introduced in 1998 (Gem 83.7%) compared to where they were introduced in 2004 (Karemo 96.7%) in 2007. Overall mean allele count (MₐC ~ 2.65) and overall unbiased heterozygosity (Hₑ ~ 0.77) remained unchanged in 1996, 2001 and 2007 in Asembo and was the same level across the two neighbouring areas in 2007. Overall parasite population differentiation remained low over time and in the three areas at Fₛₜ < 0.04. Both pairwise and multilocus linkage disequilibrium showed limited to no significant association between alleles in Asembo (1996, 2001 and 2007) and between three areas.

**Conclusions:** This study showed the *P. falciparum* high genetic diversity and parasite population resilience on samples collected 10 years apart and in different areas in western Kenya. The results highlight the need for long-term molecular monitoring after implementation and use of combined and intensive prevention and intervention measures in the region.

**Keywords:** *Plasmodium falciparum*, Population structure, Genetic diversity, ITNs, Transmission

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Background

Insecticide-treated bed nets (ITNs), including long-lasting insecticide-treated bed nets (LLINs), are an important tool for malaria control [1]. In western Kenya, the efficacy of ITNs in reducing morbidity and all-cause mortality in children under 5 years of age was demonstrated previously [2–5]. Thereafter, a nationwide scale-up campaign to distribute ITNs in all 46 districts where malaria is endemic was undertaken [6]. By 2008, the Demographic Household Survey (DHS) showed overall 61 % Kenyan households owned at least one net of any kind and 47 % of children under 5-year-old slept under ITNs [7].

ITNs reduce malaria morbidity by killing or deterring mosquito vectors, thereby reducing the number of infectious bites on human hosts [8]. To be optimally effective, ITNs require consistent and appropriate use and high community coverage in all age groups [2, 9]. Changes in malaria transmission due to the use of ITNs ultimately impact vector and parasite populations, but the effects, especially after scale-up of ITNs, on genetic diversity and parasite populations are still unclear [10, 11].

A previous study on the effects of transmission reduction by ITNs on parasite population structure using neutral MS markers in western Kenya, showed that Plasmodium falciparum maintained overall high genetic diversity and stability after 5 years of high ITN use [11] even in periods with substantial reduction in malaria transmission and decline of Anopheles gambiae [12–14]. Clinical and immunological aspects in the hosts coupled with factors such as changes in vector ecology and gene flow in vector and host migration, have been considered as potential factors affecting the parasite genetic stability [4, 11, 15, 16]. Seasonal change or geographical isolation that influence transmission may also affect P. falciparum genetic diversity and population structure [17–19].

Previous studies on genetic diversity over space and time conducted by others in Kenya reported limited time or geographical area effects on gene allelic frequencies of P. falciparum in western Kenya [20]. Although reasons for this occurrence are not clear, large local population sizes of P. falciparum with numerous reproductive units have been shown to contribute to extensive heterogeneity of the parasite with correspondingly limited or no genetic differentiation across different regions in high transmission areas in Africa [21–24]. However, other studies have shown that P. falciparum maintains a clonal structure with significant linkage disequilibrium (LD) in some high-transmission areas, indicating there are other factors influencing genetic diversity and population structure [25, 26]. Therefore, a long-term follow-up study of parasite genetic diversity and population structure in the same area and between adjacent geographic areas where ITNs were deployed more recently can help to understand the impact of transmission reduction following the scale-up of vector control programmes on parasite population.

The initial assessment of effects of ITNs on parasite genetic diversity on samples collected in 1996 and 2001 in the original study area, Asembo (ITN introduction in 1997) in Rarieda sub-county, Siaya county of western Kenya, showed P. falciparum maintained overall high genetic diversity but with locus-specific variation, which contributed to differences in population sub-structure [11]. The current study investigates further the genetic diversity of P. falciparum in samples from 2007 in Asembo and the data were compared to that from two neighbouring areas where ITNs were first introduced in 1998 (Gem, Gem sub-county) and 2004 (Karemo, Alego sub-county), respectively. Assessing differences in parasite genetic diversity between Asembo, Gem and Karemo would inform on different area effects based on different ITN coverage and/or usage, and the possible role of migration of parasites between the areas. Further, comparison of parasite diversity within Asembo in the 1996, 2001 and 2007 surveys would show any possible temporal effects of ITN application on parasite population in the same locality with decline in entomology inoculation rate (EIR) and malaria prevalence. The same eight single copy neutral microsatellite (MS) markers used previously were employed in this study [11] to assess the genetic diversity and population structure of P. falciparum. Assessments of changes between time points and between areas on P. falciparum population were quantified based on multiplicity of infection, allele frequency, unbiased heterozygosity, linkage disequilibrium (LD) and genetic differentiation.

Methods

Study areas and study samples

This was a follow-up study in Siaya county of western Kenya where a two-phase, community-based, ITN trial was conducted from 1996 to 2001 [3–5, 11]. The initial trial design and ITN introduction in the original area of Asembo in 1997 (Rarieda sub-county) and the second area of Gem in 1998 (Gem sub-county) has been described in detail previously [27]. In 2004, ITNs were implemented in the third area Karemo (Alego sub-county). During and after the ITN trial, annual malaria infection, cross-sectional surveys were conducted around the same times of the year to coincide with the rainy seasons [2, 4, 28].

This study examines parasite diversity in samples collected from a cross-sectional survey conducted in 2007 in the Asembo and compares the results with those from Gem and Karemo areas. The geographic relationship of
the three areas is shown in Fig. 1. Further, parasite diversity within Asembo in 1996, 2001 and 2007 was compared. In Asembo, after initial introduction of ITNs in 1997 the households with at least one ITN reached >95 % by 1999, and remained high through to 2008 [12]. However, while coverage was high, ITN usage was low among residents in the three study areas but the levels differed for each area. In the 2007 survey, the proportion of smear-positive participants reporting to have slept under any type of bed net (treated or untreated) the night prior to survey was 51 (Asembo), 44 (Gem) and 20 % (Karemo) while actual ITN usage was 49, 31 and 7 %, respectively [10]. In addition, following the initial introduction of ITNs in Asembo in 1997 and Gem in 1998, malaria transmission was reduced by 90 % at the early stages of ITNs trial, with the EIR falling from 61.3 infective bites per person per year to 1.3 in 2001 [4, 13]. In the 2007 survey, the EIR was estimated to be four in Asembo and Gem and 20 in Karemo (KEMRI/CDC, unpublished data). Prevalence of parasitaemia in children ≤5-year-old in Asembo was 70 and 34 % in 1996 and 2001, respectively [4, 5]. In the 2007 survey, parasitaemia prevalence in children <15-year-old was 35.8 % in Asembo, 45.4 % in Gem and 50.3 % in Karemo (KEMRI/CDC, unpublished data).

From the 2007 cross-sectional survey, a total of 235 smear-positive samples collected from children ≤15-year-old from Asembo (n = 56), Gem (n = 87) and Karemo (n = 92) were used for genetic analysis of parasites. For Asembo, the molecular data from 69 and 74 smear-positive samples collected in 1996 and 2001, respectively, from children ≤5-year-old and reported earlier were also included for further temporal comparison [11]. Dried blood spot (DBS) samples were collected on filter paper and stored at −80 ºC. Parasite genomic DNA was extracted from one blood spot for each sample using the QiAmp DNA Mini kit (Qiagen, CA, USA) as per manufacturer’s instructions. Genomic DNA was stored at −20 ºC until use.

The study was approved by the Ethical Review Committee of Kenya Medical Research Institute, Nairobi, Kenya, the Institutional Review Board of Michigan State University, East Lansing, MI, USA and the Institutional Review Board of CDC, Atlanta, GA, USA.

**Microsatellite (MS) markers and genotyping**

The genetic diversity of *P. falciparum* parasites was assessed by scoring eight single copy neutral MS loci located on different chromosomes for all the samples as reported previously [29]. The selected MS markers, the primer sequences and amplification conditions used in this study have been described previously [11, 29, 30]. Briefly, five neutral markers (Poly-α, PIPK2, ADL, TAA60 and TAA109), one MS marker linked to the protein expressed during the gametocyte maturation stages of *P. falciparum* (Pfg377) and two MS linked to genes of asexual stage antigens under possible natural immune selection (EBP and P195) were used. All MS scoring in base length and peak height, and quantification of multiple alleles used the same method as described previously [11]. Briefly, MS base pair length and peak height were quantified by GeneMapper software (ABI). For each locus, allele identity was obtained from all peaks above 200 fluorescent units (fu). The highest peak was identified as the predominant allele, while minor alleles were
determined at peak heights of ≥30 % of the predominant allele meeting the 200 fu criteria. Amplification for the eight MS ranged from 90 to 100 % and samples failing to amplify for any of the MS was reported as missing and not used for haplotype definition.

Parameters measured and data analysis
All microsatellite raw data were managed using the Excel Microsatellite Tool Kit [31] and consequently formatted for other genetic analyses software programs. For multiple infections, both the predominant and minor alleles were counted to quantify the proportion of infections with more than one allele (M_A), while the highest number of allele count detected by any of the MS comprised the mean allele counts (M_AC). Differences in both M_A and M_AC between time points or between areas were assessed using Pearson’s Chi square and one way analysis of variance (ANOVA). Conversely, only the predominant allele in each locus was used to analyse all other parameters of genetic diversity and population structure, including unbiased heterozygosity (H_o) and allele richness calculated as the average number of alleles per locus, LD and genetic differentiation (F_ST) [11]. Multiple comparisons were corrected using Bonferroni correction for all tests where applicable. Allele richness and allele frequency were obtained using FSTAT [32]. Unbiased heterozygosity (H_o), sampling variance of H_o, was calculated as described previously [33] with p-levels obtained from z absolute values from the standard error (SE) of sampling variance. The LD measures the degree of association between gene pairs or among gene loci (structured population when LD is significant) assuming a null hypothesis of no association in random genetic recombination (population admixture when LD is insignificant). Pairwise LD, measuring the degree of association between MS, was obtained using ARLEQUIN [34]. Multilocus LD, measuring non-random association among all loci, was assessed with the index of association (F_is) by using LIAN program [35]. Multilocus LD tests the differences in variance of observed (V_d) and the variance expected (V_e) at LD, assuming a null hypothesis of no association in random genetic recombination (population admixture when LD is insignificant). Pairwise LD, measuring the degree of association between MS, was obtained using ARLEQUIN [34]. Multilocus LD, measuring non-random association among all loci, was assessed with the index of association (F_is) by using LIAN program [35].

Results
Since the Asembo 2007 survey comprised children up to 15 years of age, initial data were stratified by age (≤5- and >5-year-old) and tested for differences in parasite genetic diversity (Additional file 1: Table S1, Additional file 2: Table S2). As no significant differences in parasite diversity were detected by age, the molecular data from the 2007 survey were combined and analysed as one population in comparison with 1996 and 2001 surveys. In addition, a previous study conducted by us reported no difference in multiple infections between 1996 and 2001 [11] and initial temporal analysis in this study showed no significant variations in H_o but there were some differences in LD and F_ST between parasite populations from the three time points. For brevity, therefore, temporal data presented below focused on comparison of the parameters of multiple infection and genetic diversity only between 2001 and 2007 while comparison of LD and F_ST of parasite populations among 1996, 2001 and 2007 surveys are presented.

Multiple infections
Overall proportion of infections with more than one allele (M_A) by any of the eight MS in the three study areas was over 80 %, a reflection of a highly polyclonal P. falciparum parasite population. In the different area analyses, the overall M_A was significantly higher in Karemo at 96.7 % compared to Asembo (87.7 %) and Gem (83.7 %) (p = 0.01). In contrast, the overall mean allele counts (M_AC) were similar (p = 0.53) at 2.76 (Asembo), 2.55 (Gem) and 2.68 (Karemo). For individual MS, only P195 showed significantly higher M_A and M_AC in Karemo compared to both Asembo and Gem (p = 0.01). This pattern was reversed for both Pfg377 and PfPK2 where both M_A and M_AC were significantly lower in Karemo compared to both Asembo and Gem as shown in Table 1a. No differences were detected for all other loci for the same measures in the three study areas.

For the temporal effect analysis within Asembo, the overall M_A dropped from 95.9 % in 2001 to 87.7 % in 2007 (p = 0.03), but the reduction in the overall M_AC (3.1 in 2001 to 2.8 in 2007) was not statistically significant. At individual MS, there were significant decreases in M_A and M_AC from 2001 to 2007 at the P195 (47.5 %, 1.62–29.6 %, 1.37; p < 0.05), EBP (61.1 %, 2.10–41.2 % and 1.49; p < 0.05), and TAA109 (76.7 %, 2.26–44.4 % and 1.48; p < 0.05), respectively. No other significant changes, including Pfg377 MS, were observed between the two time periods as shown in Table 1b.

Genetic diversity
Allele size and composition for the eight MS in parasite populations from each area in the 2007 survey is shown
in Additional file 3: Figure S1. The number of alleles per locus based on allele size reflected the extensive and high genetic diversity in *P. falciparum* population in the three study areas. Allele numbers per locus ranged from a low of five for the Pfg377 locus in Asembo and Karemo to a high of 19 for the Poly-α locus in Karemo. The overall $H_e$...
was approximately 0.8 in the three study areas as shown in Table 2a. Within the individual MS, \( H_e \) was significantly lower at P195 locus in Gem (\( H_e = 0.65 \)) than in both Asembo (\( H_e = 0.74 \)) and Karemo (\( H_e = 0.74 \)). No significant difference in \( H_e \) for other individual MS markers between the areas was detected (Table 2a). Similarly, no significant differences in overall and loci specific \( H_e \) were observed in the samples from Asembo area between the 2001 and 2007 time points (Table 2b).

**Pairwise and multilocus LD**

Overall, results of 28 pairwise comparisons for each area showed that LD was significant (\( p \leq 0.0018 \)) for 16, 14 and 15 MS pairs in Asembo, Gem and Karemo, respectively. Of note, Pfg377, the MS flanking the gene relating to gametocyte maturation, had the least number of significant pairwise LD (only paired with P195 and ADL; \( p \leq 0.0018 \)) in Gem and Karemo, respectively, while no significant LD was observed in Asembo. Conversely, the remaining MS had at least ten of the significant pairwise LD in all three areas as shown in Additional file 4: Table S3.

Within Asembo, the 16 MS pairs showed significant LD in the 2007 survey while only six MS pairs had significant LD in the 2001 survey. The high number of significant pairwise LD in 2007 was similar to that observed in the baseline survey (1996) where 14 pairs of MS showed significant LD. However, despite these overall changes in the number of significant pairwise LD in the different time points, LD at Pfg377 locus again showed the least number with only four significant pairs in the three time points [P195 and ADL in 1996, P195 and EBP in the 2001 and no pairs in 2007 survey (\( p \leq 0.0018 \); Additional file 4: Table S3 and Additional file 5: Table S4)]. This suggests possible consistent locus specific diversity at the Pfg377, which shows higher random association with other MS alleles and therefore less LD.

Multilocus LD, testing non-random association on all loci, among the three study areas showed diverse results. In Asembo, the variance in observed (\( V_d \)) LD in all three areas as shown in Additional file 4: Table S3.

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| Locus | Asembo | Gem | Karemo | p value | Asembo/Gem | Asembo/Karemo | Karemo/Garemo |
|-------|--------|-----|--------|---------|-----------|---------------|---------------|
| Polya | 16 (15.94) | 0.91 (0.0193) | 17 (16.78) | 0.89 (0.0279) | 19 (18.85) | 0.88 (0.0315) | 0.620 | 0.469 | 0.814 |
| Pfg377 | 5 (4.99) | 0.47 (0.0726) | 6 (5.74) | 0.41 (0.0648) | 5 (4.72) | 0.32 (0.059) | 0.472 | 0.073 | 0.316 |
| PIPK2 | 10 (10) | 0.85 (0.0243) | 11 (10.44) | 0.82 (0.0209) | 12 (11.32) | 0.84 (0.0221) | 0.353 | 0.749 | 0.530 |
| ADL | 12 (12) | 0.88 (0.0177) | 14 (13.71) | 0.90 (0.0114) | 16 (15.28) | 0.91 (0.0089) | 0.205 | 0.075 | 0.550 |
| EBP | 8 (8) | 0.82 (0.0562) | 14 (13.4) | 0.88 (0.0388) | 11 (10.94) | 0.83 (0.0232) | 0.426 | 0.886 | 0.312 |
| P195a | 7 (6.99) | 0.74 (0.0249) | 6 (5.83) | 0.65 (0.0206) | 6 (5.99) | 0.74 (0.0209) | 0.005 | 0.957 | 0.0023 |
| TAA60 | 8 (7.98) | 0.81 (0.0249) | 9 (8.42) | 0.79 (0.0206) | 7 (6.96) | 0.79 (0.0209) | 0.561 | 0.550 | 0.983 |
| TAA109 | 9 (8.97) | 0.79 (0.0249) | 12 (11.28) | 0.83 (0.0169) | 14 (13.17) | 0.83 (0.0239) | 0.124 | 0.178 | 0.993 |
| Overall | 0.78 (0.0495) | 0.77 (0.0060) | 0.77 (0.0060) | 0.77 (0.0060) | 0.77 (0.0060) | 0.77 (0.0060) | 0.77 (0.0060) | 0.77 (0.0060) | 0.77 (0.0060) |

**Table 2 Genetic diversity of parasites in (a) Asembo, Gem and Karemo areas, 2007 survey and, (b) Asembo 2001 and 2007 surveys**

| Locus | Asembo-2001 | Asembo-2007 | p value |
|-------|-------------|-------------|---------|
| Polya-a | 17 (16.92) | 0.92 (0.0243) | 16 (15.94) | 0.91 (0.0193) | 0.653 |
| Pfg377 | 3 (3.00) | 0.57 (0.0984) | 5 (4.96) | 0.47 (0.0726) | 0.48 |
| PIPK2 | 12 (11.92) | 0.83 (0.0497) | 10 (10.00) | 0.85 (0.0243) | 0.752 |
| ADL | 14 (13.96) | 0.89 (0.0191) | 12 (12.00) | 0.88 (0.0177) | 0.623 |
| EBP | 6 (5.99) | 0.82 (0.0193) | 8 (8.00) | 0.82 (0.0562) | 0.976 |
| P195 | 11 (10.91) | 0.75 (0.0241) | 7 (6.99) | 0.74 (0.0249) | 0.73 |
| TAA60 | 17 (16.87) | 0.85 (0.0322) | 8 (7.98) | 0.81 (0.0249) | 0.344 |
| TAA109 | 11 (10.94) | 0.77 (0.0372) | 9 (8.97) | 0.79 (0.0249) | 0.691 |
| Overall | 0.79 (0.0380) | 0.78 (0.0495) | 0.78 (0.0495) | 0.78 (0.0495) | 0.625 |

Asembo, Gem and Karemo denotes years after introduction of ITNs; 10, 9 and 3 years, respectively.

Comparison of genetic diversity between areas and between years was based on the number of alleles, allele richness (between areas only), unbiased heterozygosity (\( H_e \)) and standard error (SE) [31]. Standard error was calculated to generate a \( p \) value for statistical testing of differences in \( H_e \). \( p \) value <0.05 are in italics.

* Denotes locus with significantly different \( H_e \) between areas.
of 1.268 was significantly higher than the variance expected (Ve) which was 1.133 (p = 0.03) with an index of association (IA) of 0.017, suggesting a significant multilocus LD. In contrast, multilocus LD was not significant in either Gem or Karemo where IA was −0.003 and 0.001, respectively (Table 3a). The results suggest a more structured P. falciparum population in Asembo while parasite population in Gem and Karemo show more admixtures in 2007.

Within Asembo, the multilocus LD reflected the previous pattern observed in the pairwise LD. Multilocus LD was significant in 2007 survey (IA = 0.017) in contrast to the previous 2001 survey (IA = 0.001) but similar to the 1996 baseline survey (IA = 0.016) as shown in Table 3b. These results suggest that the P. falciparum population while structured in 1996, had more admixture in 2001, but was more structured 10 years after the introduction of ITN use in Asembo.

Genetic differentiation
In assessing different area effects, the overall genetic differentiation within Asembo, Gem and Karemo was low (FST = 0.021). When individual MS were analysed, only P195 MS showed moderate genetic differentiation (FST ≥ 0.05 < 0.15) between any two areas in 2007. All other individual MS showed low differentiation (FST < 0.05) that was not significant between the three study areas (Table 4a). A number of the FST negative values were observed in the comparisons between the three areas. This indicated different parasite populations being closer to each other between than within areas.

For assessing the temporal effects on genetic differentiation, the overall genetic differentiation was relatively higher in the 2001 and 2007 time points (FST = 0.040) compared to the 1996/2007 testing where FST was 0.026. Incidentally, as reported previously, FST was also low in the 1996 and 2001 testing at FST 0.027 [11]. The overall FST results show that over the three time points spanning 10 years there was only limited differences in allele frequencies resulting in insignificant parasite population differentiation in Asembo area. At the individual MS, P195 locus, as in the different areas analyses, showed consistently moderate differentiation for the paired time point comparisons between the years 1996, 2001 and 2007 (Table 4b). Although differentiation at this locus could have contributed to the differences in overall FST in Asembo, the effect of a single locus in the overall population differentiation remained low considering the 10-year period since introduction of ITNs.

Discussion
This study was aimed at assessing changes on P. falciparum population genetic diversity after scale-up of ITNs in three adjacent geographic areas: Asembo, Gem and Karemo, where ITNs were introduced at different times: Asembo in 1997, Gem in 1998 and Karemo in 2004. The study further examined temporal changes on parasite diversity within Asembo. Overall proportion of multiple
Table 4 Genetic differentiation index (F_{ST}) for *P. falciparum* populations (a) in Asembo, Gem and Karemo areas, 2007 survey and, (b) in Asembo between 1996 and 2007, and 2001 and 2007 surveys

(a) Locus | Area1 | Area2 | F_{ST} | Levels of differentiation
--- | --- | --- | --- | ---
Poly-α | Asembo | Gem | −0.003 | Low
 | Asembo | Karemo | 0.001 | Low
 | Gem | Karemo | −0.004 | Low
Pfg377 | Asembo | Gem | −0.003 | Low
 | Asembo | Karemo | 0.005 | Low
 | Gem | Karemo | −0.002 | Low
PIPK2 | Asembo | Gem | 0.007 | Low
 | Asembo | Karemo | 0.001 | Low
 | Gem | Karemo | 0.009 | Low
ADL | Asembo | Gem | 0.003 | Low
 | Asembo | Karemo | 0.001 | Low
 | Gem | Karemo | 0.006 | Low
EBP | Asembo | Gem | 0.018 | Low
 | Asembo | Karemo | 0.003 | Low
 | Gem | Karemo | 0.002 | Low
P195 | Asembo | Gem | 0.059 | Moderate
 | Asembo | Karemo | 0.100 | Moderate
 | Gem | Karemo | 0.133 | Moderate
TAA60 | Asembo | Gem | −0.006 | Low
 | Asembo | Karemo | −0.011 | Low
 | Gem | Karemo | −0.009 | Low
TAA109 | Asembo | Gem | 0.032 | Low
 | Asembo | Karemo | 0.022 | Low
 | Gem | Karemo | −0.002 | Low
ALL | Asembo | 0.021 | Low
 | Gem | 0.021 | Low
 | Karemo | 0.021 | Low

(b) Locus | Survey year | F_{ST} | Levels of differentiation
--- | --- | --- | ---
Poly-α | 1996 | 0.005 | Low
 | 2001 | −0.006 | Low
Pfg377 | 1996 | 0.022 | Low
 | 2001 | 0.022 | Low
PIPK2 | 1996 | 0.009 | Low
 | 2001 | −0.001 | Low
ADL | 1996 | 0.006 | Low
 | 2001 | 0.009 | Low
EBP | 1996 | 0.007 | Low
 | 2001 | 0.013 | Low
P195 | 1996 | 0.105 | Moderate
 | 2001 | 0.141 | Moderate
TAA60 | 1996 | 0.037 | Low
 | 2001 | 0.057 | Moderate
TAA109 | 1996 | 0.037 | Low
 | 2001 | 0.028 | Low
Overall | 1996 | 0.026 | Low
 | 2001 | 0.040 | Low

Genetic differentiation index (F_{ST}) was assessed at each MS between (a) any two areas of Asembo, Gem and Karemo and (b) in Asembo between 1996, 2001 and 2007 surveys’ parasite populations. This was based on the null hypothesis that alleles are drawn from the same distribution in any of the parasite populations tested. The levels were defined as little-to-low F_{ST} (<0.05), moderate (≥0.05 to <0.15) and great differentiation (≥0.15) as described previously [37].

Moderate F_{ST} was highlighted in bold.
infections ($M_A$) dropped from 95.9% in 2001 to 87.7% in 2007. The $M_A$ levels were similar in Asembo (87.7%) and Gem (83.7%) but significantly higher in Karemo (96.7%) in 2007. However, the overall mean allele count $M_{AC}$ remained unchanged at around 2.65 in the three areas and at the different time points. Further, after 10 years of sustained ITNs use (1997–2007), the genetic diversity measured by $H_e$ remained unchanged at approximately the same level over time and in the three areas ($H_e \sim 0.78$). Additionally, there was low parasite population differentiation for the three areas ($F_{ST} = 0.021$) and over time ($F_{ST} < 0.04$). The only slight difference observed was that in Asembo there was less significant pairwise LD and insignificant multilocus LD in 2001 compared to 1996 (baseline) and 10 years later (2007).

Initial hypothesis of this study was that malaria transmission reduction, mainly by ITNs, would decrease parasite diversity. However, in spite of relative differences in duration of ITN implementation, use of ITNs and EIR between Karemo and Asembo (also Gem), the overall $H_e$ observed in the three study areas remained high. The similarly high $H_e$ for *P. falciparum* using neutral MS markers was reported in Kombewa and other areas of western Kenya, including Kapsulu, Kodera, Rangwe, Ringa, and Rota villages in surrounding counties, although no data on ITN usage were presented [20, 38]. The high $H_e$, coupled with low overall genetic differentiation between areas in this study suggest the possible existence of vibrant reproductive units that maintain the high diversity within *Plasmodium* parasite pools. The high diversity and limited genetic differentiation also suggest gene flow is likely to be a major factor in maintaining vast parasite pools in the geographic region. The negative $F_{ST}$ results observed in this study further illustrate the extent of admixture and cross-breeding within parasite populations in the three areas. Gene flow due to human migration was reported previously as a contributing factor to a resilient *Plasmodium* parasite population in western Kenya [39]. Demographic data also confirm steady migration in the study areas, with an average of 130 per 1000 person years out-migrating, and 20 per 1000 person years in-migrating annually [40]. In addition, sub-microscopic infection and gametocyte reservoirs could indirectly contribute to genetically diverse, yet stable, parasite population observed here in the three study areas. Microscopically detectable parasitaemia, including both asexual and sexual stage parasites, could significantly underestimate the true level of parasite transmission. For example, with scale-up of malaria controls in western Kenya, the proportion of sub-microscopic infections at community level remains high and sub-microscopic gametocyte carriers are substantial in both Asembo and Karemo areas (Zhou et al., in prep) that could serve as potential transmission reservoirs, consequently maintaining parasite diversity. Indeed, a model on transmission dynamics of *P. falciparum* from hosts with a large pool of sub-microscopic asexual parasites and gametocytes predicted high uninterrupted transmission even with scaled-up LLIN coverage [41]. This robust but obscure transmission, coupled with possible over-representation of stable parasite reproductive units and gene flow due to geographical proximity of the study areas, may explain the overall genetic stability in the three study areas.

Within Asembo the overall $H_e$ remained high and stable in 1996, 2001 [11] and 2007 surveys in which period malaria prevalence declined from 70 to 36% and EIR from 61 to 4. There was also no difference in $M_{AC}$ and the overall level of population differentiation ($F_{ST}$) remained low over the three time points. A notable change was observed only in 2001 in the LD parameters with less significant pairwise LD and insignificant multilocus LD compared to 1996 (baseline) and 10 years later (2007). It is possible that sudden changes in parasite population due to the initial transmission reduction by introduction of ITNs in 1997 could allow minor populations with different allele frequencies to become dominant which might result in admixture parasite population (insignificant LD) in 2001. It is also likely that sulfadoxine-pyrimethamine (SP) and chloroquine (CQ) resistance contributed to malaria transmission [42], resulting in sustainability of parasite diversity although malaria prevalence measured by microscopy declined over the time. A study conducted in Papua New Guinea showed a strong association between multiplicity of infections and genetic diversity which was not related to prevalence, and the genetic diversity was maintained at high levels with no visible seasonal variation [43]. Other studies show that scaled-up malaria control and reduced transmission result in focal clusters of high transmission, which act as consistent parasite reservoirs [42]. Taken together, this suggests lack of direct correlation between declining prevalence (or EIR) and decreased genetic diversity [44]. Therefore, molecular monitoring is critical especially where prevalence as measured by microscopy has reduced significantly yet sub-microscopic infection that contributes malaria transmission continues [45].

While overall $H_e$ was similar in the three areas and stable over time, there were differences in overall $M_A$. In Karemo, where ITNs were introduced since 2004 (the shortest time) and with the lowest use at 20%, $M_A$ was significantly higher at 96.7% than in Asembo at 87.7% (ITN introduction in 1997 with 51% use), or Gem 83.7% with ITN introduction in 1998 and 44% use. Similarly, where temporal effects were assessed in Asembo, $M_A$ also significantly decreased from 2001 (95.9%) to 2007 (87.7%) after 10 years since introduction of ITNs.
However, the temporal and area differences in MA were not substantial to affect the overall $H_o$, suggesting that multiple infections could be confounded by within-host competition among the parasite clones that are under selection by drug pressure, host immune pressure or parasites in different species of mosquito vector, all of which would be influenced by various malaria control measures. The results also suggest that measuring multiple infections could serve as an early indicator for change of malaria transmission.

In this study, there were also a few significant differences by different measures, suggesting that the $P_{195}$ could be robust in reflecting alteration of parasite population due to subtle differences in the host’s immunity influenced by malaria exposure. In addition, $Pfg377$ MS locus, another important marker linked to a protein gene exclusively expressed during maturation of gametocytes [47], showed the least number of significant pairwise LD in three survey time points and across three areas. The results suggest a high random association of $Pfg377$ with other MS alleles to adjust gametocyte-related diversity as the parasites adapt to changes in transmission, which further indicates this marker could potentially be used as an adaptive marker for measuring change in transmission in future.

This study has a few limitations. The effect on parasite diversity was extrapolated based on neutral MS markers that may not fully capture the dynamism of the parasite population in the face of different control measures that include ITN use and drug pressure, which would further shape host immunity. Geographic proximity of the three study areas could have limited the ability to detect significant area divergences in the parasite populations. This is a cross sectional survey and while ITN coverage was is a cross sectional survey and while ITN coverage was high the actual use in the nights before sampling showed low usage (all below 50 %) which limited dissecting the impact of ITNs on parasite diversity. The temporal comparison was also limited as only one area had at least 10 years of ITN use. Further studies on parasite genetic diversity/structure for longer periods and in wider geographical regions, as well as use of other unique and robust genetic markers of parasites [48, 49], will be necessary to understand transmission dynamics and other factors that continue to sustain the high parasite diversity despite the use of ITNs/LLINs and case management by drug therapy in western Kenya.

Conclusion
This study has shown the overall high genetic diversity and stability of $P. falciparum$ over 10 years and across three different areas after scale-up of ITNs. The parasite resilience was reflected by a change in LD in Asembo at mid-point (5 years) but not at the 10-year time point. In addition to the gene flow between areas, other possible factors that might be attributed to the high and stable diversity of parasite population mainly are sub-microscopic infection and large gametocyte reservoir. Theoretically, a dramatic transmission reduction as a result of using multiple and intensive prevention and intervention measures can decrease parasite genetic diversity by creating a bottleneck effect on parasite population; for this to happen in western Kenya, such combined and intensive prevention and intervention measures must be sustained and cover wide geographic areas.


d Additional files

Additional file 1: Table S1. Comparison of the frequency of multiple alleles in Asembo 1a), Gem 1b) and Karemo 1c) by age.

Additional file 2: Table S2. Unbiased expected heterozygosity for three study areas by age.

Additional file 3: Figure S1. Allele sizes and compositions (base pairs, X-axis) and frequency distribution (Y-axis) of eight individual MS for $P. falciparum$ parasite populations from Asembo, Gem and Karemo areas, 2007.

Additional file 4: Table S3. Comparison of pairwise LD for $P. falciparum$ populations in a) Asembo, Gem, and Karemo Areas, 2007 survey and, b) Asembo 1996, 2001 and 2007 surveys.

Additional file 5: Table S4. Number of gene copies and number of alleles per locus used in LD analyses for Asembo, Gem and Karemo.

Authors’ contributions
WG carried out genotyping work and genetic data analysis. CO, MH, LS and SK designed and conducted 2007 cross sectional survey and WH, FTK, PPH, DJT and RN implemented and conducted 1996–2001 ITN trial including collection of samples and epidemiological data. Ni assisted in statistical data analysis and MS supported data management. YO participated in sample processing and MS supported data management. YO participated in sample processing for the genetic analysis. JG and EW participated in the design of this study. YPS was responsible for the design of this study and participated in data analysis. WG and YPS wrote manuscript. All authors read and approved the final manuscript.

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Competing interests
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References
1. WHO. World malaria report. Geneva: World Health Organization; 2008. p. 25.
2. Hawley WA, Phillips-Howard PA, ter Kuile FO, Terlouw DJ, Vulule JM, Ombok M, et al. Community-wide effects of permethrin-treated bed nets on child mortality and malaria morbidity in western Kenya. Am J Trop Med Hyg. 2003;68:121–2.
3. Phillips-Howard PA, Nahlen BL, Kolczak MS, Hightower AW, ter Kuile FO, Alai AJ, et al. Efficacy of permethrin-treated bed nets in the prevention of malaria in young children in an area of high perennial malaria transmission in western Kenya. Am J Trop Med Hyg. 2004;69:23–9.
4. Lindblade KA, Eisele TP, Gimnig JE, Alaii JA, Odhiambo F, ter Kuile FO, et al. Sustainability of reductions in malaria transmission and infant mortality in western Kenya with use of insecticide-treated bednets: 4 to 6 years of follow-up. JAMA. 2004;291:2571–80.
5. ter Kuile FO, Terlouw DJ, Phillips-Howard PA, Hawley WA, Friedman JF, Kolczak MS, et al. Impact of permethrin-treated bed nets on malaria and all-cause morbidity in young children in an area of intense perennial malaria transmission in western Kenya: cross-sectional survey. Am J Trop Med Hyg. 2003;68:100–7.
6. Hightower A, Kiptui R, Manya A, Wolkon A, Vanden Eng JL, Hamel M, et al. Bed net ownership in Kenya: the impact of 3.4 million free bed nets. Malar J. 2010;9:183.
7. Kenya National Bureau of Statistics NACC, National AIDS/STD Control Programme, Ministry of Public Health and Sanitation, Kenya Medical Research Institute, ICF Macro, USAID, United Nations Population Fund, United Nations Children’s Fund: Kenya Demographic and Health Survey 2008–2009. 2010:167.
8. Curtis CF, Jana-Kara B, Maxwell CA. Insecticide treated nets: impact on vector populations and relevance of initial intensity of transmission and pyrethroid resistance. J Vector Borne Dis. 2003;40:1–8.
9. Vanden Eng JL, Thwing J, Wolkon A, Kulkarni MA, Manya A, Enkine M, et al. Assessing bed net use and non-use after long-lasting insecticidal net distribution: a simple framework to guide programmatic strategies. Malar J. 2010;9:133.
10. Shah M, Omosun Y, Lal A, Odero C, Gatei W, Otieno K, et al. Assessment of molecular markers for anti-malarial drug resistance after the introduction and scale-up of malaria control interventions in western Kenya. Malar J. 2015;14:75.
11. Gatei W, Karuki S, Hawley W, ter Kuile F, Terlouw D, Phillips-Howard P, et al. Effects of transmission reduction by insecticide-treated bed nets (ITNs) on parasite genotypes population structure I. The genetic diversity of Plasmodium falciparum parasitema by microsatellite markers in western Kenya. Malar J. 2010;9:353.
12. Bayoh MN, Mathias DK, Odiere MR, Mutuku FM, Kamau L, Gimnig JE, et al. Anopheles gambiae: historical population decline associated with regional distribution of insecticide-treated bed nets in western Nyanza Province, Kenya. Malar J. 2010;9:62.
13. Gimnig JE, Kolczak MS, Hightower AW, Vulule JM, Schoute E, Kamau L, et al. Effect of permethrin-treated bed nets on the spatial distribution of malaria vectors in western Kenya. Am J Trop Med Hyg. 2003;68:115–20.
14. McCann RS, Ochoho E, Bayoh MN, Vulule JM, Hamel MJ, Gimnig JE, et al. Reemergence of Anopheles funestus as a vector of Plasmodium falciparum in western Kenya after long-term implementation of insecticide-treated bed nets. Am J Trop Med Hyg. 2014;90:597–604.
15. Kariuki SK, Lal AA, Terlouw DJ, ter Kuile FO, Ong’echa JM, Phillips-Howard PA, et al. Effects of permethrin-treated bed nets on immunity to malaria in western Kenya II. Antibody responses in young children in an area of intense malaria transmission. Am J Trop Med Hyg. 2003;68:108–14.
16. O’Meara WP, Mwangi TW, Williams TN, McKenzie FE, Snow RW, Marsh K. Relationship between exposure, clinical malaria, and age in an area of changing transmission intensity. Am J Trop Med Hyg. 2008;79:185–91.
17. Babiker HA. Unstable malaria in Sudan: the influence of the dry season. Plasmodium falciparum population in the unstable malaria area of eastern Sudan is stable and genetically complex. Trans R Soc Trop Med Hyg. 1998;92:585–9.
18. Noranate N, Prugnolle F, Jouin H, Tall A, Marrama L, Sokhna C, et al. Population diversity and antibody selective pressure to Plasmodium falciparum MSP1 block2 locus in an African malaria-endemic setting. BMC Microbiol. 2009;9:219.
19. Susomboon P, Iwagami M, Tungpukdek K, Krusood S, Looareesuwan S, Kano S. Differences in genetic population structures of Plasmodium falciparum isolates from patients along Thai–Myanmar border with severe or uncomplicated malaria. Malar J. 2008;7:212.
20. Prugnolle F, Durand P, Jacob K, Razakandrainibe F, Arnathau C, Villareal D, et al. A comparison of Anopheles gambiae and Plasmodium falciparum genetic structure over space and time. Microbes Infect. 2008;10:269–75.
21. Anderson TJ, Haubold B, Williams JT, Estrada-Franco JG, Richardson L, Molinero P, et al. Microsatellite markers reveal a spectrum of population structures in the malaria parasite Plasmodium falciparum. Mol Biol Evol. 2000;17:1467–82.
22. Bogreau H, Renaud F, Bouchiba H, Durand P, Assi SB, Henry MC, et al. Genetic diversity and structure of African Plasmodium falciparum populations in urban and rural areas. Am J Trop Med Hyg. 2006;74:953–9.
23. Lectric MC, Durand P, de Meeus T, Robert V, Renaud F. Genetic diversity and population structure of Plasmodium falciparum isolates from Dakar, Senegal, investigated from microsatellite and antigen determinant loci. Microbes Infect. 2002:685–92.
24. Bruce MC, Macheso A, McComnachie A, Molynieux ME. Comparative population structure of Plasmodium malariae and Plasmodium falciparum under different transmission settings in Malawi. Malar J. 2011;10:38.
25. Durand P, Michaelakis Y, Gestier S, Oury B, Leclecic M, Tibayrenc M, et al. Significant linkage disequilibrium and high genetic diversity in a population of Plasmodium falciparum from an area (Republic of the Congo) highly endemic for malaria. Am J Trop Med Hyg. 2003;68:345–9.
26. Razakandrainibe FG, Durand P, Koella JC, De Meeus T, Rousset F, Ayala FJ, et al. “Clonal” population structure of the malaria agent Plasmodium falciparum in high-infection regions. Proc Natl Acad Sci USA. 2005;102:17388–93.
27. Phillips-Howard P, ter Kuile F, Nahlen B, Alai AJ, Gimnig J, Kolczak M. The efficacy of permethrin-treated bed nets on child mortality and morbidity in western Kenya II. Study design and methods. Am J Trop Med Hyg. 2003;68:10–5.
28. Hamel MA, Atzazu K, Obar D, Sewe M, Vulule J, Williamson JM, et al. A reversal in reductions of child mortality in western Kenya, 2003–2009. Am J Trop Med Hyg. 2011;85:597–605.
29. Su X, Wellemes TE. Toward a high-resolution Plasmodium falciparum linkage map: polymorphic markers from hundreds of simple sequence repeats. Genomics. 1996;33:40–44.
30. Anderson TJ, Su XZ, Bockarie M, Laopong M, Day KP. Twelve microsatellite markers for characterization of Plasmodium falciparum from finger-prick blood samples. Parasitology. 1999;119:113–25.
31. Shabt I, Lattorff HMG, Moritz RFA. A microsatellite DNA toolkit for studying population structure in Apis mellifera. Mol Ecol Resour. 2008;8:1034–6.
32. Gouedet J. FSTAT (version 1.2): a computer program to calculate F-statistics. J Hered. 1995;86:485.
33. Nei M. Molecular evolutionary genetics. New York: Columbia University Press; 1987.
34. Excoffier L, Laval G, Schneider S. Arlequin ver. 3.0: an integrated software package for population genetics data analysis. Evol Bioinform Online. 2005;1:47–50.
35. Haubold B, Hudson RR. LINA; 3.0: detecting linkage disequilibrium in multilocus data. Linkage analysis. Bioinformatics. 2000;16:847–8.
36. Rousset F. GENEPOP 2007: a complete re-implementation of the GENEPOP software for Windows and Linux. Mol Ecol Resour. 2009;8:103–6.
37. Balloux F, Lugon-Moulin N. The estimation of population differentiation with microsatellite markers. Mol Ecol. 2002;11:155–65.
38. Zhong D, Afrane Y, Githeko A, Yang Z, Cui L, Menge DM, et al. Plasmodium falciparum genetic diversity in western Kenya highlands. Am J Trop Med Hyg. 2007;77:1043–50.
39. Bonizzoni M, Afrane Y, Baliraine F, Amenya D, Githeko A, Yan G. Genetic structure of Plasmodium falciparum populations between lowland and highland sites and antimalarial drug resistance in Western Kenya. Infect Genet Evol. 2009;9:806–12.
40. Odhiambo FC, Laserson KF, Sewe M, Hamel MJ, Feikin DR, Adazu K, et al. Profile: the KEMRI/CDC health and demographic surveillance system-western Kenya. Int J Epidemiol. 2012;41:977–87.
41. Karl S, Gurarie D, Zimmerman PA, King CH, St Pierre TG, Davis TM. A sub-microscopic gametocyte reservoir can sustain malaria transmission. PLoS One. 2011;6:e20805.
42. Snow RW, Kibuchi E, Karuri SW, Sang G, Gitonga CW, Mwandawiro C, et al. Changing malaria prevalence on the Kenyan Coast since 1974: climate, drugs and vector control. PLoS One. 2015;10:e0128792.
43. Barry AE, Schultz L, Senn N, Nale J, Kiniboro B, Siba PM, et al. High levels of genetic diversity of Plasmodium falciparum populations in Papua New Guinea despite variable infection prevalence. Am J Trop Med Hyg. 2013;88:718–25.
44. Escalante AA, Ferreira MU, Vinetz JM, Volkman SK, Cui L, Gamboa D, et al. Malaria molecular epidemiology: lessons from the International Centers of Excellence for Malaria Research Network. Am J Trop Med Hyg. 2015;93:79–86.
45. Canier L, Khim N, Kim S, Suydts V, Heng S, Dourng D, et al. An innovative tool for moving malaria PCR detection of parasite reservoir into the field. Malar J. 2013;12:405.
46. Holder AA, Lockyer MJ, Odink KG, Sandhu JS, Riveros-Moreno V, Nicholls SC, et al. Primary structure of the precursor to the three major surface antigens of Plasmodium falciparum merozoites. Nature. 1985;317:270–3.
47. Alano P, Read D, Bruce M, Aikawa M, Kaido T, Tegoshi T, et al. COS cell expression cloning of Pfg377, a Plasmodium falciparum gametocyte antigen associated with osmiophilic bodies. Mol Biochem Parasitol. 1995;74:143–56.
48. Daniels R, Volkman SK, Milner DA, Mahesh N, Neafsey DE, Park DJ, et al. A general SNP-based molecular barcode for Plasmodium falciparum identification and tracking. Malar J. 2008;7:223.
49. Preston MD, Campino S, Assela SA, Echeverry DF, Ocholla H, Amambua-Ngwa A, et al. A barcode of organellar genome polymorphisms identifies the geographic origin of Plasmodium falciparum strains. Nat commun. 2014;5:4052.