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SIGNIFICANT LINKAGE DISEQUILIBRIUM AND HIGH GENETIC DIVERSITY IN A POPULATION OF *PLASMODIUM FALCIPARUM* FROM AN AREA (REPUBLIC OF THE CONGO) HIGHLY ENDEMIC FOR MALARIA

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Abstract. A study based on 28 microsatellite loci was performed on 32 isolates of *Plasmodium falciparum* from Pointe Noire (Republic of the Congo) and compared with a cosmopolitan sample of 21 isolates collected from different countries in Africa, Latin America, and Asia. The Pointe Noire population exhibited very high genetic diversity ($A = 7.8 \pm 2.6$, $He = 0.79 \pm 0.11$). Significant linkage disequilibria were observed in 28 of 378 pairs of microsatellite loci. This result could be explained by two non-exclusive hypotheses: 1) uniparental propagation (i.e., selfing), leading to non-panmictic associations, and/or 2) a Wahlund effect (i.e., spatial population genetic heterogeneity). These observations are in agreement with data previously obtained from isozyme loci of the same isolates, but contrast with other population genetic analyses conducted in other hyperendemic zones.

INTRODUCTION

Malaria due to *Plasmodium falciparum* is present in 102 countries that are mainly distributed in the tropical zones of Africa, Asia, Latin America, and Central America. While the greatest burden of malaria mortality (> 90%) is encountered in children inhabiting sub-Saharan Africa, current estimates of disease risk remain poorly defined.[1] Despite the considerable economic and research efforts made against malaria, disease prevalence has not decreased sharply in most endemic areas, and except for areas displaying low transmission rates (i.e., Asia and Latin America), the population dynamics and population genetics of these parasites in most malaria foci still remain unclear. The debate concerning the genetic variability, population structure, and mating patterns of *P. falciparum* is very controversial,[2–9] and probably reflects a variety of situations. However, the knowledge of population structure is essential for understanding analyses of epidemiologic surveys (i.e., target choices for disease control), the evolution of vector/parasite compatibility, and the dynamics of drug resistance.[9]

Many studies have been carried out on surface antigenic markers (i.e., merozoite surface protein-1 [MSP-1] and MSP-2, circumsporozoite surface protein [CSP], and glutamate-rich protein [GLURP]),[8] which are *a priori* under strong selection due to host immune responses.[9] However, these studies involved selected markers, rather than neutral ones (e.g., microsatellite loci), which are more appropriate for investigating population structure.[10] Recently, 800 microsatellite markers were described for the *P. falciparum* genome.[11] Few studies to date have used these genomic markers to investigate the population genetics of *P. falciparum*.[7,12] A recent study of *P. falciparum* genetic diversity based on 12 microsatellite loci from 465 blood samples collected in different areas (Africa, South America, and Southeast Asia) described the genetic diversity observed at the level of local populations and on a global scale.[7] According to these investigators, *P. falciparum* exhibits a range of population structures, characterized by strong linkage disequilibrium, low diversity, and extensive population differentiation in low transmission regions, while at high levels of transmission linkage disequilibrium and population differentiation are low, while genetic diversity is high.[7]

In the present study, we analyzed blood samples collected from individuals living in Pointe Noire, Republic of the Congo and characterized *P. falciparum* isolates at 28 microsatellite loci. We 1) quantified the level of genetic diversity and analyzed the genetic structure of this local population of *P. falciparum*; 2) compared its diversity with the species-wide diversity of *P. falciparum* represented by a cosmopolitan sample of 21 isolates from different continents; and 3) compared its diversity and genetic structure with samples from other countries located in a globally hyperendemic area (Democratic Republic of the Congo, Uganda, and Zimbabwe[13]). This last comparison is interesting because 10 of the 12 microsatellite markers used by Anderson and others[7] are a subset of our 28 markers.[13] Despite several characteristics common to all samples, i.e., same markers, all samples situated in areas of high transmission, all samples exhibiting high genetic diversity, our analysis showed that our sample from the Republic of the Congo, similar to their samples from Zimbabwe in contrast to their samples from the Democratic Republic of the Congo and Uganda, had significant levels of linkage disequilibrium. We discuss possible interpretations of this discrepancy and their implications in malaria epidemiology.

MATERIALS AND METHODS

***Plasmodium falciparum* isolates.** We studied *P. falciparum*-infected blood samples previously analyzed with isozymes and random amplified polymorphic DNA.[14] A sample of 32 DNA isolates from a free clinic in Pointe Noire, Republic of the Congo (4°46′S, 11°53′E) was analyzed together with a cosmopolitan sample of 21 isolates collected from 10 locations in different continents: Africa (Benin: Ibrahim; Cameroon: TA10, TB3, TD4, TE9, and TH10; The Gambia: Banjul, M13, and SGE1; Ghana: NF7, and Uganda: UPA), Latin America (Honduras: HB3 and Venezuela: V5may, V7may, V8may, V11jul, and V12jul provided by L. Urdaneta),[15] Asia (Thailand: Indochina-1, TE.94, and TE.100 and Papua New Guinea: MAD20). These samples were previously cultured for 24–36 hours according to the protocol of Trager and Jensen.[16] The DNA was extracted from cultured parasites and used in previous studies.[14,15] Since multiple genotypes were previously detected from individual isolates,[14] we analyzed only isolates displaying a single genotype from each amplification.

Ethical clearance. The Republic of the Congo blood-samples analyzed were collected by the "Centre Médical Sanitaire d'Elf-Aquitaine" in Pointe Noire. This clinic is accessible to all patients and is not restricted to Elf-Acquitaine employees. All patients provided informed consent before donating blood samples to be used in this study. This study was reviewed and approved by the biomedical official of the Centre Médical Sanitaire d'Elf-Aquitaine, in agreement with international ethical guidelines for biomedical research involving human subjects. The legal representatives were informed about the objectives of the study and were included only after providing consent. The other isolates were provided by the Biomedical Committee of Malariology (Caracas, Venezuela), the Laboratoire Central de Virologie (Geneva, Switzerland), the Department of Medical Microbiology (Nijmejen, The Netherlands), and the Centers for Diseases Control and Prevention (Atlanta, GA).

Microsatellite alleles and polymerase chain reaction. We used 28 microsatellite loci distributed throughout the *P. falciparum* genome that were chosen from the described microsatellite markers (Table 1).[11] The amplified microsatellite loci were perfect, compound, and interrupted with di-, tri-, hexa-, or nona- nucleotide repeats.

Microsatellite loci were amplified from 10 ng of total DNA in a 20-μL reaction volume containing 80 μM of each deoxynucleoside triphosphate, 6 pmol of each primer, 2 μL of 10× buffer (Promega, Madison, WI), 1.5 mM Mg^{2+} (Promega), and 1.3 units of *Taq* DNA polymerase (Promega) in the buffer supplied by the manufacturer. Thermocycling was performed in a PTC100 96-well thermocycler (MJ Research, Waltham, MA) with an initial denaturation at 94°C for 2 minutes, 30 cycles at 94°C for 20 seconds, 45°C for 10 seconds, and 40°C for 20 seconds, and a final elongation step at 60°C for 30 seconds.[11] After amplification, 10-μl aliquots of the reaction mixture were subjected to electrophoresis on an 8% Long Ranger™ acrylamide gel (FMC BioProducts, Rockland, ME) with 1× Tris-borate buffer. The DNA bands were visualized by silver staining.[17] The measurement of allele length was estimated using a 10-base pair DNA ladder (Invitrogen Life Technologies, Cergy Pontoise, France).

TABLE 1
Characteristics of microsatellite loci of *Plasmodium falciparum*

Code	Marker‖	Chromosome location	dbSTS or GenBank accession number*	Number of alleles	Size range (base pairs)
L01	TA35	4	G38826	9	157–190
L02	TA111	10	G38830	5	160–196
L03	PJ2	7	G37826	15	110–230
L04	TA80	10	G38857	3	150–156
L05	TA119	11	G38863	8	213–255
L06	TA31	11	G38864	10	79–245
L07	TA125	11	G38868	7	143–164
L08	TA22	14	G38886	13	142–190
L09	IMP	2	G37800	10	142–188
L10	POLY2	3	G37805	9	98–122
L11	MDR1	5	G42769	8	137–173
L12	Pf2802	5	G37818	9	138–170
L13	ARA6	8	G37833	7	108–126
L14	ARA3	12	G37855	9	138–189
L15	ARP2	13	G37793	5	166–181
L16	POLYα	4	G37809	14	130–190
L17	TA60	13	G38876	7	79–100
L18	ARA2	11	G37848	7	64–88
L19	Pfg377	12	G37851	6	95–109
L20	PfPK2	12	G37852	8	162–186
L21	TA87	6	G38838	11	82–124
L22	TA109	6	G38842	8	147–195
L23	TA81	5	G38836	7	109–135
L24	TA42	5	G38832	5	183–243
L25	2490	10	*T02490*	5	81–96
L26	C1M25	1	G37999	8	116–155
L27	C1M8	1	G38013	13	145–214
L28	PfMAL3P2	3	*AL034558*	10	211–253

dbSTS = database sequence-tagged sites.
Number in italics is GenBank accession number.

Data analyses. Genetic polymorphism was measured by the number of alleles per locus (A) and Nei's unbiased expected heterozygosity (He) adapted to haploid data using F-STAT, version 1.2.[18] Differences in expected heterozygosities were tested by an exact Wilcoxon rank sum test using the S-PLUS software.[19]

Genotypic linkage disequilibrium (LD = non random association of genotypes occurring at different loci) was tested by the exact probability test performed using GENEPOP software, version 3.2d.[20] The null hypothesis is that genotypes at one locus are independent from genotypes at the other locus. This test computed unbiased estimates by randomization (4,000,000 iterations) and by the Markov-chain method for the exact probabilities of random association for all contingency tables corresponding to all possible pairs of loci within each population.

The previous method measures the degree of association between pairs of loci. Haubold and others[21] elaborated on another method, first proposed by Brown and others,[22] that measured non-random association among all screened loci. This method is implemented by the LIAN 3.0 software.[23] This software tests the null hypothesis of statistical independence of alleles at all loci. It also computes the distribution of the number of loci at which pairs of haplotypes within a population differ, and then calculates the variance, V_D, of these pairwise differences. The sample variance is then compared with the variance expected under linkage equilibrium, V_E. A distribution of V_E is generated by Monte Carlo simulations, and its percentiles provide 95% confidence intervals.[22] We performed 10,000 iterations to generate this distribution. The output file gives V_D and V_E –values, as well as a standardized index of association ($I_{AS} = (V_D/V_E - 1)/(1 - r)$, where r is the number of loci,[24] a measure of haplotype-wide linkage and the 95% confidence limits determined by Monte Carlo simulations (L_{MC}).[23]

For the measures of polymorphism (A and He) for multilocus linkage disequilibrium and assignment methods, we considered the Republic of the Congo sample and a cosmopolitan sample of all other isolates pooled, giving a total of 32 and 18 isolates, respectively.

RESULTS

Data analyses were performed for the 32 isolates from the Republic of the Congo and for only 18 isolates from the other samples because we found three identical multilocus genotypes for the samples Uganda (UPA), Gambia (SGE1), and Thailand (Indochina-1) isolates.

Allelic distribution and heterozygosities. The twenty-eight microsatellite loci surveyed were polymorphic in all isolates (Table 1). The number of alleles observed per locus ranged from three (L04 locus) to 15 (L03 locus). The total number of alleles detected was 236 for the 28 loci. In the sample from the

Republic of the Congo, 217 alleles were scored. They represented 92% of all detected alleles. The mean ± SD number of alleles per locus (A ± SD) were 7.75 ± 2.62 for the Republic of the Congo sample and 4.79 ± 1.52 for the cosmopolitan sample.

Unbiased expected heterozygosity (He ± SD) was high in the Republic of the Congo sample (He = 0.786 ± 0.111) and comparable with that found elsewhere in samples from Uganda, Zimbabwe, and the Democratic Republic of the Congo.[7] The expected heterozygosity in the cosmopolitan sample was 0.687 ± 0.109.

Linkage disequilibrium. Statistical tests for LD were conducted for all pairs of microsatellite loci; 28 and 201 of the 378 possible tests showed significant results ($P < 0.05$) in the Republic of the Congo and the cosmopolitan samples, respectively.

None of the significant pairwise associations involved loci located on the same chromosome. Thus, these loci are only statistically linked, not physically linked.

The LD estimated by the LIAN program was also significant in the Republic of the Congo sample (observed mismatch variance $V_D = 5.5$, expected mismatch variance $V_E = 4.3$, standardized index of association $I_{AS} = 0.01$, simulated 5% critical value $L_{MC} = 4.9$, $P = 9.10^{-4}$) and in the cosmopolitan sample ($V_D = 30.1$, $V_E = 5.3$, $I_{AS} = 0.17$, $L_{MC} = 6.5$, $P = 10^{-4}$).

The extensive LD detected by both methods in the cosmopolitan sample can be explained by a Wahlund effect, i.e., the mixing of several populations with different genotypic frequencies. The much lower, but significant, linkage disequilibrium detected in the Republic of the Congo sample will be subsequently discussed.

DISCUSSION

In the local population of Pointe Noire, Republic of the Congo, the number of alleles per locus ranged from three to 14; Anderson and others[7] also found a variable allelic distribution among 12 microsatellite loci (between five and 16 alleles per locus) in 53 *P. falciparum* samples collected from a clinic in Kimpese, Democratic Republic of the Congo. Ten microsatellite loci were common in the present study and in the study of Anderson and others[7] (Table 2). The number of alleles per locus was similar between the two studies, and no significant differences were detected between pairwise unbiased expected heterozygosities ($P = 0.272$, by the exact Wilcoxon rank sum test). It is also worth mentioning that we found no multiple genotypes in the sample from the Republic of the Congo. If we restrict the analysis to the 10 loci common to the previous study[7] and our study, this remains true. The absence of multiple genotypes is strong evidence against a clonal structure in the population from the Republic of Congo.

TABLE 2

Number of alleles (A) and unbiased expected heterozygosities (He) corrected for haploid data of the 10 shared loci between the two microsatellite data sets*

	n (a)	A (a)	He (a)	n (b)	A (b)	He (b)
Marker	(a and b)					
POLYα	53	16	0.9305	32	11	0.8629
TA60	53	9	0.8342	32	7	0.7984
ARA2	52	10	0.8718	30	6	0.8207
Pfg377	51	7	0.6866	32	5	0.7520
PfPK2	51	10	0.8886	32	8	0.8307
TA87	53	10	0.8855	32	11	0.8709
TA109	51	10	0.8593	32	6	0.6875
TA81	53	9	0.8535	32	7	0.8064
TA42	51	10	0.5807	32	4	0.4637
2490	53	5	0.5292	32	5	0.7137

* n = sample size; a = Kimpese, Democratic Republic of the Congo; b = Pointe Noire, Republic of the Congo (present study).

Despite the high genetic diversity, we also found significant genetic linkage disequilibria in the local *P. falciparum* population of Pointe Noire as previously reported for this sample based on isozyme data.[14] The amount of linkage disequilibrium observed in our study is much weaker than that expected in the case of a clonal population structure. This result is expected in an area of *a priori* high transmission and for a sample of such high genetic diversity.

After their investigation on *P. falciparum* population genetic structures, Anderson and others concluded that "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, where transmission is intense".[7] The pattern exhibited by the sample from the Republic of the Congo surveyed here clearly does not fit this categorization. The discrepancy between our results and those of Anderson and others cannot be explained by a difference in the markers used in the two studies. Indeed, we found significant linkage disequilibria in our sample from the Republic of the Congo even when we considered only the 10 loci shared by the two studies presented in Table 2 (GENEPOP: 3,000,000 iterations; P value of the exact binomial test = 0.024 and LIAN: $V_D = 2.07$, $V_E = 1.67$, $I_{AS} = 0.03$, $L_{MC} = 1.87$, $P = 0.001$).

There are two possible explanations for this discrepancy: selfing and a Wahlund effect. Selfing could explain our results either because our sampling method may have biased sampling in favor of *Plasmodium* lineages that have undergone at least one generation of self-fertilization or because some amount of selfing, capable of generating small but significant levels of linkage disequilibrium, occurs even in areas of high transmission. A bias could arise because of our sampling method. Indeed, to avoid problems related to genotyping strains in the presence of multiple infections, we chose to use only isolates that after culture displayed a single genotype (see Anderson and others for an alternative method).[7] Thus, it could be argued that we used only malaria lineages that resulted from at least one self-fertilization event. However, we do not believe that this method induces a bias for two reasons. First, selfing does not generate linkage disequilibrium but maintains it, i.e., if the whole population is in linkage equilibrium, examining only lineages resulting from a single self-fertilization event will not reveal significant LD. Second, Anderson and others[7] showed that their results with respect to LD remained unchanged whether they considered multiple infections or only single infections. Thus, for both *a priori* and *a posteriori* reasons, the bias hypothesis cannot explain our results.

The Wahlund effect could also explain our results because although samples were collected at the same site, our results cannot rule out the presence of local population genetic structures for *P. falciparum* in the Republic of the Congo. Indeed, we have no information on the residence of the patients treated at the clinic where our samples were collected; the patients could live in areas harboring genetically differentiated *Plasmodium* populations.

Both explanations imply that at some spatial level within the Republic of the Congo *P. falciparum* mating is not random. Interestingly, one of the populations in Zimbabwe[7] exhibited a similar pattern; while situated in a high transmission area, both *a priori* and based on the percentage of multiple infections and mean number of clones per individual host, this population shows significant levels of linkage disequilibrium, despite a high genetic diversity. In discussing the case of the Zimbabwe sample, Anderson and others[7] examined the two explanations mentioned earlier: selfing and a Wahlund effect. The latter was an excellent *a priori* candidate, since the Zimbabwe sample was actually composed of two sub-samples. However, the two sub-samples were not significantly differentiated, and significant linkage disequilibria were found within both samples. This situation could also arise either from selfing, despite high transmission, or from a Wahlund effect. Indeed, a Wahlund effect could still explain the pattern revealed by the Zimbabwe sample despite the absence of genetic differentiation between the two sub-samples if the two clinics from which the two sub-samples originate receive with equal probability patients from areas harboring genetically differentiated *P. falciparum* populations. We do not know how plausible this is for the Zimbabwe sample.[7] However, this possibility underlines the importance of knowing precisely the geographic origin of samples collected from human hosts to infer the population genetic micro-structure of *P. falciparum* populations.

At the local population scale, the opportunity for random mating depends on the possibility of several *Plasmodium* strains (*sensu* Hastings and Wedgwood-Oppenheim[25]) to co-infect a mosquito, which depends mainly on parasite diversity within infected human individuals. This, in turn, depends on the parasite density in the area under consideration. Thus, linkage disequilibrium observed in *P. falciparum* populations seems to be due mainly self-fertilization in regions where only a few different multilocus genotypes are present,[25] which leads to a situation considered as genetic clonality.[26] How large can the proportion of selfing be in an area of high transmission, and can it generate small but significant levels of linkage disequilibrium?

The proportion of selfing in a *P. falciparum* population will be equal to the probability of single infections in mosquitoes plus the product of the probability of multiple infections of mosquitoes by the proportion of selfed oocysts in multiply infected mosquitoes. Assuming random mating within mosquitoes,[26] this latter quantity will be equal to the sum over all haplotypes of the squares of haplotype frequencies. For example, if two *P. falciparum* haplotypes infect a mosquito at equal frequencies, on average half of the oocysts will be outcrossed and half will be selfed. Deviations from equal frequencies would lead to lower frequencies of outcrossed oocysts. The data of Taylor[27] from Tanzania, a highly endemic area, provide the basis for a rough estimate of the selfing rate. Using polymorphism data of surface proteins, Taylor reported that approximately 30% of the infected mosquitoes carried a single infection, while the mean number of *Plasmodium* genotypes carried by multiply infected mosquitoes was 2.38 (this latter number is inferred from her table). Assuming that all co-infecting strains are at equal frequencies (thus yielding a minimal selfing rate estimate), and that multiple infections consist of either two or three different genotypes, we obtain a selfing rate of 0.61 corresponding to $F_{is} = 0.44$. The selfing rate estimate could be overestimated because we assumed that all co-infecting strains were at equal frequencies, and underestimated because surface proteins polymorphism could have lower resolution than microsatellites,[28] thus underestimating the number of coinfecting strains. Whatever its weaknesses, however, these are the only relevant data available. It is at present unclear on theoretical grounds whether this amount of selfing (0.6) can lead to the low but significant LD observed in our sample and in the Zimbabwe sample[7] (see the discussion of Vitalis and Couvet).[29]

The evidence presented here and the Zimbabwe sample[7] and the arguments provided to explain it show that even in highly endemic areas and despite high genetic diversity, small deviations from random mating, either through weak population differentiation of partial selfing, may exist. Thus, the generalization of Anderson and others[7] should be slightly modified. However, we do believe that the major differences raised by them between areas of high and low endemicity remain valid. Nevertheless, the small deviations from panmixia observed in areas of high endemicity show that our understanding of the population biology of this parasite is still incomplete in such areas. Further advances will probably come from more detailed studies of *P. falciparum* sampled from mosquitoes.

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