

# "Clonal" population structure of the malaria agent *Plasmodium falciparum* in high-infection regions

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The population genetic structure of *Plasmodium falciparum*, the agent of malignant malaria, has been shown to be predominantly "clonal" (i.e., highly inbred) in regions of low infectivity; in high-infectivity regions, it is often thought to be panmictic, or nearly so, although there is little supporting evidence for this. The matter can be settled by investigating the parasite's genetic makeup in the midgut oocysts of the mosquito vector, where the products of meiosis can directly be observed. The developmental stages of *P. falciparum* are haploid, except in the oocysts of infected mosquito vectors, where two gametes fuse, diploidy occurs, and meiosis ensues. We have investigated genetic polymorphisms at seven microsatellite loci located on five chromosomes by assaying 613 oocysts in 145 mosquitoes sampled from 11 localities of Kenya, where malignant malaria is perennial and intense. There is considerable allelic variation,  $16.3 \pm 2.1$  alleles per locus, and considerable inbreeding,  $\approx 50\%$  on the average. The inbreeding is caused by selfing ( $\approx 25\%$ ) and nonrandom genotype distribution of oocysts among mosquito guts (35%). The observed frequency of heterozygotes is  $0.43 \pm 0.03$ ; the expected frequency, assuming random mating, is  $0.80 \pm 0.05$ . Linkage disequilibrium is statistically significant for all 21 pairwise comparisons between loci, even though 19 comparisons are between loci in different chromosomes, which is consistent with strong deviation from panmixia and the consequent reproduction of genomes as clones, without recombination between gene loci. This is of considerable evolutionary significance and of epidemiological consequence, concerning the spread of multilocus drug and vaccine resistance.

epidemiology | evolutionary genetics | parasitism | population genetics

Malaria is a devastating disease, caused by four species of the parasitic protozoan *Plasmodium*, endemic in tropical regions of >100 countries and territories. *Plasmodium falciparum*, the agent of the most malignant form of malaria, accounts for 300–600 million episodes of clinical malaria (1) and 1.5–2.7 million deaths each year (2), mostly of African children (3). *P. falciparum* is transmitted by species of *Anopheles* mosquitoes. During the mosquito bloodmeal, male and female gametocytes are ingested, which rapidly mature into gametes that fuse to form an ookinete, which encysts in the mosquito gut, forming an oocyst where meiosis rapidly ensues (4). A single mosquito may carry several oocysts, each resulting from the fusion of two gametes. Oocysts are thus the only "diploid" stage in the life cycle of *P. falciparum*, because only oocysts contain the results of gamete mixing and the products of a single meiosis. Elsewhere in the mosquito, as everywhere in the human host, *P. falciparum* exists only as a haploid organism.

The population genetic structure of *P. falciparum* is generally ascertained by investigating the haploid stages encountered in infected human blood, where pairs of genomes derived each from a single meiosis cannot be distinguished from genomes derived from multiple infections, even in regions with low endemicity (5–7). The incidence of clonality or inbreeding versus outbreeding or panmixia is estimated indirectly by measuring genetic diversity, linkage disequilibrium, and the like (2, 6, 8–16). Hence the con-

flicting evidence favoring different levels of clonality or panmixia, although it is often accepted that *P. falciparum* displays different population structures in different places, ranging from clonality to panmixia in approximate correlation with the increasing intensity of transmission (2, 5, 6, 8–16).

Conflicting interpretations of the population structure of *P. falciparum* also arise because of a failure to distinguish between two meanings of the phrases "sexual reproduction" and, correspondingly, "clonal reproduction." Sexual reproduction as a physiological phenomenon is predicated of individuals that each develop from a zygote formed by the fusion of gametes and that each experiences meiosis and produces gametes. As a genetic phenomenon, sexual reproduction refers to genetic recombination between loci, whereas clonal reproduction refers to the reproduction of whole genomes as units, without genetic recombination. It seems likely that physiological sexuality evolved as an adaptive mechanism for producing genetic recombination, but physiological and genetic sexuality have repeatedly become separated in the evolution of many organisms. In terms of long-term evolutionary consequences, it is genetic sexuality (i.e., genetic recombination between dissimilar genomes) that is of consequence (see ref. 17). To determine the genetic structure of a population, what must be ascertained is the extent to which reproduction departs from panmixia and approaches clonal reproduction of genomes. *P. falciparum*, physiologically, is an obligate sexual organism, because fusion of gametes, zygote formation, and meiosis must occur in the mosquito gut for the parasite's life cycle to be completed. But, with respect to its population structure and evolutionary pattern, what needs to be determined is the extent of the parasite's genetic clonality relative to panmixia, because genetic recombination impacts the genetic makeup of genomes; physiological sexuality, by itself, does not. The genetic composition and evolutionary change of a population is of great import to ascertain the ecophysiology of pathogens, host–pathogen interactions, and the evolution of a pathogen's drug resistance.

We have sought to unravel the population structure of *P. falciparum* by investigating directly the genetic makeup of oocysts, where diploid genotypes can be determined. We carry out our investigation in a region of intense infectivity, where panmixia is often assumed to prevail. Two previous population-genetic investigations of *P. falciparum* in mosquito oocysts have considered only two (18) or three (8) gene loci and have not directly analyzed the causes of inbreeding. Moreover, the loci studied are clearly subject to selection by the host; selectively neutral gene markers are more suitable for settling the issues at hand, namely the extent of random relative to nonrandom mating (19). Herein,

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nonrandom distribution of genotypes among mosquito guts; thus,  $F_{ST}$  quantifies the differentiation between subpopulations (mosquito guts) in the total population (locality). Finally,  $F_{IT}$  measures overall inbreeding, resulting both from effects combined, i.e., deviations from panmixia among all oocysts from all mosquitoes in a given locality:  $F_{IT} = 1 - (1 - F_{IS})(1 - F_{ST})$ .

Linkage disequilibrium between pairs of loci is measured with the coefficient of correlation  $R$  generalized for the multiallelic case; the statistical significance of linkage disequilibrium is assessed with a randomization test. Genotypes at two loci are associated at random a number of times, and the log likelihood  $G$  test statistic is recalculated on the randomized data set. Because this procedure was repeated on all pairs of loci, we have applied a Bonferroni correction to the  $P$  values ( $P$  value times the number of tests).  $F$  statistics and linkage disequilibrium analyses are performed with FSTAT, version 2.9.4 (23).

## Results

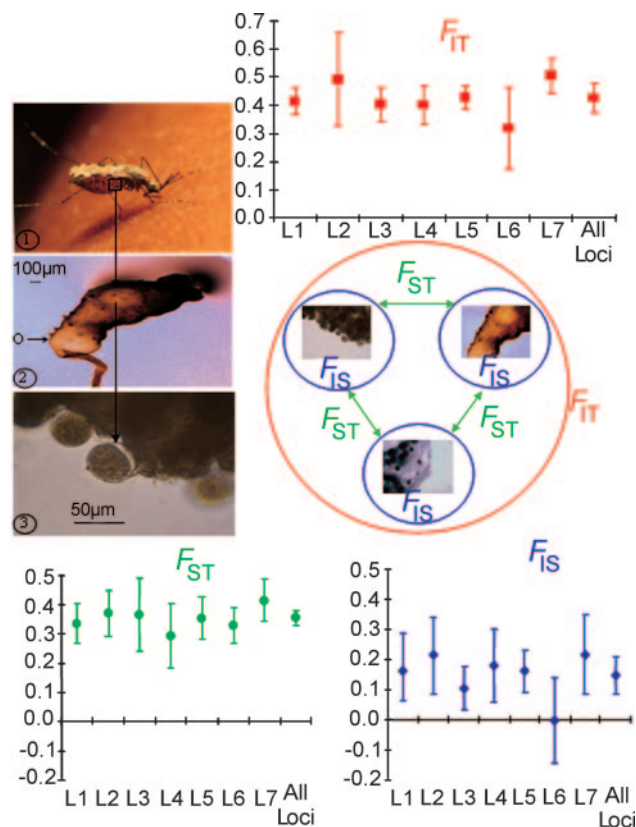
We captured 4,602 mosquitoes in 11 villages from western Kenya (Fig. 1) during 2002 and 2003, of which 181 (3.9%) mosquitoes were infected by *P. falciparum* oocysts, with  $6.0 \pm 0.7$  oocysts per infected mosquito (Table 1), values similar to those obtained in previous studies of the same region (24, 25). The allele frequencies (Table 2) are based on 613 oocysts from 145 mosquitoes; some oocysts were lost during extraction from the mosquito guts.

The mean parasitic load is  $m = 0.24$ , with a variance  $s^2 = 4.58$ , values that imply a highly aggregated distribution of the parasites as measured by the coefficient  $\beta = s^2/m = 19$ . This positive aggregation reflects that most oocysts are contained in few mosquito individuals, whereas most mosquitoes remain uninfected.

Table 2 displays the allele frequencies at the seven microsatellite loci in the 11 Kenyan populations. The frequency data for 2002 and 2003 have been combined; there is no significant difference in allele frequencies between the 2 years. A total of 613 oocysts were genetically analyzed, obtained from 145 infected mosquitoes, an average of 4.23 oocysts per mosquito. This average is lower than the average of 6.0 oocysts, observed in 181 mosquitoes in the full sample (Table 1), because some oocysts were lost during dissection. In 60 of the 145 mosquitoes, only one oocyst was successfully analyzed (although some of the 60 mosquitoes harbored more than one oocyst).

The average number of alleles per locus is  $16.3 \pm 2.1$ , ranging from seven alleles (*Pfg377*) to 23 (*POLYa*) at each locus (Table 2). High allele diversity is typically observed at microsatellite loci in regions of perennial malaria transmission (5). The average observed heterozygosity ( $H_{obs} = 0.43 \pm 0.03$ ) per population (sites; Fig. 1) is much lower than expected ( $H_{exp} = 0.80 \pm 0.05$ ). This discrepancy is approximately similar for each locus separately and for each population with a sample of 10 or more oocysts. The large disparity between observed and expected heterozygosity indicates a considerable amount of inbreeding, even in this region of high infectivity.

We use  $F$  statistics for further exploring the pattern of inbreeding in these populations (Fig. 2). If we treat all oocysts as members of one population, the departure from panmixia is 0.46. Little of this departure can be attributed to the pooling of localities and years (Wahlund effect): the inbreeding within each village and year is  $F_{IT} = 0.43$  [95% confidence interval (CI) = 0.37–0.48],  $P < 10^{-4}$  for each locus separately, as well as for all loci. The inbreeding attributed to nonrandom distribution of genotypes among mosquitoes is also high,  $F_{ST} = 0.36$  (CI = 0.33–0.38;  $P < 10^{-4}$ ). Finally, the rate of inbreeding within a mosquito gut is  $F_{IS} = 0.147$  (CI = 0.062–0.209,  $P < 10^{-4}$ ). We estimate the rate of selfing within each mosquito gut as  $s = 0.25$  (CI = 0.16–0.32;  $P < 10^{-4}$ ), where  $s = 2F_{IS}/(1 + F_{IS})$ . This value is based on 553 oocysts from 85 mosquitoes (60 mosquitoes each with only one oocyst are not included in the calculation of  $F_{IS}$ ).



**Fig. 2.** Inbreeding statistics for *P. falciparum* oocysts collected from *A. gambiae* mosquitoes. L1 to L7 refer to the seven microsatellite loci sequentially listed in Table 2.  $F_{IS}$  measures the deficiency of heterozygous genotypes as a result of self fertilization within individual mosquito guts;  $F_{ST}$  measures the deficiency of heterozygotes resulting from nonrandom distribution of oocyst genotypes among mosquito guts;  $F_{IT}$  is the overall deficiency of heterozygotes in a population, resulting from both effects combined. Photographs are of (1) a bloodfeeding *A. gambiae* female (adapted with permission from Ekisei Sonoda), (2) a mosquito midgut with oocysts (O), and (3) oocysts encysted in a mosquito midgut.

Linkage disequilibrium is significant for all 21 pairs of loci,  $R = 0.50$ – $0.60$  between any two loci,  $P < 0.05$  for each of the 21 pairs, with the Bonferroni correction. This cannot be attributed to close physical linkage between loci, because the seven loci are distributed among five different chromosomes, so that all but two pairs among the 21 involve loci located on different chromosomes (moreover, the loci on the same chromosome are far apart, 45.8 cM for the pair on chromosome 12 and 36.5 cM for the pair on chromosome 6). Rather, linkage disequilibrium is a consequence of inbreeding, which reduces the effectiveness of recombination because of selfing and strong relatedness among oocysts within each mosquito. That is, multilocus genotypes within a given gamete are transmitted together even when loci are located on different chromosomes (26, 27).

## Discussion

Rich *et al.* (26) investigated the highly polymorphic circumsporozoite (*Csp*) gene in 25 DNA sequences from the worldwide distribution of *P. falciparum* and conjectured that the reproduction of the parasite is prevalently clonal, that is, that haplotypes predominantly reproduce as units, with rare recombination events between loci. Conway *et al.* (28) concluded that meiotic recombination is frequent in African populations of *P. falciparum* because linkage disequilibrium in the large (5.1-kb) *msp1* gene decreases with distance along the gene. This evidence, however, is far from conclusive, because

*msp1* contains several repetitive regions, including short DNA sequences repeated in tandem, where intragenic recombination occurs independently of meiosis, at frequencies orders of magnitude higher than the mutation rate; linkage disequilibrium between sites along the gene may arise as a consequence of intragenic recombination (16, 27). Further, the results do not imply that recombination is effectively breaking haplotype structure. Indeed, strong linkage disequilibrium has been observed between physically unlinked markers (29), as well as significant deficit of heterozygous oocysts in populations of Tanzania (18) and Papua New Guinea (8), where overall inbreeding coefficients have been estimated at 0.34 and 0.90, respectively (12). Anderson *et al.* (5) observed significant linkage disequilibrium among 12 microsatellite loci in all localities with low infectivity and in two of five populations from regions with high transmission frequencies. Other results have been interpreted as indicating that *P. falciparum* reproduction may be genetically clonal in low-infectivity regions but predominantly panmictic in high-infectivity regions (13, 14). Nevertheless, Durand *et al.* (30) found significant linkage disequilibrium in two African populations from the Republic of the Congo, where infectivity is high.

We sought to provide additional insight into the question whether recombination is effective in high-infectivity African populations by investigating the genetic makeup at seven microsatellite loci of *P. falciparum* in mosquito oocysts, the only stage of the parasite's life where diploidy and the immediate products of meiosis can be observed. The Kenya populations of *P. falciparum* harbor ample genetic variation, such as is typical for regions with intense perennial transmission of malignant malaria. The average number of alleles at the seven microsatellite loci is  $16.3 \pm 2.1$ , and the average expected heterozygosity per locus in a population is huge,  $0.80 \pm 0.05$ , with relatively little variation from one to another population among the eight populations for which at least 10 oocysts have been sampled. The observed heterozygosity, however, is much lower,  $0.43 \pm 0.03$ , indicating that there is a considerable

amount of inbreeding. Evidence of inbreeding exists within each mosquito with two or more oocysts ( $F_{IS} = 0.147 \pm 0.031$ ). There is considerable inbreeding as well when the oocysts from each mosquito gut are considered as a subpopulation ( $F_{ST} = 0.355 \pm 0.013$ ) and also within each locality ( $F_{IT} = 0.425 \pm 0.026$ ). Quite relevant for our purposes is that linkage disequilibrium is significant for all 21 pairwise comparisons between seven genetic markers located in five different chromosomes. This implies that the reproduction of *P. falciparum*, even in this African region of perennial infection, is prevalently clonal. A contributing factor is the observed high rate of self fertilization ( $s = 0.25$ ).

Reduced genetic recombination is consistent with the observation that linkage disequilibrium is associated with the haplotypic spread of chloroquine resistance in diverse populations of *P. falciparum* from two foci in Asia and South America (31). Current models of the spread of resistance to antimalarial drugs have shown that a positive association between the intensity of transmission and the degree of the parasite's inbreeding may be involved (32–34); low rates of inbreeding may lead to a slow spread of resistance in regions with high infectivity (33, 34). Be that as it may, accurate knowledge of the population genetic structure of *P. falciparum* is of great import for the successful control of malignant malaria. Although a variety of population structures may prevail in different regions, as a consequence at least in part of the rates of malaria transmission, it must be kept in mind that a clonal population structure of *P. falciparum* may prevail in regions of perennial transmission as well as in regions of low infectivity.

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