Microsatellite Markers Reveal a Spectrum of Population Structures in the Malaria Parasite *Plasmodium falciparum*

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Multilocus genotyping of microbial pathogens has revealed a range of population structures, with some bacteria showing extensive recombination and others showing almost complete clonality. The population structure of the protozoan parasite *Plasmodium falciparum* has been harder to evaluate, since most studies have used a limited number of antigen-encoding loci that are known to be under strong selection. We describe length variation at 12 microsatellite loci in 465 infections collected from 9 locations worldwide. These data reveal dramatic differences in parasite population structure in different locations. Strong linkage disequilibrium (LD) was observed in six of nine populations. Significant LD occurred in all locations with prevalence <1% and in only two of five of the populations from regions with higher transmission intensities. Where present, LD results largely from the presence of identical multilocus genotypes within populations, suggesting high levels of self-fertilization in populations with low levels of transmission. We also observed dramatic variation in diversity and geographical differentiation in different regions. Mean heterozygosities in South American countries (0.3–0.4) were less than half those observed in African locations (0.76–0.8), with intermediate heterozygosities in the Southeast Asia/Pacific samples (0.51–0.65). Furthermore, variation was distributed among locations in South America (F_{ST} = 0.364) and within locations in Africa (F_{ST} = 0.007). The intraspecific patterns of diversity and genetic differentiation observed in *P. falciparum* are strikingly similar to those seen in interspecific comparisons of plants and animals with differing levels of outcrossing, suggesting that similar processes may be involved. The differences observed may also reflect the recent colonization of non-African populations from an African source, and the relative influences of epidemiology and population history are difficult to disentangle. These data reveal a range of population structures within a single pathogen species and suggest intimate links between patterns of epidemiology and genetic structure in this organism.

Introduction

Multilocus genotyping has been used extensively to investigate the genetic structure of bacterial pathogens in the past 20 years. This approach superseded genetic typing systems utilizing surface antigens and provided many fundamental insights into the spread of epidemics, the extent of recombination, and the degree of population differentiation in bacterial pathogens (Caugant et al. 1986; Maynard-Smith et al. 1993; Haubold et al. 1998; McGee, Koornhof, and Caugant 1998; Feil et al. 1999; Souza et al. 1999). In particular, this approach demonstrated that while some bacteria, such as *Neisseria gonnhorrea*, show high levels of recombination, others, such as *Escherichia coli* and Salmonella, have a predominantly clonal population structure. Classical multilocus approaches have not been widely applied to the protozoan pathogen *Plasmodium falciparum*, the causative agent of the most pathogenic of the human malarias. In fact, previous work on the molecular population genetics of this parasite have utilized a limited number of surface antigen loci, such as merozoite surface antigens and circumsporozoite surface proteins (Paul et al. 1995; Babiker et al. 1997). These proteins form the basis for a number of candidate malaria vaccines, and there is abundant evidence from patterns of substitution that these proteins are under strong natural selection (Hughes 1992; Hughes and Hughes 1995). Interpretation of population structure using data derived from these loci is problematic, since it is not clear whether the patterns observed reflect population history or natural selection. As a consequence, there is currently uncertainty about many aspects of the population genetics of *P. falciparum.*

Plasmodium falciparum is a hermaphroditic protozoan, with haploid asexual replication in the human host and a brief diploid sexual phase in the mosquito vector. Haploid parasites divide mitotically in the human host, and some cells differentiate into male and female stages. Male and female gametes fuse in the mosquito host to form a short-lived diploid zygote. Meiotic divi-

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sion then gives rise to haploid cells that develop into infective sporozoites, which migrate to the mosquito salivary glands and infect humans during mosquito bloodfeeding. Fusion of male and female gametes from the same clone (selfing) results in no effective recombination, while fusion of gametes from different clones (outcrossing) may result in recombination. While Plasmodium has a well-established sexual phase in its life cycle and genetic crosses have been performed (Walker-Jonah et al. 1992; Su et al. 1999), there is ongoing discussion about the level of effective recombination in natural malaria populations (Rich et al. 1998; Conway et al. 1999). Rich et al. (1998) used substitution patterns in the circumsporozoite antigen to argue that *P. falciparum* populations are predominantly clonal (Rich, Hudson, and Ayala 1997). This conclusion was based on the absence of any decay in linkage disequilibrium (LD) with distance across the locus studied. This claim was clearly refuted by Conway et al. (1999), who showed a rapid decay in LD with physical distance along a chromosome and argued for high levels of recombination, at least in African locations. However, the situation is by no means clear: while some authors have observed no evidence for LD between physically unlinked antigen loci (Babiker et al. 1994; Paul et al. 1995), others have reported strong LD (Abderrazak et al. 1999). Other aspects of population structure are also debated. Data on levels of geographical genetic differentiation among parasite populations are conflicting, with two antigen loci (MSP-1 and MSP-2) indicating low levels of global genetic structure ($F_{ST} < 0.2$) (Conway 1997) and a third locus, the gametocyte surface antigen pfs48/45, suggesting strong subdivision ($F_{ST} > 0.7$) (Drakeley et al. 1996). Once again, interpretation of these data is hampered by the use of small numbers of strongly selected loci.

To clarify our understanding of *P. falciparum* population genetics, we employed microsatellite genotyping. Recently, microsatellite markers have been shown to be extremely widespread in *P. falciparum*, occurring every 2–3 kb throughout the genome (Su and Wellems 1996; Su et al. 1999). We adapted 12 loci bearing trinucleotide repeats for use with the minimal amounts of template present in *P. falciparum*–infected blood samples (Anderson et al. 1999) and used these loci to measure allelic variation in 465 *P. falciparum*–infected blood samples collected from nine different locations worldwide. The samples were collected from regions with high levels of transmission in Africa and Papua New Guinea, and also from areas with low levels of transmission in Thailand and in three countries in South America. For all samples, we constructed infection ''haplotypes'' using the predominant allele present in the PCR products from each locus, while we also documented the frequency of infections containing multiple clones. These data clearly reveal a spectrum of population structures in a single parasite species. Strong LD, low diversity, and extensive population differentiation are seen in regions with low levels of transmission, while linkage equilibrium, high diversity, and low levels of differentiation are observed in regions with high levels of transmission.

Materials And Methods

Collection of Parasite Samples

Blood samples were collected from people infected with *P. falciparum* from nine different locations, including three African countries (Uganda, Democratic Republic of Congo, and Zimbabwe), three South American countries (Colombia, Bolivia, and Brazil) and three sites in the Asia/Pacific region (Thailand and two villages in Papua New Guinea). The population samples from South America and Thailand were from areas with low levels of transmission (<1% infection rate), while the populations from Africa and Papua New Guinea were from regions with higher levels of transmission (>1%–80% prevalence). In eight locations, blood samples were obtained by piercing the skin with a sterile lancet and absorbing a drop of blood (≅50 μl) on a piece of filter paper. These samples were then air-dried and stored at room temperature. At one site (Brazil), blood samples were collected by vacutainer and frozen. Further details about the study sites and sampling protocols for each location are given below.

In Uganda, *P. falciparum*–infected blood samples were collected (April 1996–June 1997) from HIV-infected individuals living in a 15-km radius of a clinic near Entebbe. This area is hyperendemic for malaria, with ≈10% of adults being blood-slide-positive (one or more parasites observed in fields containing 200 white blood cells). In the Democratic Republic of Congo, blood samples were obtained from asymptomatic pregnant women visiting a clinic in Kimpese in 1993. This area is holoendemic for malaria: 70% of women in their first pregnancy were slide-positive for *P. falciparum*, while 13% of multigravidae were slide-positive (Jackson et al. 1991). In Zimbabwe, blood samples were collected in April and May 1998 from symptomatic patients visiting health clinics in the Mutare and Mutasa districts near the border with Mozambique. In this region, transmission is seasonal with a prevalence of 2%–4.6%. In Papua New Guinea, blood samples were collected during cross-sectional surveys in Mebat, Madang Province (November 17 and December 16, 1997) and in Buksak, 80 km away (September 22 and December 8, 1997). In Mebat 38% of samples were slide-positive, while in Buksak, 51% were positive. In Thailand, samples were collected between December 15, 1997, and January 11, 1998, from symptomatic patients in Shoklo, Tak Province. A survey in January 1998 showed a prevalence of 0.6%. In Colombia, samples were collected from symptomatic individuals visiting the clinic in El Bagre (Province of Antioquia). In Bolivia, blood samples were collected from symptomatic individuals during an outbreak in May–June 1994 in Guayaramerín (Department of Beni). In Brazil, 22 infected blood samples were obtained from symptomatic individuals visiting the clinic in Porto Velho, Rondônia, in July–September 1998, while a further 10 samples were obtained in July 1997. The sites in Bolivia and Brazil are approximately 350 km apart. For South American locations, we used Pan American Health Organization incidence data for *P. falciparum* to estimate cross-sectional prevalence. This was

done by dividing the yearly number of reported cases by the population size and dividing the resulting figure by 12 (duration of infection was estimated at 1 month). For Porto Velho, Brazil, in 1997, 4,426 cases were observed in a population of 294,327, giving an estimated prevalence of 0.125%. In Guayanamerín, Bolivia, in 1997, 1,644 cases were observed in a population of 44,950, giving an estimated prevalence of 0.305%. In El Bagre, Colombia, in 1998, 3,391 cases were observed in a population of 50,204, giving an estimated prevalence of 0.563%.

Scoring of Microsatellite Length Variation

In eight locations, parasite DNA was prepared from finger-prick blood samples (≅50 μl) absorbed onto Whatman filter paper using a chelex extraction protocol (Wooden, Kyes, and Sibley 1993), and re-eluted in 100 μl TE. Brazilian DNA samples were prepared from 200 μl of whole blood. Levels of parasite infection vary by two or three orders of magnitude between infected individuals and are frequently very low. As a result, levels of parasite DNA template available for PCR are also variable and may be as low as 1 pg. We used a two-round hemi-nested PCR strategy to amplify microsatellite loci from *P. falciparum*, and fluorescently labeled PCR products were sized on polyacrylamide gels by comparison with internal size standards. For samples from Buksak (PNG), primers were end- labeled with γ-P^{32}, and products were sized by reference to M13 sequence ladders. Primers, PCR conditions, primer specificity, and reproducibility of the techniques used have previously been described (Anderson et al. 1999). The 12 loci used are distributed throughout the *P. falciparum* genome. The loci are Polyα (Chr4), TA42 (Chr5), TA81 (Chr5), TA1 (Chr6), TA109 (Chr6), TA87 (6), TA40 (Chr10), 2490 (Chr10), ARAII (Chr11), pfG377 (Chr12), PfPk2 (12), and TA60 (Chr13). Four loci (Polyα, ARAII, pfG377, and PfPk2) are in coding sequences from GenBank. The other eight loci were drawn from a genomic library (Su and Wellems 1996), and their function is unknown. We used GENESCAN and GENOTYPER software (Applied Biosystems) to automate measurement of allele length and to quantify peak heights. We discarded data from samples that amplified poorly for particular loci (maximum peak height < 200 fluorescent units).

Measurement of Diversity, Effective Population Size, and Geographical Structure

We measured allele frequencies using only the predominant allele present at each locus within each infection. The predominant allele at each locus was defined as the highest peak in electropherogram traces. This procedure results in unbiased estimation of allele frequencies within a population, if we assume the composition of PCR products is representative of the composition of templates. We measured expected heterozygosity (H) at each locus in each location as $H = [n/(n - 1)][1 - \Sigma_{i=1}^{n} p_i^2]$, where n is the number of infections sampled and p_i is the frequency of the ith allele. We also measured the variance in allele size (V_{SZ}) and counted the number of alleles (A) at each locus in the nine populations. We compared levels of diversity in different populations using ANOVA or nonparametric tests.

We estimated effective population size using observed H and mutation rate estimates. We used a microsatellite mutation rate (μ) for *P. falciparum* of 1.59×10^{-4} (95% confidence interval: 6.98×10^{-5}, 3.7×10^{-4}). This was estimated from the observation of five unique nonparental alleles in 35 progeny of a genetic cross that were genotyped for 901 microsatellite loci (Su et al. 1999). Confidence intervals were estimated from chi-square tables using standard methods for distribution of Poisson-distributed variables (Johnson, Kotz, and Kemp 1992). Since 5 of the 12 loci (Polyα, TA42, TA1, TA109, and TA40) used in the present study show patterns of variation which are inconsistent with a pure stepwise mutation model (Anderson et al. 2000*b*), we used estimates based on both the infinite-alleles model (IAM) and the stepwise mutation model (SMM). For IAM, we used the relationship $N_e\mu = H/4(1 - H)$, while for SMM we used the relationship $N_e\mu = \frac{1}{8}\{[1/(1 - H)]^2 - 1\}$ (Schug, Mackay, and Aquadro 1997).

We measured population subdivision using Weir and Cockerham's (1984) unbiased estimator of Wright's F statistics, while confidence intervals were estimated by bootstrapping over loci 10^4 times using the program GDA (Lewis and Zaykin 2000). We examined correlations between pairwise values of genetic divergence (θ) and geographical distance between locations using the Mantel test. The significance of the observed correlation was estimated by permuting the order of taxa in the data matrices 10^5 times and computing the frequency with which the correlation observed between the permuted data sets was greater than or equal to that observed between the original data sets. We measured Nei's (1978) genetic distance between parasite populations while we examined the genetic relationships among individual parasite haplotypes by counting the proportion of alleles shared between 12-locus haplotypes (*Ps*) and using the measure ($1 - Ps$) as a simple distance measure (Bowcock et al. 1994). All trees were constructed using PHYLIP (Felsenstein 1993).

Assessment of Multiple Infections

Blood samples are frequently infected with two or more haploid clones of *P. falciparum*, resulting in the detection of two or more alleles at polymorphic loci. This may result from superinfection and therefore provides a surrogate indicator of the level of transmission within populations, as well the opportunity for recombination between unlike malaria clones (Hill and Babiker 1995). We scored multiple alleles per locus if minor peaks were >33% the height of the predominant allele present for each locus. Multiple infections were defined as those in which at least one of the 12 loci contained more than one allele. This method has the advantage of being very simple. However, when populations differ in levels of heterozygosity, this method may be biased, since multiple infections are easy to detect in popula-

Table 1
Patterns of Diversity in Nine Malaria Populations

Population	$n \pm$ SE	$A \pm$ SE	$H \pm$ SE	$V_{SZ} \pm$ SE
South America				
Colombia	29.67 ± 0.33	2.42 ± 0.34	0.30 ± 0.08	14.24 ± 6.92
Bolivia	31.50 ± 0.38	2.17 ± 0.21	0.37 ± 0.06	28.66 ± 16.72
Brazil	32.17 ± 0.44	2.50 ± 0.29	0.40 ± 0.05	25.77 ± 12.12
Thailand				
Shoklo	36.08 ± 0.36	4.92 ± 0.57	0.51 ± 0.08	36.30 ± 8.97
Papua New Guinea				
Buksak	61.67 ± 0.14	6.00 ± 0.71	0.62 ± 0.07	62.32 ± 25.74
Mebat	57.58 ± 0.19	6.58 ± 0.61	0.65 ± 0.06	63.15 ± 27.99
Africa				
Uganda	91.08 ± 0.29	10.00 ± 1.11	0.76 ± 0.05	102.28 ± 40.48
Congo	52.00 ± 0.28	10.17 ± 0.86	0.80 ± 0.04	98.02 ± 32.27
Zimbabwe	65.75 ± 0.45	10.67 ± 0.97	0.80 ± 0.02	105.24 ± 39.62

NOTE.—A = mean number of alleles per locus; H = effective heterozygosity; V_{SZ} = variance in allele length. The sample size (n) is averaged over all loci. Samples sizes for each locus are shown in the appendix.

tions with high levels of heterozygosity and more difficult to detect in populations with low heterozygosity. We therefore also used a maximum-likelihood procedure (Hill and Babiker 1995) to estimate the mean number of multiple infections using data from each of the 12 loci. For this analysis, we assumed a positive Poisson distribution of parasite clones among hosts (Hill and Babiker 1995).

Multilocus Linkage Disequilibrium

We used the predominant allele detected at each locus to construct ''infection haplotypes.'' Where blood samples contain a single parasite clone this, results in recovery of true parasite haplotypes. Where two or more clones are present, the infection haplotypes may be a composite of alleles from two or more clones. This may impose additional recombination on the data and bias the data against detection of LD. We conducted analyses of both the complete data set and a curtailed data set in which multiple infections (see above) were removed. We used a permutation procedure to test the null hypothesis of random association among loci for each parasite population (Souza et al. 1992; Haubold et al. 1998). The program LIAN, version 3 (Haubold and Hudson 2000), was used to compute the number of alleles shared (D) between all pairwise comparisons of complete 12-locus haplotypes and to measure the variance of this distance measure (V_D). To investigate if the observed data differed from random expectations, we compared the observed V_D with the distribution of V_D values in 10,000 simulated data sets in which alleles at each locus were randomly reshuffled among genotypes. Significant LD was detected if the observed V_D was >95% of the values generated in the reshuffled data sets. We used the index of association (I_A) to measure the strength of LD. The ''classical'' I_A was defined as $I_A = (V_D/V_e - 1)$, where V_e is the mean variance of the reshuffled data sets (Maynard-Smith et al. 1993). However, since this statistic scales with $r - 1$, where r is the number of loci analyzed (Hudson 1994), we used a ''standardized'' I_A statistic (I_A^S), calculated as $I_A^S = (V_D/V_e - 1)/(r - 1)$.

Inferring Population History

We used Goldstein's $(\delta\mu)^2$ (Goldstein et al. 1995) distance, which is related linearly to time, to estimate divergence times between parasite populations. For this purpose, the five ''complex'' loci (Anderson et al. 2000*b*) that show deviations from SMM were excluded (see above). The divergence time between populations was estimated using the relationship $(\delta\mu)^2 = 2\mu t$, where μ is the mutation rate and t is the number of generations elapsed since divergence (Goldstein et al. 1995). The mutation rate estimation used in this calculation is described above.

Results

The complete data set comprised multilocus genotypes from 465 malaria-infected blood samples. There were 415 samples with full 12-locus genotypes, while in the remaining 50 samples, genotype data from more than eight loci were available. Sample sizes ranged from 30 to 92 for each location. Numbers of alleles per locus in the total sample ranged from 5 (locus 2490) to 24 (Polyα). Allele frequencies and sample sizes are shown in the appendix.

Genetic Diversity

Levels of genetic diversity showed dramatic heterogeneity among locations (table 1). All three measures of diversity showed the highest diversity in African locations, intermediate levels of diversity in samples from Papua New Guinea and Thailand, and the lowest diversity at the three South American sites. Up to 18 alleles per locus were found within African locations (in Polyα in Zimbabwe and Uganda), while a maximum of five alleles per locus were observed within South American locations (also in Polyα). Similarly, H ranged from 0.8 in Zimbabwe and Congo to a minimum of 0.3 in Colombia. Variance in allele size was also greater in African locations, and the heterogeneity among populations was highly significant.

Table 2
Estimates of Effective Sizes (N_e) of *Plasmodium falciparum* Populations

	IAM		SMM	
South America				
Colombia ..	682	(293, 1,554)	830	(357, 1,891)
Bolivia	917	(394, 2,089)	1,185	(509, 2,699)
Brazil	1,051	(452, 2,394)	1,402	(603, 3,194)
Thailand				
Shoklo	1,653	(710, 3,766)	2,523	(1,084, 5,746)
Papua New Guinea				
Buksak	2,586	(1,111, 5,891)	4,713	(2,025, 10,736)
Mebat	2,931	(1,259, 6,676)	5,662	(2,433, 12,898)
Africa				
Uganda	4,900	(2,106, 11,162)	12,535	(5,387, 28,555)
Congo	6,137	(2,637, 13,980)	18,114	(7,784, 41,263)
Zimbabwe .	6,491	(2,789, 14,786)	19,888	(8,547, 45,304)

NOTE.—Estimates of N_e are based on mean effective heterozygosity and the estimated mutation rate (see *Materials and Method*) and assume either an infinite-alleles model (IAM) or a stepwise mutation model (SMM) of microsatellite evolution. The values in parentheses are the estimates obtained when the upper and lower confidence intervals for the mutation rate are used to estimate N_e.

We used observed levels of *H* and mutation rates estimated from a genetic cross to compare the effective population sizes (N_e) (Schug, Mackay, and Aquadro 1997) of *P. falciparum* in different locations (table 2). Estimated levels of N_e were 10–24 times as high in African populations than in South American populations, depending of whether estimates were based on IAM or SMM.

Geographical Differentiation

There were striking differences in the distribution of genetic variation within continents. We calculated coancestry coefficients (θ) (Weir and Cockerham 1984) for all pairwise comparisons between populations (fig. 1). Parasites from three African sites separated by >2,000 km either were indistinguishable or showed very low levels of differentiation. In contrast, parasite populations from Bolivia, Brazil, and Colombia, which are separated by comparable geographical distances, were highly differentiated. To phrase this in a different way, the majority of the observed variation was found within locations in Africa and Papua New Guinea, while in South America, variation was distributed between sites. Mantel tests (Manly 1994) revealed a significant correlation between genetic differentiation (θ) and geographical distance ($r = 0.4835$, $P = 0.00047$), consistent with an isolation-by-distance model of *P. falciparum* population structure. Population trees support these findings (fig. 2). African populations form a closely related cluster with strong bootstrap support, as do populations from Papua New Guinea and Thailand. However, the Colombian population is well differentiated from the other two South American populations, and there is only weak support for a monophyletic South American clade. We also used a distance-based approach (Bowcock et al. 1994) to inspect the relationships between individual parasite haplotypes. We observed strong clustering of haplotypes by location of origin (fig. 3). The tree reveals a dramatic contrast between South American populations, in which multiple alleles are shared between haplotypes and branch lengths are short, and other populations in which few alleles are shared and branch lengths are long.

Multiple Infection

Figure 4 summarizes patterns of infection with multiple clones in different locations. Both the propor-

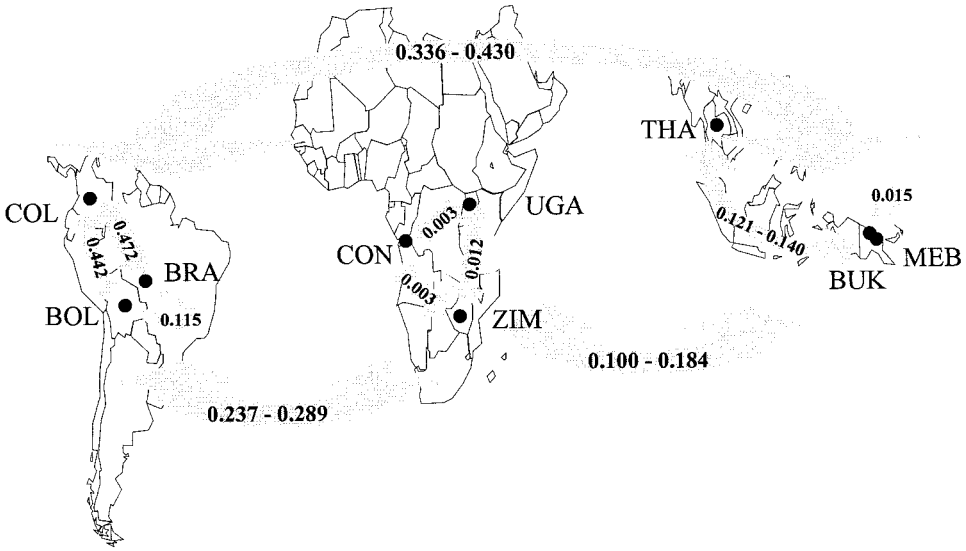

FIG. 1.—Population differentiation in *Plasmodium falciparum.* The numbers marked on the arrows describe levels of genetic differentiation measured using coancestry coefficients (θ) between parasite populations. Ranges of values of θ are shown in comparisons between groups of populations from different geographical regions. Bootstrapping revealed significant ($P < 0.001$) differentiation between populations in all but 1 of the 36 pairwise comparisons. No significant differentiation was observed between the Uganda and Congo populations. Furthermore, no significant differentiation was observed between samples collected from Porto Velho, Brazil, in successive years or among samples collected from Mutasa and Mutare in Zimbabwe. Populations from Brazil and Zimbabwe are therefore treated as single populations in all analyses.

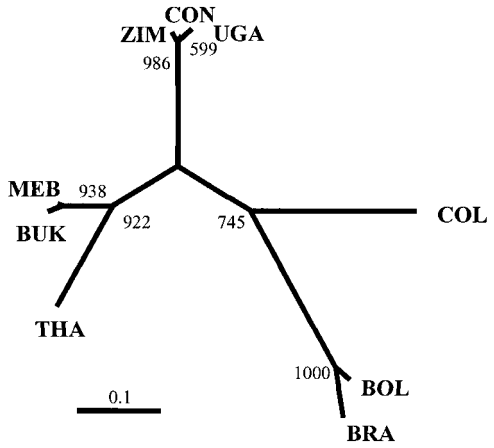

FIG. 2.—Unrooted neighbor-joining tree showing the relationships between the nine parasite populations. Bootstrap support (1,000 replications) for the nodes are shown, while abbreviated population names are shown at the branch tips: Congo (CON), Uganda (UGA), Zimbabwe (ZIM), Colombia (COL), Bolivia (BOL), Brazil (BRA), Mebat (MEB), Buksak (BUK), and Thailand (THA). The tree shown is based on Nei's (1978) distance; trees based on allele sharing and chord distances give identical topologies.

tion of infections bearing multiple clones and the maximum-likelihood estimates of the mean numbers of clones per locus show substantial variation among locations. Both measures are strongly correlated (r^2 = 0.924). Less than 20% of samples from South American countries contained multiple infections; intermediate levels were observed in Thailand, while in Papua New Guinea and Africa, >45% of samples contained multiple clones. Numbers of multiple infections were significantly lower in South America and Thailand, where prevalence was <1%, than in Africa and Papua New Guinea, where infection prevalences were >1% and frequently >50%. Since detection of multiple alleles per locus depends critically on the sensitivity of the methods used, we emphasize that these data provide relative, rather than absolute, measures of multiple infection.

Linkage Disequilibrium

We used a Monte Carlo method (Haubold et al. 1998) to test the significance of LD both for the complete data set, containing data from both single-clone and multiclone infections, and for a curtailed data set from which infections containing multiple clones were removed (table 3). Both data sets gave very similar results, although the deviations from random expectations were marginally lower for the full data set (including multiple infections) than for the curtailed data set (multiple infections excluded) in seven of nine populations. Highly significant departures from random association were observed in six of the nine populations for both data sets. Significant LD was found in all regions with low transmission: on the Thai-Burmese border and in the three South American locations. Significant LD was also observed in Buksak (Papua New Guinea) and Zimbabwe, while there was no evidence for LD in Mebat (Papua New Guinea), Congo, or Uganda. Visual inspection of the data reveals one obvious cause of the observed LD. Twenty-seven multilocus genotypes are represented more than once in the data set. Multiply represented genotypes were most common in those locations with low levels of transmission. In both Bolivia and Brazil, six genotypes were represented between two and four times, while in Colombia, five genotypes were recovered from up to seven different hosts. Similarly, in Thailand, four identical clones were observed and one genotype was found in six different individuals. These identical genotypes persisted in both time and space. In Brazil, samples were collected in both 1997 and 1998, and three multilocus genotypes were found in both years of sampling. Furthermore, one of these genotypes was also found in Bolivia in 1994. We also observed some identical 12-locus genotypes in regions with relatively high levels of transmission. In Mebat (Papua New Guinea), three pairs of individuals contained identical genotypes, while in both Buksak (Papua New Guinea) and

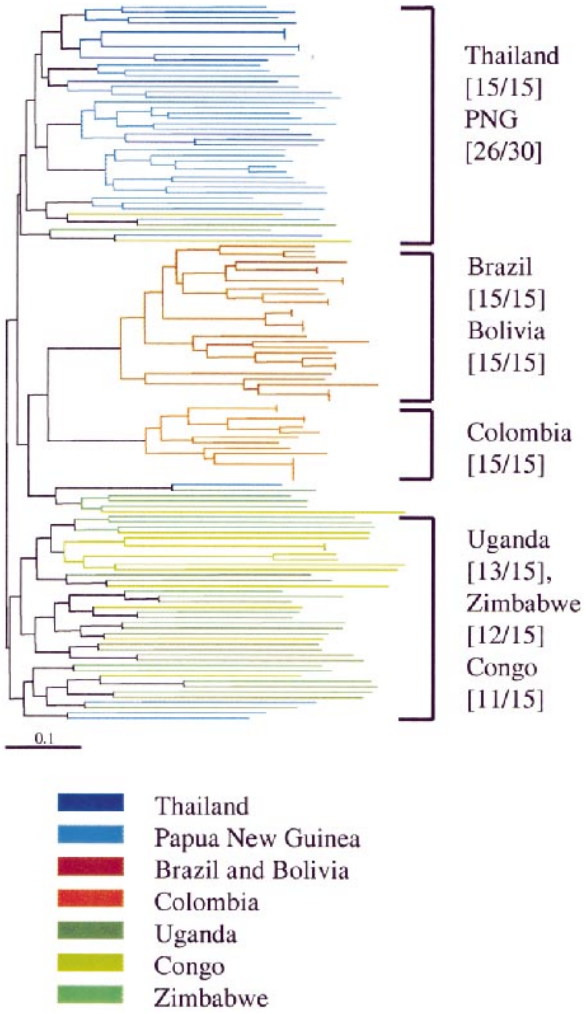

FIG. 3.—Midpoint rooted neighbor-joining tree showing the relationships between *Plasmodium falciparum* haplotypes from nine different locations (see *Materials and Methods* for details of distance measure). Fifteen randomly chosen haplotypes from each location are included, while branches are colored to show the origin of the parasites. Terminal branches of zero length mark identical haplotypes. The numbers in square brackets describe the proportion of parasites from a particular country that are found together in one cluster in the tree.

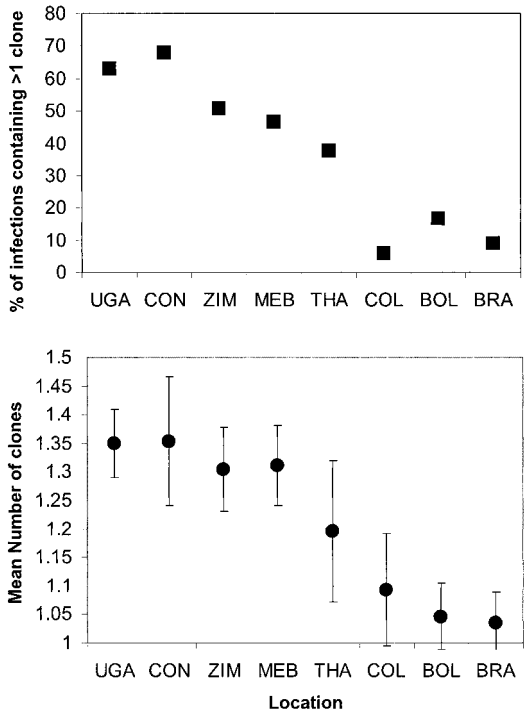

FIG. 4.—Summary of patterns of multiple infection in *Plasmodium falciparum*–infected blood samples from nine locations. *A*, The proportion of infections containing more than one parasite clone. *B*, The estimated mean number of clones per sample; the error bars represent standard deviations of the estimates derived from the 12 loci. The criteria used to score numbers of alleles per locus are described, and the statistical methods used to estimate clonal carriage are summarized in the *Materials and Methods* section. Data from Buksak were excluded from this analysis, since radioactive, rather than fluorescent, detection of alleles was used (see *Materials and Methods*). As such, the results are not directly comparable.

Zimbabwe, one genotype was recovered in three different individuals. When only unique multilocus genotypes were analyzed, significant LD remained only in populations from Bolivia and Zimbabwe.

Population History

Table 4 shows pairwise measurements of $(\delta\mu)^2$ between populations from South America and Africa and corresponding estimates of divergence time. Using the point estimate of microsatellite mutation rate, divergence times range from 385 to 1,101 years with a mean of 665. These estimates may be substantially altered by inaccuracies in the mutation rate estimate or by variation in mutation rate across loci. Using the upper and lower confidence intervals on the mutation rate, estimated divergence times range from 305 to 1,559 years.

Discussion

The microsatellite data reveal a spectrum of population structures within a single pathogen species. Strong LD, low genetic diversity, and high levels of geographical variation are observed in regions of low transmission, while random association among loci, high genetic diversity, and minimal geographical differentiation are observed in regions of Africa and Papua New Guinea, where transmission is intense. In the following paragraphs, we first discuss possible explanations for these patterns. First, we explore the reasons for the differences in levels of LD among populations. Second, we evaluate the importance of population history and disease ecology in determining the patterns of diversity and geographical structuring observed.

Linkage Disequilibrium

Significant deviations from random association among loci were observed in six of nine parasite pop-

Table 3
Multilocus Linkage Disequilibrium in Nine Malaria Populations

	All Infections		Single Clones		Unique Haplotypes Only	
Population	*n*	I_A^S	*n*	I_A^S	*n*	I_A^S
South America						
Colombia	26	0.0467****	22	0.0597****	13	0.0133
Bolivia	26	0.1084****	25	0.1123****	15	0.0368*
Brazil	26	0.0298**	26	0.0284**	17	0.0011
Thailand						
Shoklo	33	0.1180****	20	0.1328****	22	0.0071
Papua New Guinea						
Buksak	58	0.0149**	25	0.0480***	56	0.0073
Mebat	55	0.0076	31	0.0153	52	0.0055
Africa						
Uganda	83	0.0013	33	−0.0072	83	0.0013
Zimbabwe	58	0.0169****	30	0.0369****	56	0.0091*
Congo	48	−0.0046	17	0.0018	48	−0.0046

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.
**** $P < 0.0001$.

Table 4
Genetic Distances ($(\delta\mu)^2$) and Inferred Separation Times Between South American and African Populations

			Estimated Divergence Time (Years)		
		$(\delta\mu)^2$	$\mu = 1.59 \times 10^{-4}$	$\mu = 6.78 \times 10^{-5}$	$\mu = 3.47 \times 10^{-4}$
Bolivia	Congo	1.792 (0.651, 2.933)	939 (341, 1,537)	2,203 (800, 3,605)	430 (156, 704)
	Uganda	1.027 (0.591, 1.463)	538 (310, 767)	1,262 (726, 1,798)	247 (152, 351)
	Zimbabwe	1.331 (0.453, 2.209)	698 (237, 1,158)	1,636 (557, 2,715)	320 (109, 530)
Brazil	Congo	2.101 (1.115, 3.087)	1,101 (584, 1,618)	2,582 (1,370, 3,794)	505 (268, 741)
	Uganda	1.185 (0.853, 1.517)	621 (447, 795)	1,456 (1,048, 1,865)	285 (205, 364)
	Zimbabwe	1.466 (0.758, 2.174)	768 (397, 1,139)	1,802 (932, 2,672)	352 (182, 522)
Colombia	Congo	1.010 (0.024, 1.996)	529 (13, 1,046)	1,241 (29, 2,453)	243 (6, 479)
	Uganda	0.734 (0.402, 1.066)	385 (211, 559)	902 (494, 1,310)	176 (97, 256)
	Zimbabwe	0.773 (0.065, 1.481)	405 (34, 776)	950 (80, 1,280)	186 (16, 356)
Mean		1.269 (0.546, 1.992)	665 (286, 1,044)	1,559 (671, 2,488)	305 (131, 478)

Note.—Estimates of $(\delta\mu)^2$ are based on seven loci only. Values ± 1 SE are shown in brackets. Inferred separation times (±SE) are shown for the estimated mutation rate and for the lower and upper confidence intervals around the mutation rate estimation.

ulations using both the complete data set and the reduced data set from which multiple infections were removed. Maynard-Smith et al. (1993) have described a simple framework for evaluating the population structure of microbial pathogens. They distinguish between ''clonal'' organisms, such as Salmonella and *E. coli*, in which levels of recombination are insufficient to break down clonal lineages, and ''epidemic'' population structures of organisms such as *Neisseria meningitidis*, in which LD results from temporal expansion of particular clones in an otherwise sexual population. Epidemic population structures can be identified by treating multiply represented genotypes as single individuals and remeasuring LD. This procedure restores linkage equilibrium to four of the six malaria populations investigated; LD remained in populations from Zimbabwe and Bolivia. Hence, the *P. falciparum* populations studied here range from epidemic in low-transmission areas to panmixia in high-transmission areas.

In Bolivia, LD remains even when only unique genotypes are included in the data set. Two explanations are conceivable. The rate of recombination may be sufficiently low relative to mutation, such that LD is maintained. Alternatively, the populations may result from admixture with a genetically divergent parasite population, and insufficient time has passed for recombination to homogenize these two populations. We note that parasite populations in South America show strong differentiation over relatively small geographical distances, so admixture of populations may occur frequently. LD also remains in Zimbabwe, even when unique genotypes are analyzed. This is surprising, given that we observe very high levels of multiclone infection in this region, suggesting relatively high levels of transmission. The Zimbabwe sample was collected from people visiting two different clinics in Mutare and Mutasa. These samples showed no significant genetic differentiation and were therefore analyzed together. Moreover, significant LD was observed in both populations, even when only unique haplotypes were analyzed (Mutare: $n = 32$, $I_A^S = 0.0167$, $P = 0.0058$; Mutasa: $n = 24$, $I_A^S = 0.0158$, $P = 0.0461$), suggesting that combining different populations did not generate the observed LD.

The simplest explanation for the observed association between transmission intensity and LD is that *P. falciparum* utilizes a mixed mating system in which inbreeding predominates in low-transmission areas, while higher levels of outbreeding occur in regions with higher transmission. This may occur, since people are rarely superinfected with more than one parasite clone in low-transmission regions. As a result, unrelated parasites rarely co-occur in the same mosquito blood meal. Conversely, multiple-clone infections are frequent where transmission is intense. Consequently, mosquitoes frequently ingest unrelated parasites, leading to higher levels of outbreeding (Babiker et al. 1994; Paul et al. 1995). To further investigate the relationship between LD and transmission, we compared two indicators of transmission intensity (prevalence and proportion of infections containing multiple clones) with I_A^S, which measures the strength of LD (fig. 5). In general, parasites from regions with low prevalence or low levels of multiple infection show higher levels of I_A^S than those from regions with high prevalence or with high levels of multiple infections. This relationship should be viewed with some caution. Hudson (1994) has shown that I_A^S is not directly comparable between populations when N_e varies. A theoretical framework to allow interpretation of microsatellite-derived I_A^S values in terms of levels of recombination would be extremely useful. Such a model does exist for markers evolving by IAM (Hudson 1994). However, for most microsatellite data, this mutation model is likely to be inappropriate.

How frequently does outcrossing occur in populations of *P. falciparum*? Inbreeding coefficients have previously been measured in *P. falciparum* by genotyping oocysts dissected from mosquitoes. This stage of the *P. falciparum* life cycle contains the haploid products of meiosis and can be used to measure diploid genotypes and heterozygote deficits. This approach has been used in both Papua New Guinea and Tanzania, and inbreeding coefficients of 0.9 and 0.3, respectively, have been reported (Babiker et al. 1994; Paul et al. 1995). However, the prevalence of infection in mosquitoes from low-transmission regions (often <1/1,000) makes this method impractical, since literally thousands of mosquitoes

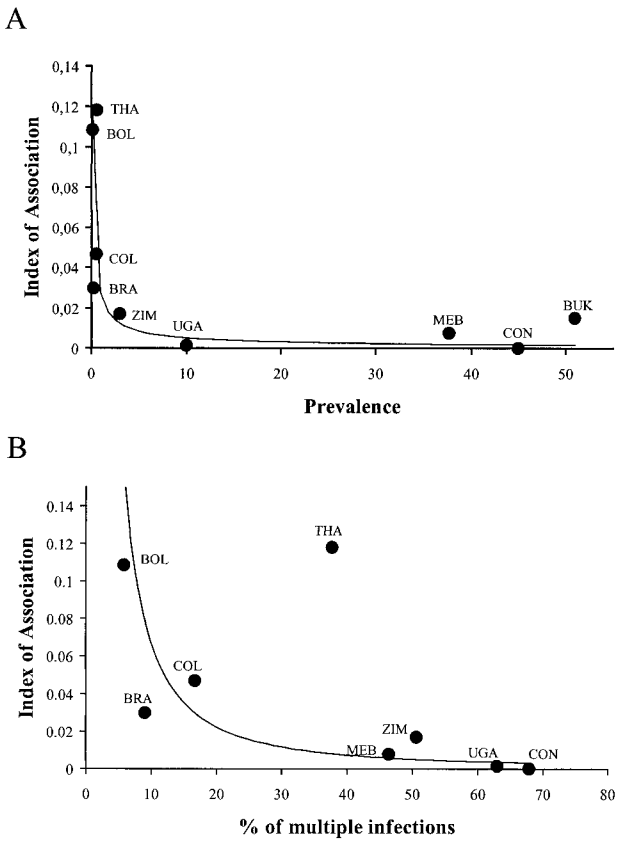

FIG. 5.—The relationship between transmission intensity and linkage disequilibrium. Two surrogate measures of transmission intensity, (*A*) infection (*B*) prevalence and proportion of people carrying multiple infection, were used to assess levels of parasite transmission. Linkage disequilibrium was assessed using the statistic I_A^S. Methods used to estimate these parameters are described in the text.

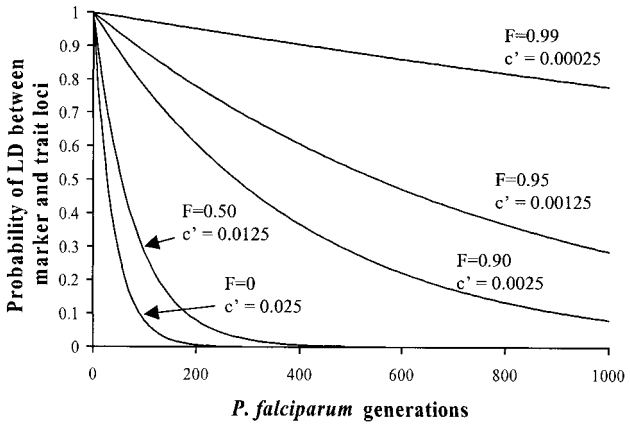

FIG. 6.—The probability of observing no recombination between a marker locus and a trait locus separated by 5 cM (≈75–150 kb in *Plasmodium falciparum*) in populations with differing levels of recombination and inbreeding. The frequency of chromosomes bearing the marker allele and the gene of interest is assumed to be 0.5, while for the case of a map distance of 5 cM, the rate of recombination (c) is 0.0025. The probability that there has been no recombination between marker and trait locus after t generations is given by $P = (1 - c')^t$, where c' is the effective rate of recombination (Lynch and Walsh 1997). The effective rate of recombination is given by $c' = c(1 - F)$, where c is the recombination rate and F is the inbreeding coefficient (Conway et al. 1999).

would need to be dissected. Furthermore, recent reanalyses of the data from Papua New Guinea have suggested the presence of nonamplifying alleles, which may lead to overestimation of inbreeding coefficients using this method (Anderson et al. 2000*a*). Estimating inbreeding from blood stage data is less straightforward, and the simplest estimates may be the most informative. In Colombia, only 3 of 30 (10%) infections contained multiple clones. Therefore, outcrossing is unlikely to occur in >10% of infected mosquitoes in this region. Similarly, the observation of the same multilocus genotype in blood samples collected 4 years apart is informative. If we assume a generation time of 2 months, this parasite genotype has been transmitted through 24 generations without change due to recombination.

Levels of LD may have important consequences for a number of aspects of *P. falciparum* biology. In particular, the rate at which recombination breaks down association between genes may influence the persistence of clonal genotypes (Paul et al. 1995; Hastings and Wedgewood-Oppenheim 1997), the maintenance of antigenically distinct ''strains'' (Gupta et al. 1996; Hastings and Wedgewood-Oppenheim 1997), sex ratio (Read et al. 1992; Dye and Godfray 1993), and the spread of drug resistance (Dye and Williams 1997; Hastings 1997; Hastings and Mackinnon 1998). The extensive LD observed has important practical consequences for malaria research, a major goal of which is to locate parasite genes underlying important phenotypes such as pathogenicity and resistance to drugs. Two resources—the sequence data emerging from the malaria genome project (Gardner et al. 1998) and a dense microsatellite map, with markers every 30–50 kb (Su and Wellems 1996; Su et al. 1999)—should simplify the location of important genes in *P. falciparum.* However, the high recombination rate (1 cM = 15–30 kb) observed in a genetic cross (Walker-Jonah et al. 1992) and the recent demonstration that LD is rarely detected between markers separated by >1 kb in African populations (Conway et al. 1999) may discourage researchers from using LD in natural populations as a mapping tool. In populations with high levels of inbreeding, the ''effective'' recombination rate will be considerably reduced. In such populations, it should be possible to locate genes encoding important parasite traits using relatively low densities of marker loci (Noorberg 2000). This approach is likely to be particularly effective for genes involved in drug resistance, since the mutations involved have occurred recently (White 1992), allowing little time for LD between marker and trait loci to have been broken down (fig. 6). For example, with 1% recombination, LD may be maintained between markers spaced 5 cM apart for 2,750 generations, which is equivalent to >400 years if we assume a 2-month generation time for *P. falciparum.* Thus, for recently evolved traits (<50 years ago) genome screens using 200–400 markers spaced at 75–150-kb intervals are likely to be successful. In comparison, in regions with 50% outcrossing, all traces of LD between loci will be lost in <60 generations (≅10 years), and marker densities one or two orders of magnitude higher would be necessary. Empirical data provide en-

couraging support for this mapping approach: LD is observed for >60 kb on either side of the putative chloroquine resistance locus (Su et al. 1997).

Population Differentiation and Diversity

We observe dramatic differences in both genetic diversity and genetic differentiation in different regions. Sequencing studies of antigen-encoding loci have shown lower levels of variation in antigen-encoding genes (Yoshida et al. 1990; Anderson and Day 2000) in South American locations. Since these loci encode antigens exposed to the immune system, it is uncertain whether the patterns observed indicate different regimes of immune selection in different regions, or whether these patterns reflect population history. The microsatellite data clarify this issue. Patterns of diversity are remarkably consistent across loci. At each of the 12 loci examined, diversity is lower in the three South American locations than in the three African populations, with intermediate levels in the populations from Papua New Guinea and Thailand. The loci were selected for use in this study on the basis of patterns of variation in 12 laboratory isolates originally isolated from worldwide locations. Therefore, the trivial explanation of ascertainment bias (Ellegren, Primmer, and Sheldon 1995) is unlikely to explain the differences observed. Differences in contemporary patterns of disease ecology and/or in population history are more likely explanations. These explanations are evaluated in the following paragraphs.

The differences in diversity may result from differences in effective population size and levels of inbreeding in low- and high-transmission regions. The intraspecific patterns of diversity, genetic differentiation, and LD observed in *P. falciparum* show a striking similarity to interspecific patterns of variation observed in plants (Schoen and Brown 1991; Awadalla and Ritland 1997) and animals (Jarne 1995) with differing levels of inbreeding. Outbred species typically show higher levels of genetic variation and lower levels of genetic differentiation than inbred species. The interplay between mating system, diversity, and differentiation is complex. Three factors are likely to result in the reduced levels of genetic variation observed in inbred populations of *P. falciparum.* First, N_e is halved in situations of complete inbreeding relative to complete outbreeding (Pollak 1987). This alone cannot account for the variation in diversity observed in *P. falciparum,* since N_e is reduced 9–23-fold in South American populations relative to African populations (table 2). Second, LD generated by selfing will increase the size of genomic regions involved in selective events, since ''hitchhiking'' either with deleterious sites (background selection) (Charlesworth, Morgan, and Charlesworth 1993) or with sites under positive selection (selective sweeps) (Hedrick 1980) will remove variation in the vicinity of the sites under selection. The size of genomic regions affected will be greatest in geographical regions in which strong LD is observed. Third, the effect of LD and inbreeding on diversity are likely to be compounded by the fact that both numbers of infected hosts and numbers of clones per individual are generally higher in areas of high transmission than in areas of low transmission. The reduced effective size of parasite populations in low-transmission areas may also explain the increased levels of genetic differentiation in regions such as South America, since allele frequencies may change rapidly in small populations owing to increased levels of genetic drift. If this explanation is correct, then we might expect to see similar numbers of alleles in both South America and Africa if sufficient populations are sampled. The fact that variation is distributed among populations in South America, while variation is distributed within populations in African locations, may give an illusion of reduced variation in parasites from the New World when the number of populations sampled is limited.

There is some supporting evidence for explanations involving disease ecology from two recent studies in which malaria parasites from isolated epidemics were genotyped for antigen-encoding loci. Arez et al. (1999) observed no genetic variation at loci in a malaria epidemic on Cabo Verde, while Laserson et al. (1999) observed no genetic variation at two antigen loci in an epidemic among Yanomani Indians in the Venezuelan Amazon. These papers suggest the importance of recent founder events associated with epidemic malaria in generating low-diversity parasite populations. Patterns of allelic distribution also provide some support for this explanation. In South American locations, the distribution of allele frequencies is flat, while in African countries the distributions are L-shaped (fig. 7). Furthermore, in two locations, Bolivia and Brazil, the modal allele frequency range is in one of the intermediate allele frequency classes (40%–50% for Bolivia and 10%–20% for Colombia). Such ''mode shifts,'' indicating a loss of rare alleles, are commonly observed in recently bottlenecked populations and appear to be indicative of populations that are not at mutation drift equilibrium (Maruyama and Fuerst 1985; Luikart et al. 1998).

Population history may also explain or contribute to the patterns of diversity and geographical differentiation observed. The high diversity in Africa may reflect the fact that this was the source for parasite populations in other parts of the world. Multiple colonization events could also explain the strong geographical structuring of South American populations if different events have led to the establishment of populations in different regions of the continent. It is thought that malaria was introduced into South America ≅500 years ago with the arrival of Europeans and that subsequent reintroduction from Africa occurred in the course of the slave trade. Pairwise measurements of Goldstein's $(\delta\mu)^2$ distances (Goldstein et al. 1995) between South American and African malaria populations range from 0.773 to 2.101, consistent with a split between South American and non–South American populations between 385 and 1,101 years ago (mean = 665 years ago), assuming a generation time of 2 months for *P. falciparum.* These figures are consistent with the historical scenario but should be viewed with caution. The range is extremely large, particularly when we consider that error around our estimate of mutation rate or variation among loci in

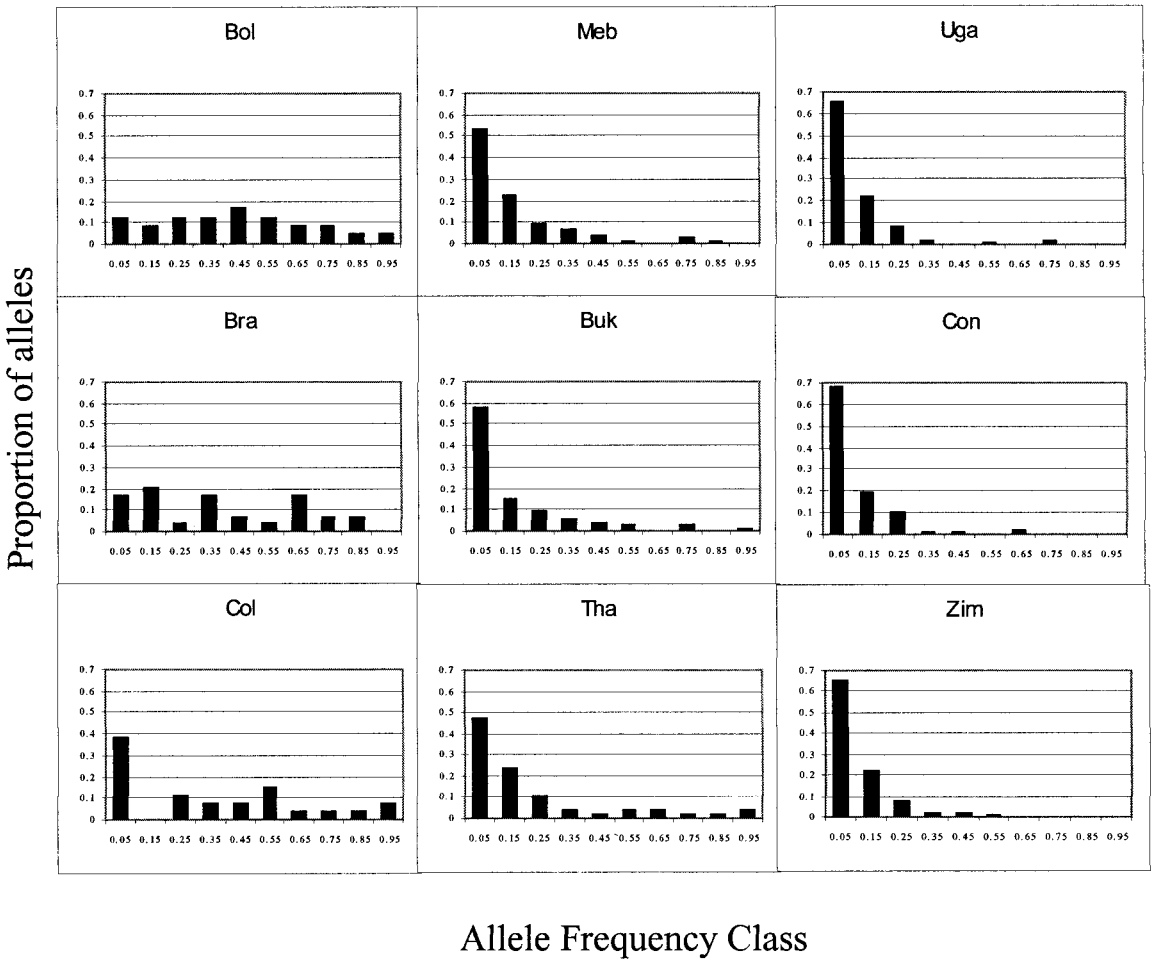

FIG. 7.—Allele frequency distributions for each of the nine populations studied. Only polymorphic loci are included.

mutation rate has not been incorporated. When confidence intervals around the mutation rate are used, mean divergence times range from as short as 131 years to as long as 2,488 years. Furthermore, $(\delta\mu)^2$ is accurate only if populations compared are in mutation drift equilibrium, which seems unlikely for parasite populations in regions of unstable epidemiology. Indeed, allelic distributions in both Bolivia and Colombia strongly suggest that these populations are not at mutation drift equilibrium (fig. 7). It will prove extremely difficult to determine the extent to which the low genetic diversity in South America reflects contemporary patterns of genetic structure and epidemiology, as argued above, or whether bottlenecks resulting from recent colonization events are responsible.

Regardless of the causes, the dramatic differences in genetic diversity, population differentiation, and LD in different locations have important consequences for our understanding of *P. falciparum* biology. In parasite populations with low microsatellite diversity, we would also expect to see reduced diversity in antigen-encoding loci (Ferreira et al. 1998) and a smaller repertoire of variant surface antigens. Hence, under a model of genotype-specific immunity (Gupta et al. 1994), we might expect effective immunity to malaria to be generated following a relatively small number of infective mosquito bites in low-transmission regions. Second, in regions with low levels of recombination, multilocus genotypes may be maintained through multiple generations. In this situation, it should be possible to track the spread of multilocus genotypes within communities, as is done for bacterial haplotypes. Furthermore, comparison of infection characteristics of multiply represented haplotypes can be used to investigate which aspects of *P. falciparum* virulence (or other traits) are a product of parasite genetics rather than host factors.

Acknowledgments

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APPENDIX

Allele Frequencies and Sample Sizes (*n*) for 12 Microsatellite Loci from 9 Populations of *Plasmodium falciparum*

	South America			Southeast Asia/Pacific			Africa		
	BOL	BRA	COL	THA	BUK	MEB	CON	UGA	ZIM
TAI									
159 ...	—	—	—	0.135	—	—	—	—	—
162 ...	—	—	—	—	—	—	0.077	0.121	0.104
165 ...	—	—	—	0.108	0.098	0.069	0.115	0.077	0.060
168 ...	—	—	—	0.649	0.262	0.310	0.077	0.165	0.134
171 ...	—	—	—	0.027	0.180	0.431	0.231	0.220	0.209
174 ...	0.700	0.667	1.000	—	0.230	0.017	0.135	0.132	0.119
177 ...	0.233	0.152	—	—	0.115	0.121	0.077	0.077	0.104
180 ...	—	—	—	—	0.066	0.017	0.115	0.066	0.075
183 ...	0.067	0.182	—	0.081	—	0.017	0.038	0.011	0.075
186 ...	—	—	—	—	0.033	—	0.077	0.099	0.060
189 ...	—	—	—	—	—	0.017	0.038	0.022	0.030
192 ...	—	—	—	—	0.016	—	0.019	—	—
195 ...	—	—	—	—	—	—	—	—	0.015
198 ...	—	—	—	—	—	—	—	—	0.015
204 ...	—	—	—	—	—	—	—	0.011	—
n	30	33	30	37	61	58	52	91	67
TA40									
201 ...	—	—	—	—	—	—	—	—	0.015
207 ...	—	—	—	—	—	—	—	0.011	0.031
210 ...	—	—	—	—	—	—	—	—	0.015
213 ...	—	—	—	—	—	—	0.020	—	—
219 ...	—	—	—	0.056	—	0.018	0.020	0.011	—
222 ...	—	—	0.033	—	—	—	0.098	0.079	0.092
225 ...	—	—	—	0.222	0.180	0.179	0.078	0.101	0.062
228 ...	—	—	—	0.139	—	—	0.098	0.090	—
231 ...	1.000	1.000	0.967	—	—	0.018	—	—	0.031
234 ...	—	—	—	—	—	0.071	0.020	—	0.123
237 ...	—	—	—	—	0.066	—	0.020	0.011	—
240 ...	—	—	—	0.361	—	0.089	0.059	0.022	—
243 ...	—	—	—	0.056	0.115	0.089	0.118	0.236	0.323
246 ...	—	—	—	0.028	0.016	—	0.216	0.236	0.154
249 ...	—	—	—	0.139	0.475	0.321	0.098	0.056	0.031
252 ...	—	—	—		—	—	0.059	0.011	0.031
255 ...	—	—	—	—	0.115	0.214	—	0.067	0.077
258 ...	—	—	—	—	—	—	—	0.022	—
261 ...	—	—	—	—	—	—	0.059	0.034	0.015
264 ...	—	—	—	—	—	—	0.020	0.011	—
267 ...	—	—	—	—	—	—	0.020	—	—
291 ...	—	—	—	—	0.016	—	—	—	—
297 ...	—	—	—	—	0.016	—	—	—	—
n	32	32	30	36	61	56	51	89	65
TA42									
182 ...	—	—	—	—	—	—	—	—	0.030
185 ...	—	—	—	—	0.033	—	0.020	—	—
188 ...	0.645	0.758	1.000	0.973	0.754	0.776	0.647	0.714	0.448
191 ...	—	—	—	—	0.098	0.017	0.059	0.022	0.075
200 ...	—	—	—	—	—	—	0.039	—	—
203 ...	0.355	—	—	—	—	—	0.059	0.099	0.179
206 ...	—	0.242	—	—	—	0.103	—	—	0.015
209 ...	—	—	—	—	0.033	—	—	—	—
218 ...	—	—	—	—	—	—	0.039	—	0.045
239 ...	—	—	—	—	—	—	—	—	0.015
242 ...	—	—	—	—	—	—	0.020	—	0.015
245 ...	—	—	—	0.027	0.082	0.034	0.078	0.033	0.164
248 ...	—	—	—	—	—	0.069	0.020	0.011	0.015
251 ...	—	—	—	—	—	—	0.020	0.121	—
n	31	33	30	37	61	58	51	91	67
TA109									
154 ...	—	—	—	—	—	—	—	—	0.015
160 ...	—	—	—	—	—	—	0.020	—	0.015
163 ...	0.531	0.091	1.000	0.973	0.984	0.894	0.255	0.228	0.239
166 ...	0.438	0.879	—	—	0.016	—	0.157	0.272	0.164
169 ...	—	—	—	—	—	0.070	—	0.011	—
172 ...	—	—	—	—	—	0.018	—	—	0.030
175 ...	—	—	—	0.027	—	—	0.137	0.120	0.254

APPENDIX
Continued

	South America			Southeast Asia/Pacific			Africa		
	BOL	BRA	COL	THA	BUK	MEB	CON	UGA	ZIM
178 ...	0.031	0.030	—	—	—	0.018	0.196	0.163	0.119
181 ...	—	—	—	—	—	—	0.059	0.011	—
184 ...	—	—	—	—	—	—	0.020	—	—
187 ...	—	—	—	—	—	—	0.118	0.076	0.045
190 ...	—	—	—	—	—	—	—	0.043	0.045
193 ...	—	—	—	—	—	—	—	0.011	—
196 ...	—	—	—	—	—	—	—	—	0.015
199 ...	—	—	—	—	—	—	0.020	0.043	0.015
202 ...	—	—	—	—	—	—	—	0.022	—
211 ...	—	—	—	—	—	—	—	—	0.030
214 ...	—	—	—	—	—	—	0.020	—	—
223 ...	—	—	—	—	—	—	—	—	0.015
n	32	33	30	37	61	57	51	92	67
Polyα									
114 ...	—	—	—	—	—	—	—	—	0.015
129 ...	—	—	—	—	—	—	—	0.011	—
132 ...	—	—	—	—	—	—	0.019	—	—
135 ...	—	—	—	—	0.097	0.053	0.075	0.056	0.015
138 ...	—	—	—	—	—	—	—	—	0.015
141 ...	—	—	—	—	—	—	0.019	0.011	0.015
144 ...	—	—	—	—	0.048	—	0.075	0.033	0.030
147 ...	—	—	—	—	—	0.018	0.057	0.011	0.076
150 ...	—	—	0.300	—	—	—	0.038	0.011	0.045
153 ...	0.323	0.138	—	0.676	0.177	0.140	0.208	0.156	0.273
156 ...	—	—	0.067	—	0.097	0.193	0.094	0.133	0.091
159 ...	—	—	0.367	—	0.097	0.088	0.094	0.122	0.030
162 ...	—	—	0.267	0.176	—	—	0.057	0.067	0.091
165 ...	—	0.034	—	0.059	—	0.105	0.075	0.033	0.106
168 ...	—	—	—	—	0.097	0.088	0.019	0.044	0.091
171 ...	—	—	—	—	0.016	—	0.038	0.089	0.030
174 ...	—	—	—	0.029	0.226	0.140	0.019	0.100	0.030
177 ...	—	0.034	—	0.029	0.065	0.123	0.038	0.089	—
180 ...	—	—	—	—	0.081	—	0.075	0.011	0.015
183 ...	0.677	0.483	—	—	—	0.053	—	0.011	0.015
186 ...	—	—	—	0.029	—	—	—	—	—
189 ...	—	0.310	—	—	—	—	—	—	—
198 ...	—	—	—	—	—	—	—	—	0.015
201 ...	—	—	—	—	—	—	—	0.011	—
n	31	29	30	34	62	57	53	90	66
TA60									
69 ...	—	—	—	—	—	—	—	0.066	0.015
72 ...	—	—	—	0.081	—	—	—	—	0.015
75 ...	0.455	0.724	—	—	0.032	0.018	0.208	0.176	0.277
78 ...	—	—	0.067	—	—	0.088	0.075	0.176	0.123
81 ...	—	—	0.067	0.027	0.048	0.035	0.057	0.022	0.015
84 ...	0.485	0.172	0.833	0.081	0.226	0.211	0.321	0.286	0.338
87 ...	0.061	0.103	—	0.514	0.403	0.474	0.113	0.165	0.108
90 ...	—	—	0.033	0.162	0.032	0.070	0.132	0.099	0.077
93 ...	—	—	—	0.054	0.226	0.035	0.057	—	0.031
96 ...	—	—	—	—	0.032	0.070	0.019	—	—
99 ...	—	—	—	0.081	—	—	0.019	0.011	—
n	33	29	30	37	62	57	53	91	65
TA81									
112 ...	—	—	—	—	—	—	0.038	—	0.030
115 ...	—	—	—	0.297	0.081	0.224	0.113	0.022	0.045
118 ...	—	0.061	—	0.216	0.323	0.241	0.189	0.196	0.164
121 ...	0.719	0.818	0.033	0.054	0.097	0.069	0.264	0.174	0.269
124 ...	0.281	0.121	0.733	0.243	0.177	0.069	0.189	0.141	0.194
127 ...	—	—	—	0.162	0.194	0.069	0.094	0.196	0.119
130 ...	—	—	0.233	0.027	0.097	0.138	0.075	0.228	0.149
133 ...	—	—	—	—	—	0.103	—	0.011	0.015
136 ...	—	—	—	—	0.016	0.069	—	0.011	0.015
139 ...	—	—	—	—	0.016	0.017	0.019	0.011	—
142 ...	—	—	—	—	—	—	0.019	0.011	—
n	32	33	30	37	62	58	53	92	67

APPENDIX
Continued

	South America			Southeast Asia/Pacific			Africa		
	BOL	BRA	COL	THA	BUK	MEB	CON	UGA	ZIM
TA87									
90 ...	—	—	—	—	—	—	—	—	0.016
93 ...	—	—	—	0.054	—	0.017	0.057	0.033	—
96 ...	—	—	—	—	0.016	—	0.019	0.022	0.097
99 ...	0.303	0.303	0.033	—	0.081	0.069	0.113	0.174	0.129
102 ...	—	—	0.500	0.027	0.161	0.293	0.189	0.109	0.097
105 ...	—	—	—	0.135	0.323	0.293	0.132	0.130	0.194
108 ...	—	—	—	0.027	0.290	0.172	0.208	0.261	0.177
111 ...	0.212	—	—	0.595	0.081	0.052	0.132	0.130	0.177
114 ...	0.485	0.697	—	0.054	0.048	0.103	0.075	0.076	0.065
117 ...	—	—	0.033	—	—	—	0.057	0.011	0.016
120 ...	—	—	0.433	0.108	—	—	—	0.033	0.016
123 ...	—	—	—	—	—	—	—	—	0.016
126 ...	—	—	—	—	—	—	0.019	0.022	—
n	33	33	30	37	62	58	53	92	62
ARAII									
63 ...	—	—	—	—	—	—	0.058	0.089	0.047
66 ...	0.900	0.697	—	—	—	—	0.038	0.078	0.016
69 ...	0.100	0.303	0.500	0.029	0.516	0.414	0.231	0.322	0.234
72 ...	—	—	—	0.176	0.226	0.276	0.135	0.256	0.266
75 ...	—	—	0.500	0.147	0.065	0.103	0.115	0.044	0.188
78 ...	—	—	—	0.382	0.145	0.172	0.231	0.100	0.109
81 ...	—	—	—	0.265	0.048	0.034	0.038	0.044	0.047
84 ...	—	—	—	—	—	—	0.038	0.033	0.031
87 ...	—	—	—	—	—	—	0.077	0.033	0.031
90 ...	—	—	—	—	—	—	0.038	—	0.031
n	30	33	26	34	62	58	52	90	64
pfG377									
89 ...	—	—	—	—	—	—	—	—	0.015
92 ...	—	—	—	—	—	—	0.020	—	—
95 ...	0.121	0.333	—	0.086	0.597	0.500	0.039	0.054	0.061
98 ...	0.879	0.667	0.967	0.743	0.306	0.310	0.275	0.250	0.318
101 ...	—	—	0.033	0.143	0.097	0.172	0.490	0.587	0.439
104 ...	—	—	—	0.029	—	0.017	0.118	0.065	0.106
107 ...	—	—	—	—	—	—	0.039	0.043	0.030
110 ...	—	—	—	—	—	—	0.020	—	0.015
113 ...	—	—	—	—	—	—	—	—	0.015
n	33	33	30	35	62	58	51	92	66
PfPK2									
159 ...	—	—	—	—	—	0.034	0.020	—	—
162 ...	—	—	—	0.108	0.081	0.103	0.098	0.066	0.075
165 ...	—	—	0.267	0.486	0.323	0.345	0.216	0.352	0.194
168 ...	—	0.364	0.067	0.054	0.452	0.328	0.196	0.132	0.224
171 ...	—	—	—	0.270	0.081	0.034	0.059	0.099	0.119
174 ...	1.000	0.636	—	—	0.016	0.138	0.118	0.143	0.134
177 ...	—	—	0.667	—	0.048	—	0.118	0.077	0.075
180 ...	—	—	—	—	—	0.017	—	0.011	0.060
183 ...	—	—	—	0.027	—	—	—	0.022	0.030
186 ...	—	—	—	—	—	—	0.078	0.022	0.015
189 ...	—	—	—	—	—	—	0.059	0.022	0.045
192 ...	—	—	—	0.054	—	—	0.039	0.055	0.030
n	29	33	30	37	62	58	51	91	67
2490									
78 ...	—	—	0.467	—	—	—	0.057	0.109	0.242
84 ...	0.500	0.531	0.533	0.857	0.758	0.759	0.208	0.109	0.136
87 ...	0.500	0.469	—	0.143	0.177	0.190	0.660	0.728	0.561
90 ...	—	—	—	—	—	0.034	0.019	0.043	0.061
93 ...	—	—	—	—	0.065	0.017	0.057	0.011	—
n	32	32	30	35	62	58	53	92	66

Note.—Allele lengths are shown in base pairs, and sampling location abbreviations are as described in figure 2. Only the predominant allele present in amplification products from infections containing multiple clones was used to estimate allele frequencies.

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